Complexation of Glycoalkaloid α-Tomatine with Sterols and Its Potential Application as an Anti-Cancer Drug

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Complexation of Glycoalkaloid α-Tomatine with Sterols and Its Potential Application as an Anti-Cancer Drug

Bishal Nepal


A Dissertation Submitted to the Graduate School at the University of Missouri – St. Louis
In Partial Fulfillment of the Requirement for the Degree
Doctor of Philosophy in Chemistry with an Emphasis in Physical Chemistry
August 2021

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ABSTRACT

Complexation of Glycoalkaloid α- Tomatine with Sterols and Its Potential Application as an Anti-Cancer Drug

Bishal Nepal, Prof. Keith J. Stine (Advisor)
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Glycoalkaloids (GAs) are secondary metabolites found mostly in higher plant species and some marine invertebrates. They are known to form complexes with 3β-hydroxy sterols such as cholesterol causing membrane disruption. So far the visual evidence showcasing the complexes formed between glycoalkaloids and sterols has been mainly restricted to some earlier studies using Brewster angle microscopy. This study aimed to develop a method for topographic and morphological analysis of sterol-glycoalkaloid complexes. Langmuir-Blodgett (LB) transfer of monolayers comprising of glycoalkaloid tomatine, sterols, and lipids in varying molar ratios onto mica followed by AFM examination was performed. The AFM method used required minimal sample preparation and allowed visualization of sterol-glycoalkaloid aggregation at nanometer resolution. While aggregation was observed in a mixed monolayer of tomatine with cholesterol and mixed monolayers with coprostanol, no sign of complexation was observed for the mixed monolayers of epicholesterol and tomatine. Similar aggregates were observed in ternary mixtures of tomatine with cholesterol and phospholipids 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or egg sphingomyelin (egg SM). Further, the effect of complexation on membrane integrity was studied using a supported bilayer composed of DMPC and cholesterol in a 7:3 molar ratio. Disruption of the bilayer was observed within five minutes of tomatine introduction into the aqueous medium above the supported bilayer.
The effectiveness of GAs against various cancer cells are reported in the literature. One major problem that prevents their practical application is the hemolytic nature of GAs. The possibility of employing mesoporous silica nanoparticles (MSNs) to circumvent this problem is explored. MSNs due to their high pore volume and tunable pore size enable loading of a large volume of molecules. Further surface modification of MSNs provides an opportunity to design a carrier system compatible with the host body and specific to target cells. Amino functionalized MSNs (MSN-NH$_2$) coated with polydopamine (PD) and polyethylene glycol (PEG) were synthesized. The hemolytic activity of the nanoparticles (NPs) was tested. PD and PEG-coated MSNs showed no sign of hemolysis. Further, the PEGylation of MSNs resulted in reduced phagocytosis. GA α-tomatine was loaded inside the pores of MSN-NH$_2$. Thermogravimetry analysis (TGA) indicated a loading of 4.7 %. The developed drug delivery system (DDS) showed a significant decrease in the hemolytic activity of α-tomatine. The effectiveness of the DDS was tested against human liver cancer (HepG2) cells. Cell viability assay suggested the effectiveness of the developed DDS was comparable to free tomatine. The cells showed signs of late apoptosis. An increase in cell viability upon the use of, QVD-OPh, a poly caspase inhibitor suggested caspase-mediated apoptosis might be a plausible cause of cell death.

AFM is a useful tool that finds its application across different branches of science. In material sciences, AFM is used to characterize the surface properties of materials such as roughness, and surface coverage. AFM was used for the characterization of nanoporous gold (NPG) the observed structure was comparable to the structure observed under SEM. Further, the ability of AFM to record three-dimensional data was utilized to confirm the presence of the anti-cancer drug doxorubicin (DOX) on the surface of NPG. Further, AFM was utilized to capture images of RBCs and HepG2 cells. The effect of α-Tomatine on HepG2 cells was also visualized using AFM.
DEDICATIONS

To my Family
ACKNOWLEDGEMENT

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AFM  Atomic force microscopy
APTES  3-(Aminopropyl) triethoxy silane
ATP  Adenosine triphosphate
BAM  Brewster angle microscopy
BET  Brunauer-Emmett-Teller
BJH  Barrett-Joyner-Halender
CTAB  Cetyltrimethylammonium bromide
DDS  Drug delivery agent
DMPC  dimyristoyl-sn-glycero-3-phosphocholine
EPR  Enhanced permeability and retention
FITC  Fluorescein isothiocyanate
GAs  Glycoalkaloids
IUPAC  International union of pure and applied chemistry
LSM  Laser scanning microscopy
LUVs  Large unilamellar vesicles
MSNs  Mesoporous silica nanoparticles
MTS  3-(4,5-dimethyltiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophe
<table>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PD</td>
<td>Polydopamine</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red blood cells</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SLBs</td>
<td>Supported lipid bilayers</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SUVs</td>
<td>Small unilamellar vesicles</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEOS</td>
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Reproduced from reference 138.

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CHAPTER 1 INTRODUCTION

1.1 Overview

Glycoalkaloids (GAs) from the plant family Solanaceae have been known for their membranolytic properties. Their affinity towards cholesterol disturbs the interaction of cholesterol with membrane lipids and hence affects the membrane-stabilizing ability of cholesterol. Despite several studies, the nature of complexes formed between GAs and cholesterol is still not understood clearly\(^1\). The research aimed to investigate the complexation between GAs and sterols employing Langmuir monolayers of various compositions of sterols and lipids as model membranes. Surface pressure-area isotherm experiments were conducted to study the sterol-GA complexes formed at the air-water interface. The structure of the complexes formed was investigated using atomic force microscopy (AFM) on monolayers transferred onto mica supports.

Several recent studies have shown the application of GAs as potential anticancer drugs. One of the obstacles in using glycoalkaloids as an anticancer drug is their hemolytic activity. To circumvent this problem, we loaded GA, tomatine inside mesoporous silica nanoparticles (MSNs) functionalized with pH-sensitive polymers Polydopamine (PD) which was further decorated with polyethylene glycol (PEG) chains, for the delivery and release inside cancer cells. The strategy has the potential to serve for targeted specific delivery of the cargo.

This thesis reports the findings of the projects. Chapter 1 introduces saponins and GAs, GAs of genus Solanum, complexes of GAs with sterols, and application of GAs in cancer therapy. The chapter also introduces various model membrane systems developed for the study of complex biological phenomena. The application of AFM for biophysical characterization of the model membrane is also briefly discussed. Finally, the application of MSNs in drug delivery is discussed. Chapter 2 covers materials and methods used
during the research. Chapter 3 presents results from the AFM study of the complexation between sterols and the GA tomatine in transferred monolayers. Chapter 4 discusses results from the synthesis, characterization, and biocompatibility study of MSNs. Chapter 5 presents the result and discussion of MSNs for loading and safe delivery of tomatine for effective treatment of HepG2 cancer cells *in vitro*. Chapter 6 presents some preliminary results in which AFM was used to image RBC and HepG2 cells. The effect of α-Tomatine on HepG2 cells is also presented. The results here are encouraging and could be potential projects for future endeavors.

1.2 Saponins and Glycoalkaloids

Saponins are secondary metabolites found primarily in higher plants but also in some marine invertebrates. These are amphipathic glycosides and get their name because of their foaming and emulsifying properties. Saponins are also known to exert hemolytic activity. The aglycone core of saponins is known as genin or sapogenin. Two types of sapogenin are known to exist: triterpene and steroid. Both triterpene and steroidal sapogenin are derived from the same 30-carbon linear precursor, 2,3-oxidosqualene. During the process of synthesis steroidal sapogenin loses three methyl groups resulting in a 27-carbon backbone while triterpene sapogenin retains all 30 carbons in its backbone. The number of sugar units attached to the aglycone core could vary from 1 to 11, but saponins with 2-5 sugar units are most common. The sugars can have either linear or branched arrangements.

Glycoalkaloids have a nitrogen atom incorporated steroidal skeleton as an aglycone unit, which shares their biosynthetic origin with steroidal sapogenin. The nitrogen atom can be present either in a ring or in the side chain. Steroidal alkaloids are found in plants of the family Solanaceae, Liliaceae, Apocynaceae, Buxaceae, and some marine invertebrates. They are generally isolated in the glycoalkaloid form. Glycosylated
steroidal alkaloids have been reported to have antimicrobial and anti-inflammatory activities. Besides this, several recent studies have suggested glycoalkaloids have anticarcinogenic activity. All these biological activities make glycoalkaloids a promising new drug candidate. These compounds are important in plants' defense mechanisms against pathogens and pests. They are known to form complexes with cholesterol and other 3β-hydroxy sterols resulting in membrane disruption and for inhibition of acetylcholinesterase and butyrylcholinesterase.

1.2.1 Glycoalkaloids of Genus *Solanum*

The glycoalkaloids from the genus *Solanum* are widely studied and are representative of this class of alkaloids. *Solanum* genus includes various commercially important plants as *S. Lycopersicum* (tomato), *S. tuberosum* (potato), and *S. melongena* (eggplant).

![Figure 1.1](image_url) Figure 1.1 Structure of potato glycoalkaloids Chaconine (a), Solanine (b), and tomato glycoalkaloid Tomatine (c) The sugar units are D-galactose (pink), D-glucose (orange), L-rhamnose (blue), D-xylose (red). The six-ring steroidal aglycone units are shown in red.
The GAs can be extracted from flowers, sprouts, stems, and leaves of the plants. The major glycoalkaloids found in potatoes are α-chaconine and α-solanine (Figure 1.1). Both α-chaconine and α-solanine have trisaccharides attached at the 3-hydroxyl position of their aglycone unit. α-chaconine has two L-rhamnose and one D-galactose while α-solanine had one D-glucose, one L-rhamnose, and one D-galactose unit. Most examinations have shown α-chaconine to exhibit higher biological activity compared to α-solanine. However, the mixture of both glycoalkaloids shows a marked synergistic effect when used in combination, especially a 1:1 mixture of the two that have shown a significant increase in membrane-disruptive activity\textsuperscript{11-12}. α-Tomatine is another important glycoalkaloid found in tomato plants (Figure 1.1). The carbohydrate chain of tomatine consists of two D-glucose, one D-galactose, and one D-xylose unit. The prefix α – suggests an intact oligosaccharide unit while the prefixes β1-, β2-, γ- and δ- are used upon hydrolysis of one (either xylose or glucose from the branch), two or three saccharide units, respectively.

The amount of tomatine is high in green tomatoes which can be up to 500 mg Kg\textsuperscript{-1} but the tomatine content decrease to about 5 mg Kg\textsuperscript{-1} as the fruit ripens\textsuperscript{13}. The tomatine content in the vines remains constant at about 500 mg Kg\textsuperscript{-1}. The GA content in the eggplants was found to be between 0.625-20.5 mg Kg\textsuperscript{-1} in the 21 varieties studied\textsuperscript{14}. The GA concentration in potatoes varies widely depending on the type and condition of cultivation\textsuperscript{15}. The concentration of GA also increases in potatoes upon injury to the plant\textsuperscript{16}. The reported GA concentration in normal potato tuber is between 12 and 20 mg Kg\textsuperscript{-1} while the concentration in green tubers is between 250-280 mg Kg\textsuperscript{-1} and green skin is reported to have GA content between 1500-2200 mg Kg\textsuperscript{-1}\textsuperscript{17}. 

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1.2.2 Complexation of GAs with Sterols

GAs are known to form 1:1 complexes with 3 β-OH sterols such as cholesterol. The stoichiometry of complexation with cholesterol has been confirmed by precipitation experiment\textsuperscript{18}. It was found that intact oligosaccharide was required and removal of one or more sugar units resulted in a reduction in the ability to form complexes with sterols. Morphological features such as protrusion and ridges formed due to the complexation between tomatine and cholesterol have been used to image the presence of unesterified cholesterol in cells by freeze-fracture scanning electron microscope\textsuperscript{19-20}.

The interaction of GAs with liposomes of different compositions has been studied. The result from these studies showed that in the presence of 3 β-OH sterols in the liposome formulation, GAs generally caused the leakage from the liposomes releasing the encapsulated contents such as dyes or enzymes. Roddick et al. observed that the release of peroxidase from the liposomes in the presence of tomatine was influenced by the pH and lipid composition of the liposomes as well as the concentration of tomatine\textsuperscript{21}. The activity of tomatine was maximal at pH 7.2 and decreased as the pH was lowered to pH 5, probably due to the protonation of steroidal N-atom. The pKa value of N-atom is reported to be 6.0\textsuperscript{13}. The N- atom is therefore protonated at lower pH and is in deprotonated form at pH > 7. Likewise, for the same concentration of α-tomatine, the activity was higher for the liposome composed of egg yolk phosphatidylcholine (PC) and cholesterol than bovine brain sphingomyelin (SM) and cholesterol. A significant increase in the release of peroxidase was observed with an increase in tomatine concentration from near 10 µM up to near 100 µM. Similarly, the release of peroxidase from multilamellar vesicles composed of egg yolk PC and three different sterols – cholesterol, stigmasterol, or ergosterol induced by solamargine and solasonine was also evaluated\textsuperscript{22}. The activity of solamargine was found to be significantly higher than solasonine. The combination of
two GAs in a 1:1 ratio showed a synergistic effect in promoting the lysis of bovine RBC. Similar synergism was also observed for the release of peroxidase from liposomes made of egg yolk PC and cholesterol and it was observed that both GAs were ineffective at pH below neutral. In another study, it was found that an equimolar mixture of GAs chaconine and solanine exhibited strong synergism for the release of peroxidase from the liposomes suggesting the formation of a ternary complex of GAs with cholesterol. In the same study it was observed that chaconine was effective at 100 μM and pH 7.2 while solanine did not show any activity under similar conditions. The activity of chaconine was maximum at pH 8 and solanine showed comparable activity at pH 9. The derivatives obtained by synthetically modifying the GA solamargine such as the opening of the ring or introduction of nitroso group did not show membrane disrupting properties.

A series of studies was conducted by Keukens et al. investigating the effect of the presence or absence of sterols in membrane disruption by GAs using large unilamellar vesicles (LUV). Various sterols including cholesterol, fucosterol, a sterol found in algae, and ergosterol, a fungal sterol was incorporated in LUVs along with egg yolk PC. The LUVs were loaded with 6-carboxyfluorescein and its release upon membrane disruption was monitored. The GAs did not cause leakage of LUVs without cholesterol. The effectiveness of different GAs to cause the leakage of cholesterol-containing LUVs followed the order tomatine> chaconine> solanidine > solanine. It was observed a minimum of 10 mol % cholesterol is required to promote membrane disruption. Further, the extent of dye release was found to depend upon the structure of the sterol used. Based on their observation they proposed a model for the action of GAs on lipid bilayer containing cholesterol. The first step involved the insertion of GAs into the lipid bilayer between the acyl chain of lipids. At the cholesterol concentration above the suggested critical value of 10 mol %, a stable GA-cholesterol complex would form due to the interaction between the
cholesterol rings and the aglycone portion of the GAs. The segregation of complex then occurs due to carbohydrate–carbohydrate interactions resulting in a region that starts to protrude outward due to steric demand. Finally, the budding of tubular structure from the membrane occurs (Figure 1.2).

Figure 1.2 Proposed model for GA induced membrane disruption. Reproduced from reference1.

The effect of GAs tomatine chaconine and solanine on membrane integrity of human RBCs, colorectal carcinoma, Caco2, cells, and mitochondria was also investigated26. The hemolytic activity of the glycoalkaloid was in the order tomatine> chaconine> solanine determined based on the concentration required to release 50% hemoglobin. The same order of effectiveness was observed for the promotion of the release of Ca^{2+} ions or lactase dehydrogenase from Caco2 cells. The observation suggested membrane disruption requires significant cholesterol content and the human RBCs contain about 50% cholesterol. The mitochondrial membrane disruption was measured by following the release of adenylate kinase.
1.2.3 Application of Glycoalkaloids in Cancer Therapy

GAs have been extensively studied for their anticancer activity. Studies have been conducted either to just determine effectiveness against cell proliferation or to assessing biochemical mechanisms leading to the observed anti-cancer activity. The results have suggested GAs act against the cancer cells by promoting apoptosis, causing cell cycle arrest, and inhibiting cell signaling pathways.

Lee et al. examined antiproliferative activities of glycoalkaloid α-solanine, α-chaconine, α-tomatine, their hydrolyzed products, β1- chaconine, β2-chaconine, γ-chaconine, β2-solanine and aglycones against human colon (HT29) and liver (HepG2) cancer cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. These results showed that all compounds were active, but the activity of hydrolyzed products was less compared to the glycoalkaloid, indicating the importance of carbohydrate side chains. It was further observed that the glycoalkaloids were more effective against the HepG2 cells than HT29 cells. The potencies of α-chaconine and α-tomatine against HepG2 were found to be higher than those observed for anticancer drugs doxorubicin and camptothecin. Friedman et al. performed MTT assays to investigate the ability of glycoalkaloid α-tomatine, dehydrotomatine, and their respected aglycone tomatidine and tomatidenol to induce cell death in breast (MCF-7), colon (HT29), gastric (AGS), and liver (HepG2) cancer cells. It was observed that tomatine extracts from green tomatoes were found to be more effective. The IC50 value was found to be lowest against AGS cells and HT29 cells at 0.03 μg mL⁻¹. In comparison dehydrotomatine, a minor component from tomato extract with a double bond present between carbon 5 and 6 of the steroidal B ring, was much less effective with IC50 values of 578 μg mL⁻¹ and 262 μg mL⁻¹ against AGS and HT29 cells, respectively.
In another experiment, Lee et al. suggested that α-tomatine might be beneficial against prostate cancer development and progression. An EC$_{50}$ value of 1.67 ± 0.3 μM was observed against PC3 prostate cancer cells after 24 h of treatment while EC$_{50}$ value for the normal prostate cell, RWPE1 was 3.85 ± 0.1 μM. The inhibitory effect was attributed to induction of apoptosis as was evident from positive Annexin V staining, increased nuclear condensation, decrease in mitochondrial membrane potential, increased cell permeability, and cytochrome c expression. Further, the result showed induced activation of caspase -3, -8, and -9 suggesting the involvement of both intrinsic and extrinsic apoptosis pathways.

Shieh et al. showed that α-tomatine exhibits antimetastatic activity on human non-small cell lung cancer (NCI-H460) cells. It was found that at a non-cytotoxic concentration (0.5-1.5 μM) α-tomatine significantly suppressed the abilities of NCI-H460 cells to adhere, migrate, and invade. The marked reduction in cell migration and invasion suggested a potential application of tomatine for the treatment of cancer metastasis. Sucha et al. observed that death of human breast adenocarcinoma, MCF7, cells in the presence of α-tomatine at 6 and 9 μM concentration was by necrosis due to membrane disruption caused by the interaction with cholesterol. The lack of apoptosis was determined by assay for proteins p53, p21, and phosphorylated p53. Further, absence in the activity of caspase-8 or caspase-9 supported lack of apoptosis. However, the treated cells showed a loss of adenosine triphosphate (ATP). It was also observed that for cells treated with 9 μM α-tomatine, proliferation was reduced greatly after the initial 24 h; however, by 72 h recovery in cell proliferation was observed. TEM observations of the cells showed no signs of blebbing a feature that is common in apoptotic cells but evidence for swelling and degeneration of nuclear and plasma membranes was visible suggesting necrosis.
1.3 Biological Membranes

Biological membranes are complex arrangements of lipid bilayers decorated with proteins, carbohydrates, and their conjugated forms including glycoproteins and glycolipids that serve as a boundary between the interior of the cell and the external environment. Biological membranes play important roles in maintaining cell homeostasis due to their selectively permeable nature. Membrane proteins and glycoconjugates attached to the membrane regulate various biological activities such as host-pathogen recognition, signal transduction, fertilization, immunity, and immune response.

Figure 1.3 Representation of biological membrane showing its complicated nature. Reproduced from reference 34.
The lipid bilayer is predominantly composed of phospholipids. Phospholipids are amphipathic molecules consisting of a hydrophobic tail made of fatty acids and a hydrophilic head with a phosphate group, which can be functionalized with organic molecules such as choline, serine, or ethanolamine. Phospholipids are of two types - glycerophospholipids and sphingophospholipids. In glycerophospholipids, two fatty acids and a phosphate group are attached to a glycerol molecule as esters while in sphingophospholipids, a fatty acid molecule and a phosphate head group are attached to the sphingosine backbone through amide and O-linkage, respectively. Sterols, another class of lipids, are an important component of the biological membrane. Cholesterol is a typical animal sterol that comprises about 30% of the animal cell membrane. Cholesterol plays important role in maintaining membrane fluidity and integrity.

**Glycerophospholipids**

**Sphingolipids**

**Sterols**

Figure. 1.4 Major lipids found in biological membranes. Reproduced from reference\textsuperscript{35}. 
Cholesterol and sphingolipids are the major components of functional nanoscale membrane domains named lipid rafts. Lipid rafts are characteristics of outer membranes and are involved in signal transduction, trafficking of membrane proteins, and regulation of neurotransmission. Cholesterol is responsible for lateral heterogeneity in lipid bilayers. This is due to the difference in interaction potential of cholesterol between saturated and unsaturated lipids. The planarity of the rigid sterol ring favors interaction with saturated lipids and disfavors interaction with unsaturated lipids. Cholesterol is also known to increase the ordering of lipid tails, thicken the bilayer and decrease the lateral area; these observations are collectively termed condensing effect.

1.4 Model Membrane System

The complex nature of biological membranes makes them challenging to study. This necessitated the development of model membrane systems that would mimic biological membranes and at the same time give researchers control over the parameters such as composition, size, and structure, making the experimental observations easier to interpret. The Liposomes of various sizes (small, large, or giant) with one (unilamellar) or multiple (multilamellar) bilayers and monolayers of lipids at air-water interface are two models often used to mimic biomembranes. Liposomes are also studied for their application as drug delivery vehicle.

1.4.1 Monolayers as a Model Membrane System: Fabrication and Methods of Study

Monolayers of lipids are assembled by depositing lipid solution onto the water surface and subsequent evaporation of the solvent. The choice of solvent is important to ensure the uniform spreading of the monolayer. Ideally, a solvent should be volatile, chemically inert, free of impurities, and with solubilization power enough to dissolve the solutes under study. It is important to select solvents that are insoluble in the subphase.
Chloroform and the mixture of chloroform with methanol, ethanol, or acetone are the commonly used solvents. Other solvents include cyclohexane, benzene, and hexane. Ultra-pure water or buffer solutions of different pH and compositions are used as subphase. The uniformly spread single molecule thick film of lipids thus formed is called Langmuir monolayer named after Irving Langmuir, who pioneered the technique together with Katharine B. Blodgett. The lipid composition of the monolayer is chosen to mimic one of two leaflets of a lipid bilayer.

The monolayer formed then can be studied using different methods some of which include surface pressure – Area (\(\Pi - A\)) isotherm, surface potential measurement, Brewster angle microscopy (BAM), transfer of Langmuir film for study by atomic force microscopy (AFM), fluorescence microscopy, X-ray reflectivity, and scattering, and infrared spectroscopy.

The surface tension of the water molecule on the subphase decreases when the monolayer is deposited at the air-water interphase. The difference in surface tension before and after the deposition of the monolayer is known as surface pressure, \(\Pi\). The plot of change in \(\Pi\) Vs \(A\) as the monolayer is compressed by closing the barrier at constant pressure gives \(\Pi - A\) isotherm. \(\Pi - A\) isotherm provides information about molecular arrangements, phase transitions, compressibility, and stability of the monolayer. A typical surface pressure isotherm with different phase transition regions is shown in Figure 1.4. The phase change is noticed by a change in slope in the surface pressure isotherm. The molecules go from a highly compressible gas-like phase with random orientation of the hydrophobic tails to a highly oriented solid-like phase in which the hydrophobic tails are vertically oriented with very low compressibility. Further compression of the molecules results in the collapse of the monolayer which is visible as a rapid decrease in surface pressure. Phase diagrams can be constructed from the isotherms recorded at various
temperature. Thermodynamic calculations like the excess Gibbs free energy of mixing ($\Delta G^{\text{exc}}$) and free energy of mixing ($\Delta G^{\text{mix}}$) can be made from data gathered from the $\Pi - A$ isotherms.

The monolayer formed can be transferred onto the solid support for further characterization. CaF$_2$ plates are used as solid support for transmission infrared spectroscopy while SiGe and ZnSe plates are used for internal reflection spectroscopy. A freshly cleaved mica provides an atomically flat surface for the transfer of monolayer to be studied using AFM. AFM is useful for the visualization of co-existing phases with great resolution down to a few nanometers. The monolayer can be transferred onto the solid support using two different techniques- a vertical transfer known as Langmuir-Blodgett (L-B) method and a horizontal transfer known as Langmuir-Schaefer (L-S) method. Two different approaches can be used during L-B transfer depending upon the nature of the substrate (hydrophobic or hydrophilic). Hydrophilic substrates are immersed into the subphase before spreading the monolayer. The monolayer then is compressed to desired surface pressure and the substrate is lifted out of subphase at a suitable transfer rate while maintaining a constant surface pressure. The hydrophobic substrate is immersed in the subphase after deposition of the monolayer. A series of upward and downward passes can be conducted to generate multilayer films. If a layer is deposited in every upward and downward pass, then such transfer is known as Y-type transfer. The other types of transfer are X-type in which only downward pass is transferred and Z-type transfer in which only upward pass is transferred.
Figure 1.5 Various steps involved in the preparation and transferring of Langmuir monolayer. Reproduced from reference\textsuperscript{43}.
1.4.2 Supported Lipid Bilayers (SLBs) as Model Membrane System

SLBs are another commonly used biomimetic membrane system. SLBs are two-dimensional films of lipid bilayer attached to the underlying solid support. It was first fabricated by Tamm and McConnell in 1985. SLBs has advantages over its predecessor bilayer membrane, black lipid membrane, in which bilayer is formed across an aperture (0.1 mm - 1 mm) on hydrophobic materials (polyethylene or Teflon) partitioning two sides of a chamber filled with an aqueous solvent, for being robust, stable, and amenable to several characterization techniques.

Fused silica, mica, oxidized silicon, and borosilicate glass are commonly used substrates for the fabrication of SLBs. L-B transfer or combination of L-B and L-S transfer technique can be used for the formation of SLBs on flat supports. However, the formation of SLBs by adsorption and fusion of small unilamellar vesicles (SUVs) is commonly practiced (Figure 1.5). SUVs are prepared either by extrusion of multilamellar vesicles through a polycarbonate membrane with a pore size less than 200 nm or by sonication and ultracentrifugation of aqueous lipid suspensions. The process of bilayer formation begins with the adsorption of SUVs onto the solid support. The vesicles then rupture and fuse giving a planar lipid bilayer (Figure 1.5). The presence of divalent cations such as Ca$^{2+}$ or Mg$^{2+}$ accelerates the process of adsorption and helps in the formation of a lipid bilayer with fewer defects and complete coverage of the substrate. It is also important to keep the temperature of lipid suspension above the main phase transition temperature of the lipid used during the entire process of SLB fabrication.
Figure 1.6 Different methods used for the fabrication of SLBs (right) (a) combination of L-B and L-S transfer technique, (b) vesicle fusion (C) L-B/L-S transfer followed by vesicle fusion. Different processes occurring adsorption and fusion of SUVs on the solid support. Reproduced from reference 45.
1.5 Atomic Force Microscopy (AFM)

AFM is a type of scanning probe microscope that is used for imaging with nanometer resolution. AFM was developed in collaboration between IBM (G. Binnig, and C. F. Quate) and Stanford University (C. Gerber) and the first commercial AFM was developed by Stanford researchers in 1988. AFM consist of a tip of diameter 5-10 nm attached at the end of the cantilever acting as a probe. The tip scans the surface of the sample, and the movement of the tip is detected by a position-sensitive detector and is reconstructed as an image. Based on the nature of the sample the images can be captured on contact mode (for rigid samples), tapping mode, or non-contact mode (for soft biological samples).

In contact mode, the cantilever tip is in constant contact with the surface and is subjected to lateral forces which might result in damage of tip or scratching of the sample. It is therefore important to determine the minimum setpoint force to be used for acquiring the images. The selection of a cantilever is also important. Generally, a soft and flexible cantilever with a low spring constant is required for imaging in contact mode. Since the tip and sample are always in contact the tip always experiences repulsive force while operating in contact mode. Determination of cantilever sensitivity is another important step if quantitative information about tip surface interaction is required. The AFM instrument does the measurements in terms of potential (V). An accurate cantilever sensitivity which is the measure of change in potential per unit change in cantilever motion (V nm\(^{-1}\)) is therefore important. Multiplying the cantilever sensitivity with the measured potential allows us to determine the accurate distance between the tip and the sample. Next, it is also important to calibrate the cantilever spring constant before each measurement as the spring constant provided by the manufacturer is not always accurate. The thermal noise method developed by Hutter and Bechhofer is the common calibration method that is
incorporated with most commercially available AFM instruments and has an uncertainty of 5-10%. The thermal noise method for calibrating effective spring constant is governed by the equation:

\[ K = \frac{k_B T}{\langle q(t)^2 \rangle} \]

The unknown parameter \( \langle q(t)^2 \rangle \), mean squared displacement of the cantilever is obtained by integrating the area under the power spectral density curve, which is the measure of the frequency distribution of \( \langle q^2(t) \rangle \). After which it is possible to measure interaction forces between the tip and the sample based on Hook’s law \( F = -x \) Spring constant.

Another common mode developed later for imaging soft biological samples vulnerable to deformation while imaging in contact mode is tapping mode. In this mode, the cantilever tip is oscillated at its resonance frequency and as the name suggests gently taps the sample surface. Hard tips with high spring constant and resonance frequency are used for tapping mode. Initial tuning of the cantilever to determine its resonance frequency is required. The amplitude of vibration changes as the force experienced by the tip changes as it comes across different regions of samples with different properties. This difference in amplitude is then converted to an image.

Images can also be acquired in a non-contact mode in which the tip is brought close enough to the sample such that an attractive force is established between the tip and the sample. The change in this attractive force as the tip raster over the sample is then converted to an image. While the non-contact mode is useful for imaging soft biological samples it has disadvantages such as high sensitivity to external vibrations and low image resolution. Therefore, tapping mode is the preferred choice for imaging soft biological samples.
AFM has several advantages over other imaging techniques such as SEM, TEM, fluorescent, and confocal laser scanning microscope (LSM). The first advantage is that the images can be acquired either in air, vacuum, or in an aqueous medium unlike in the cases of SEM and TEM which require high vacuum. The second advantage is the ease of sample preparation as it does not require fluorescent labeling or coating of conductive materials. Further, AFM can provide quantitative information such as height distribution of the sample which is not possible by other methods. Furthermore, the possibility of live cell imaging under physiological conditions and the ability to combine AFM with other optical imaging platforms such as fluorescence or confocal LSM has attracted its use in the biological field.
AFM is not only used for capturing topographical images. It can also be used to generate force-distance curves. The force curves than can be used to gather nanomechanical properties of biological sample\textsuperscript{55,56}. Properties such as stiffness have been measured in terms of Young’s modulus for a wide range of sample types including globular proteins, protein filaments, liposomes, viruses, microbes, and eukaryotic cells. Moreover, the advancement of AFM instrumentation has made it possible to get information about the interaction between the molecules at the single-molecule level. Force spectroscopy experiments give valuable fingerprint information on conformational changes of single polymers\textsuperscript{57}, unfolding and refolding of protein\textsuperscript{58}, type of glycosidic linkage in particular polysaccharide\textsuperscript{59}, and to measure the forces required to deform and separate a double-stranded DNA into single strands\textsuperscript{60}.

Figure 1.8 Illustration of different types of force-distance curves showing penetration of layer upon indentation and rupturing force upon pulling molecule attached to cantilever tip. Reproduced from reference\textsuperscript{53}.  

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AFM has also been utilized effectively to visualize the surface topography of model membrane lipid domains. The phase-separated domains when two or more lipids with different melting points are mixed can be visualized using AFM at nanometer resolution. The force curves generated on SLBs give information about the force required to rupture the lipid bilayer, breakthrough force \( F_b \), which helps understand the strength of bilayers and how it is affected upon varying composition of lipids or during the interaction of lipid with other biomolecules such as proteins or drugs. Further, it is also possible to follow such lipid-protein, lipid-drug interaction, or interaction of lipid bilayer with any molecules of interest in general over time.

1.6 Mesoporous Silica Nanoparticles (MSNs)

According to the International Union of Pure and Applied Chemistry (IUPAC) materials with pore sizes between 2 and 50 nm are categorized as mesoporous. The materials with pore size < 2 nm are designated as microporous and those with pore size > 50 nm are labeled as macroporous materials. MSNs were first synthesized by Kresge et al. from Mobile Research and Development Corporation. Their method was based on “liquid crystal templating” in which hexagonally arranged cylindrical micelles were utilized as a template for the deposition of the silica layer. The template was then removed by calcination. The resulting a porous material with uniformly arranged hexagonal pores was dubbed Mobil composition of matter No-41 (MCM-41). The surface area of the synthesized material was > 1000 m\(^2\) g\(^{-1}\) and the pore volume was between 0.7 – 1.2 cm\(^3\) g\(^{-1}\). The pore size of the material could be tuned from 3 – 10 nm by varying the alkyl chain length of the surfactant.

Since then, different variety of MSNs with different pore orientations and sizes have been synthesized. Some of which include MCM-48 which has a cubic arrangement and MCM-50 which has a lamella-like arrangement of pores. Another type of MSNs
named Santa Barbra Amorphous type material (SBA-15) was synthesized by a group from the University of California, Santa Barbra. The group used non-ionic triblock co-polymer like poly (ethylene oxide) oligomeric surfactant and poly (alkylene oxide) block copolymer as a template in an acidic medium. SBA-15 differs from MCM-41 in that they have a larger pore of size 4.6 – 30 nm. Several other MSNs, SBA-11 (3D cubic), SBA-12 (3D hexagonal), and SBA-16 (cubic cage structure) were also synthesized the pore symmetry of which depended upon the type of surfactant used. Other types of MSNs include KIT-5 (Korea Advanced Institute of Science and Technology) with cage type cubic structure and COK-12 (Center for Research Chemistry and Catalysis) with hexagonal pore symmetry.

![Figure 1.9 Illustration of the mechanism of formation of MSNs. Reproduced from reference](image)

1.6.1 Application of Mesoporous Nanoparticles to Cancer Therapy

Two types of strategies are employed to deliver drug-loaded mesoporous nanoparticles to the cancer tissues, passive, and active targeting. Passive targeting takes advantage of the abnormal vascular structure of tumor tissue, the so-called enhanced permeability, and retention (EPR) effect. The blood vessels around tumors have a gap between endothelial cells allowing small nanoparticles with sizes between 30 to 400 nm to leak into the interstitial space of solid tumor tissue. However, the vasculature
around all tumors does not exhibit the EPR effect therefore passive targeting is not a reliable approach.

The limitation of passive targeting demanded the development of advanced active targeting strategies. Cancer cells overexpress folate and transferrin receptors due to their high folate and iron demand for rapid proliferation. Nanoparticles grafted with folate and transferrin or antibody specific to these receptors can be used for active targeting of the cancer cells. Carbohydrates, glycoproteins, aptamers such as sgc8 for targeting human protein kinase-7 (PTK7), antibody fragments targeting Her-2 receptor overexpressed in lungs or breast cancer cells are other possible conjugates that could be attached to nanoparticles for selectively binding with receptors overexpressed on cancer cells.

The nanoparticles can be cleared from the blood vessels by phagocytic cells before they reach their targets. The phagocytic cells are triggered by the layer of protein deposited on the surface of NPs upon their contact with blood. The process of adsorption of protein is known as opsonization and the layer of protein deposited is known as the corona. This problem can be circumvented by coating the nanoparticles with polymers like polyethylene glycol (PEG). PEGylation of NPs shields the NPs from opsonization preventing phagocytosis and prolonging the systemic circulation time. Once nanoparticles reach the cell surface, they are taken in by endocytosis. The microenvironment near a tumor has a pH of 0.5 – 1 units lower than around normal cells and pH inside the endosome inside cells can be as low as 4.5. The difference in pH can be used as a trigger to release drugs. Nanoparticles coated with the pH-sensitive polymer can be used for the release of drugs.
1.6.2 Application of MSNs in Drug Delivery

Mesoporous silica nanoparticles (MSNs) because of their high specific surface area and large pore volume can encapsulate a variety of therapeutic agents. Silica is approved by FDA as a Generally Recognized as Safe (GRAS) substance and is widely used in cosmetic products and as a food additive. The fabrication of MSNs is cost-effective, scalable, and simple. All these properties make MSNs promising as candidates for a drug-delivering agent.

![Diagram of drug delivery](image)

Figure 1.10 Strategies for delivering drugs to cancer site using nanomaterials. Reproduced from reference.

The first application of MSNs for drug delivery was reported in 2001 when anti-inflammatory drug ibuprofen was loaded inside MSNs. Since then MSNs have been used as a drug delivery system for several pharmaceutical drugs with different properties such as water solubility, molecular weight, and biomedical effects against different diseases including diabetes, bone/ tendon tissue engineering (repairing of the cavity defects in rabbit femur and Achilles tendon healing in rat), and inflammation. Mellaerts et al. loaded MSNs with poorly water-soluble drug itraconazole and showed that the release of drug from MSNs in an aqueous environment was faster than the dissolution of a pure...
drug\textsuperscript{90}. They also carried out in vivo experiment in dogs and rabbits and the result showed a significant increase in systemic availability of the drug. In another experiment, Gu et al. functionalized the MSNs with carboxyl group for effective loading of cisplatin due to complexation with the platinum atom\textsuperscript{91}. The drug delivery system showed markedly prolonged and pH-responsive release of cisplatin. Chen et al. utilized MSNs to co-deliver doxorubicin (DOX) and siRNA to treat multidrug-resistant cancer cells. They modified the surface of Dox-loaded MSNs with generation 2 polyamidoamine (PAMAM) dendrimers which were then complexed with siRNA. In their experiment, they found the efficacy of Dox increased 132x compared to free Dox to induce cell apoptosis into multidrug-resistant human ovarian (A2780/AD) cells.
CHAPTER 2 MATERIALS AND METHODS

2.1 Materials

Cholesterol (>99%) was purchased from Sigma-Aldrich (St-Louis, MO, USA). 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, >99%) and egg sphingomyelin (SM, >99%) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA). Coprostanol and epicholesterol were purchased from Steraloids, Inc. (Newport, RI, USA). α-Tomatine (>95%) was purchased from ChromaDex (Irvine, CA, USA). Cetyltrimethylammonium bromide (CTAB, ≥99.0%), tetraethyl orthosilicate (TEOS, >99.0%), (3-aminopropyl) triethoxysilane (99%, APTES), Dopamine hydrochloride (98%) were purchased from Sigma Aldrich, St. Louis, USA. 5(6)-fluorescein isothiocyanate mixed isomer (FITC, >95%) was purchased from ThermoFisher Scientific, USA. Tomatine (> 80.0%) was purchase from TCI America. Amine polyethylene glycol (mPEG-NH2 MW 5000) was purchased from Laysan Bio Inc., USA. Single donor human red blood cells (RBC) were purchased from Innovative Research, Inc., Novi, USA. Sodium hydroxide (NaOH, 1N), Hydrochloric acid (HCl, 37%) were purchased from Fisher Scientific, USA. Reagents for cell culture Eagle’s minimum essential medium (EMEM), fetal bovine serum (FBS), penicillin-streptomycin solution (Pen-Strep), phosphate-buffered saline (PBS, 1x), dimethyl sulfoxide (DMSO) were purchased from American Type Culture Collection (ATCC®) Manassas, USA. All the solvents used, ethanol, methanol, and toluene were HPLC grade and were purchased from Fisher Scientific, USA. Ultrapure deionized water with 18.2 MΩ resistivity was obtained using Simplicity® UV water purification system (Millipore Sigma, USA).

2.2 Sample Preparation

All stock solutions were prepared using HPLC grade solvents and were stored at -20°C. A fresh batch of solutions was prepared every 15 days. The solutions of sterols and
Lipids were prepared in chloroform and the solution of α-tomatine was prepared in ethanol. Appropriate volumes of lipids and sterols or lipids, sterol, and tomatine were added to get the mixtures with desired ratios. The mixtures containing α-tomatine were stirred for an hour before depositing them onto the subphase. The final percent by volume of ethanol in the mixtures containing α-tomatine was kept between 8 to 10%.

2.2 AFM Study of Interaction of Tomatine with Lipid Monolayer and Bilayer

AFM measurements of transferred monolayers were done using NanoScope III microscope (Digital Instruments, Inc., Santa Barbara, CA) operated in the tapping mode in air. Silicon probe TAP300AI-G (Ted Pella, Inc., Redding, CA) having a nominal resonance frequency of 300 kHz, tip radius of < 10 nm, and force constant of 40 N m⁻¹ were used to record topographic images. The ratio of setpoint amplitude to free amplitude (Aₛₛ/ₐₒ) was kept >0.8 to assure softer tapping and avoid sample damaging. Images were captured at a scan rate of 1 Hz using a D-type scanner. The scanner was calibrated using calibration grating TGQ1 (Ted Pella, Inc.).

BioScope Resolve™ AFM (Bruker, Santa Barbra, CA, USA) was used to observe complexation formed in the bilayer. Images were captured using SNL-C probes with a nominal spring constant of 0.12 N/m and a resonance frequency of 23KHz. The images were acquired using Peak-Force mode in the fluid cell. After determining the deflection sensitivity, the spring constant of the cantilevers was calibrated using the thermal noise method. Peak force was set between 1 and 3 nN and images were acquired at a scan rate of 1 Hz. Force curves were captured by continuously extending and retracting the cantilever along the z-axis at the rate of 0.5 μm s⁻¹. Images were acquired using a micro-volume fluid cantilever holder (Bruker, Santa Barbra, CA, USA).
2.3 Preparation of Langmuir Monolayer

The mixture of α-tomatine and sterols or α-tomatine, sterols, and lipids were spread as monolayers on a Langmuir trough containing Milli-Q water as a subphase. The spread monolayers were then compressed to a surface pressure of 15 mN m\(^{-1}\) after allowing the spreading solvent to evaporate for 10 min and then transferred onto the freshly cleaved mica surface using the Langmuir-Blodgett transfer technique. The transferred layers were then analyzed using AFM operated in tapping mode.

2.4 Langmuir – Blodgett Transfer of Monolayer

Langmuir Blodgett trough (Nima Technology, Coventry, England) was used to transfer the deposited layer of lipids, sterols, or the mixtures after compressing to surface pressure of 15 mN m\(^{-1}\). Surface pressure was measured using the Wilhelmy plate method with a piece of filter paper used as the Wilhelmy plate. Compression was done at a compression rate of 20 cm\(^2\) min\(^{-1}\). The initial surface area of the trough before compression was 250 cm\(^2\). A strip of freshly cleaved mica 10 mm wide and 30 mm long was dipped into the subphase before injection of samples. The compressed monolayers were transferred onto the mica at a transfer rate of 4 mm min\(^{-1}\). All the experiments were conducted at room temperature. The procedures were repeated at least twice to assure reproducibility.
2.5 Preparation of Small Unilamellar Vesicles (SUVs)

DMPC and cholesterol were mixed in a 7:3 molar ratio from a stock solution of concentration 10mg mL$^{-1}$ prepared in chloroform. The chloroform was evaporated under a gentle stream of nitrogen gas. The mixture was further dried under a vacuum for one hour. After one hour the dried mixture was hydrated with 20 mM pH 7.5 HEPES buffer to get a final concentration of 1 mM. The mixture was incubated for one hour at room temperature with agitation in between. It was then vortexed to get a milky suspension. The suspension was passed through three cycles of freeze and thaw. The obtained suspension was then passed 11 times through a polycarbonate membrane with 50 nm pore diameter (Nucleopore, Whatman) using an Avanti mini-extruder at 40 °C. The obtained SUV suspension was then stored at 4 °C and was used within 3 days.
Figure 2.2 Schematics showing different steps during the preparation of SUVs (A). Scheme of different components of mini extruder (B) and completely assembled mini extruder from Avanti Polar Lipid, Inc used for the preparation of SUVs (C).

2.6 Preparation of Supported Lipid Bilayer

180 μL of pre-warmed SUVs suspension and 20 μL of 50 mM CaCl$_2$ were deposited onto freshly cleaved mica solid support and were incubated for 20 minutes at 40°C. After 20 minutes the SLB was washed three times with 200 μL of 20 mM pH 7.5
HEPES buffer. The SLB was then observed under AFM using pH 7.5 20 mM HEPES buffer as an imaging solution.

2.8 Preparation of Surface for Immobilization RBC and HepG2 Cells for AFM study

Circular glass coverslips (8 mm diameter) were coated with poly-L-lysine (MW 30-70 kDa, Sigma Aldrich, St. Louis, USA). The coverslips were incubated for 18 h at 37 °C in 0.1 mg mL\(^{-1}\) solution of poly-L-lysine prepared in Milli-Q water. 1 mL of the solution was used to coat the surface of 25 coverslips, the volume was estimated from the manufacturer protocol which recommended 0.5mL of 0.1 mg mL\(^{-1}\) for a surface of 25 cm\(^2\). After 18 h the solution was vacuum aspirated, and the coverslips were then rinsed with a copious amount of Milli-Q water. The coverslips were then dried and stored at 4 °C until further use.

For immobilization of RBC, 100 µL 5% (v/v) dilute RBC was pipetted on the on poly-L-lysine coated glass surface. The RBC solution was kept for 30 min at room temperature allowing the RBCs to adhere to the surface. It was then rinsed with PBS and was fixed with 3.7% glutaraldehyde for 5 min. It was then rinsed with PBS to remove glutaraldehyde.

Similarly, for AFM imaging of HepG2 cells, the cells were incubated in 24 well plates at a seeding density of 75,000 cells well\(^{-1}\) in complete EMEM for 24 h. The cells were then treated with 1 mg mL\(^{-1}\) α-tomatine solution prepared in 0.1% DMSO for 1 h. The untreated cells were used as a control. After 1h the growth medium was aspirated, the cells were then incubated in 100 µL of Trypsin- EDTA for 1 min. The detached cells were collected in a centrifuge tube and were centrifuged at 0.2 rpm for 10 min the supernatant was discarded the cells were then resuspended in 100 µL 3.7 % formaldehyde for fixing
for 5 min. After 5 min the cells were centrifuged, the formaldehyde was discarded, and the cells were resuspended in PBS. The cells were then immobilized in a poly-L-lysine coated glass surface as described above.

AFM images were acquired in the fluid cell using BioScope Resolve™ AFM (Bruker, Santa Barbra, CA, USA) and PBS was used as an imaging buffer. The images were acquired in contact mode using SNL-C probes with a nominal spring constant of 0.12 N m⁻¹ and a resonance frequency of 23 KHz.

2.7 Synthesis of MSNs

MSNs were synthesized using the modified Stober method. Briefly, 1 g of CTAB, 480 mL of H₂O, and 7 mL of 1 N NaOH were taken in a round-bottom flask, the mixture was heated to 80 °C and stirred vigorously until CTAB was completely dissolved giving a clear solution. After that, 5 mL of TEOS was added to the mixture dropwise while continuous magnetic stirring resulting in a white cloudy solution. The obtained mixture was left stirring for 2 h at 80 °C. The mixture was then vacuum filtered, washed with a copious amount of H₂O and ethanol. The obtained product was left to dry under a vacuum for 24 h. 1 g of the dried powder was then calcined at 600 °C for 1 h followed by acid extraction, refluxed with 100 mL of 1 % HCl in ethanol for 24 h, to remove CTAB. Thus, synthesized MSNs was vacuum filtered and washed with a copious amount of ethanol, dried under vacuum for 24 h, and was stored at 4 °C until further use.
For the study of the internalization of NPs by different cells by confocal laser scanning microscopy (LSM), fluorescein-labeled MSNs were synthesized. For this purpose, 3.8 mg of FITC was mixed with 330 μL of ethanol and 70 μL of APTES and was magnetically stirred for 2 h. After 2 h, 100 μL of the reaction mixture was added after 5 mL of TEOS as described above.

2.8 Synthesis of Amino Functionalized MSNs (MSN-NH$_2$)

500 mg of MSNs was mixed with 0.6 mL of APTES and 10 mL of toluene and was stirred for 24 h at 100 ºC under reflux. The mixture was then vacuum filtered and washed with a copious amount of toluene, ethanol, and methanol. The obtained product was then dried under vacuum for 24 h and stored at 4 ºC until further use.

2.9 Loading of Tomatine in MSN-NH$_2$ (MSN-NH$_2$@Tom)

2 mL of a highly concentrated solution of tomatine, 7.2 mg mL$^{-1}$ was added to 50 mg of MSN-NH$_2$ giving the mass ratio of MSN-NH$_2$ to tomatine at 3.5: 1. The mixture was sonicated for 30 minutes and then was refluxed for 18 h. The mixture was then centrifuged
and washed three times with ethanol to get rid of excess tomatine. The prepared NPs were dried under a vacuum for 24 h and stored at 4 °C until further use.

2.10 Polymer coating of MSN-NH$_2$-Tom, MSN-NH$_2$-Tom@PD, and MSN@Tom

Polydopamine coating of tomatine-loaded NPs was done by adding 50 mg of MSN-NH$_2$-Tom to 50 mL of 1 mg mL$^{-1}$ dopamine prepared in 10 mM pH 8.5 Tris-HCl buffer immediately before MSN-NH$_2$-Tom addition. The mixture was then magnetically stirred for 18 h followed by centrifugation and washing with Milli-Q H$_2$O three times to remove excess polydopamine, dried under vacuum for 24 h, and stored at 4 °C until further use. NPs without tomatine were also coated with polydopamine using the same protocol and are designated as MSN-NH$_2$@PD.

PEGylation of MSN-NH$_2$-Tom@PD was achieved by adding 20 mg MSN-NH$_2$-Tom@PD to 8 mL of 5 mg mL$^{-1}$ mPEG-NH$_2$ prepared in 10 mM pH 8.5 Tris-HCl buffer immediately before MSN-NH$_2$-Tom@PD addition. The mixture was then magnetically stirred for 18 h followed by centrifugation and washing with Milli-Q H$_2$O three times to remove excess mPEG-NH$_2$, dried under vacuum for 24 h, and stored at 4 °C until further use. NPs without tomatine were also PEGylated using the same protocol and are designated as MSN-NH$_2$@PD/PEG.

2.11 Characterization techniques

Scanning electron microscopy was done using JEOL JSM-6320F field emission SEM (JEOL USA, Inc., MA, USA) images were acquired at a working distance of 8 mm and an acceleration voltage of 8 kV. The morphology of NPs, arrangement of mesopores, and polymer coating were studied using Thermo Fisher Scientific G2 field emission gun transmission electron microscopy (FEG-TEM) operated at 100 kV. Powder X-ray
diffraction (XRD) was carried out using Ultima IV, Rigaku X-ray diffractometer equipped with Cu Ka radiation (λ=1.5406 Å). Brunauer-Emmett-Teller (BET) surface area analysis and Barrett-Joyner-Halenda (BJH) pore size and pore volume analysis were done using Beckman Coulter SA-3100 Gas Adsorption Surface Area and Pore Size Analyzer (Beckman Coulter, Inc., CA, USA). Thermogravimetric analysis (TGA) was performed in TGA Q500 (TA Instrument, Inc., DE, USA) with 15 °C min⁻¹ heating ramp from 25 °C to 700 °C. FTIR- attenuated total reflection (ATR) (Thermo Fisher Scientific, MA, USA) was used to characterize functionalization and tomatine loading.

2.12 Hemolysis Assay

1 mL of RBC was centrifuged at 2000 rpm for 5 min, then the supernatant was discarded, and the process was repeated until a clear supernatant was obtained. The packed RBC was further diluted to a 5% (V/V) solution. 200 μL of 5% RBC dispersion was added to 800 μL of nanoparticle suspensions of different concentrations prepared in PBS. The mixture was incubated for 2 h at 37 °C with gentle mixing by inversion every 30 min. After 2 h the mixtures were centrifuged, and the absorbance of the supernatant was measured at 541 nm using a Cary 50 UV–Vis spectrophotometer (Agilent, CA, USA). PBS and DI water were used as the negative and positive control, respectively. Solutions of a-tomatine were prepared in 0.1% DMSO. Hemolytic activity of 0.1% DMSO was tested as vehicle control.

2.13 Cell Culture

HepG2 cells (ATCC®, Manassas, VA) were seeded into each well of 24-well plates at a seeding density of 75,000 cells well⁻¹ in 1 mL EMEM containing 10% FBS and 1% penicillin-streptomycin. The cells were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO₂ to allow attachment to wells. The cells were then incubated with
NPs at varying concentrations for 24 h. Cells incubated without nanoparticle and with 0.05% TritonX-100 were used as a positive and negative control, respectively.

For confocal LSM cells were first seeded on 35 mm MatTek glass bottom dishes with 14 mm coverslip at a seeding density of $2 \times 10^5$ cells per dish. Confocal images were acquired using Zeiss LSM 900 and analyzed using ImageJ.

2.14 Cell Viability Assay

Cell viability was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). The reagent contains a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS), and an electron coupling reagent, phenazine ethosulfate (PES). PES has enhanced chemical stability, which allows its combination with MTS to form a stable solution. Tetrazolium compound, MTS is reduced by viable cells to generate a colored formazan dye that is soluble in cell culture media. The absorbance of formazan dye was quantified at 490 nm. Briefly, 30 μL of assay reagent was added after 24 h of incubation with tested materials to each well and was further incubated for 3 h. After 3 h the absorbance at 490 nm was measured with Wallac 1420 multilabel counter (PerkinElmer™, Finland).

Solutions of α-tomatine were prepared in 0.1% DMSO and cells treated with 0.1% DMSO was used as vehicle control. IC$_{50}$ was calculated by fitting the data to a four-parameter non-linear dose-response regression model using GraphPad Prism 9.1.0.

2.15 Study of Cellular Internalization of MSN-NH$_2$@PD/PEG

The internalization of NPs by cells was confirmed by visualization with confocal LSM. 100 μg mL$^{-1}$ of fluorescein-labeled MSN-NH$_2$@PD/PEG were incubated for 3 h with HepG2 cells. After 3 h cells were washed three times with sterile PBS and fixed with 3.7%
formaldehyde. The actin filaments were stained with Phalloidin-iFluor 555 and DAPI was used to stain nuclei.

2.16 Study of Phagocytosis of NPs by THP-1 Macrophages

Phagocytosis of NPs was studied using differentiated human peripheral blood monocytes, THP-1, cells (ATCC®, Manassas, VA). Briefly, THP-1 cells were incubated in an RPMI medium containing 10 nM Phorbol 12-myristate-13-acetate (PMA) for 24 h. After 24 h the medium was changed, and cells were incubated with 100 μg mL⁻¹ of fluorescein-labeled NPs for 3 h. To ensure the formation of protein corona NPs were incubated in the growth medium with 10% FBS for 1 h before incubating with THP-1 macrophages. Nanoparticle suspension prepared in a growth medium without FBS was used as a control (non-corona forming condition). The cells were then fixed, stained, and visualized with confocal LSM as above.

2.17 Apoptosis/Necrosis assay

Apoptosis/Necrosis Detection Kit (Abcam Inc.) was used to monitor apoptotic, necrotic, and healthy cells following the manufacturer’s protocol. Briefly, after 24 h of incubation with NPs, HepG2 cells were washed with 100 μL assay buffer provided with the kit twice after which 200 μL of assay buffer was added to the cell followed by 2 μL of Apoxin Green indicator, 1 μL of 7-AAD and 1 μL of CytoCalcein 450. The cells were incubated for 30 min at room temperature and then washed with assay buffer finally 200 μL of assay buffer was added to the cells and were visualized using confocal LSM.

2.18 Caspase Inhibition Assay

The effect of caspase inhibition on cell viability was studied using QVD-OPh, a caspase inhibitor (Abcam Inc.). Briefly, 1μM of QVD-OPh was added to cells along with MSN@Tom, and the viability of cells was tested after 24 h of incubation.
2.19 Data analysis

AFM images were analyzed using Gwyddion software\textsuperscript{93}. Images were first flattened and corrected for scan line artifacts applying a polynomial function of first to third order. Height histograms were extracted after initial image processing. Solver, a Microsoft Excel add-in program, was used to fit the height distribution to a multi-peak Gaussian model using the non-linear least square minimization method \textsuperscript{94}. The first peak of the fitted curve was set to zero. The standard deviation of measurements was obtained from the fitted parameter.

Results are presented as mean ± SEM. Statistical significance of variables between two groups was determined using unpaired t-test and two-way ANOVA followed by post hoc Tuckey’s test was done for statistical evaluation of data with more than two groups. A value of p < 0.05 was considered significant.
Chapter 3 ATOMIC FORCE MICROSCOPY STUDY OF THE COMPLEXATION OF STEROLS AND THE GLYCOALKALOID α-TOMATINE IN SUPPORTED MONOLAYERS AND BILAYERS

3.1 Introduction

Glycoalkaloids (GAs) are a class of natural products containing an N-incorporated steroidal skeleton coupled to one or more saccharide units. The pharmacological properties of GAs have been well established. They are known to exhibit antifungal, anti-inflammatory, and antimicrobial activity. In recent years, the potential application of GAs against cancerous cells is being explored extensively. Several in vitro and in vivo studies carried out on various cancer cell and animal models have shown promising results. Cellular assays have been performed to understand the mechanism of cytotoxicity. These studies have revealed glycoalkaloids can induce apoptosis, cause cell cycle arrest, and inhibit various cell signaling pathways and proteins.

GAs are also known to form complexes with 3β-hydroxy sterols such as cholesterol. Their affinity towards cholesterol disturbs the interaction of cholesterol with membrane lipids and hence affects the membrane-stabilizing ability of cholesterol, compromising the membrane integrity. Earlier experiments showing leakage of enzymes or dyes encapsulated in the liposomes consisting of 3β-hydroxy sterols upon treatment with GAs are suggestive of this fact. In more recent studies, membrane disruption of cancer cells resulting in its necrosis was attributed to complex formation between cholesterol and the GA. Despite several studies, there is very little literature reporting high-resolution visual evidence of the complexes formed between GAs and sterols. Earlier studies using Brewster angle microscopy (BAM) of
tomatine injected beneath spread monolayers of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and cholesterol clearly showed increases in surface pressure together with the onset of aggregate formation at the water-air interface but the method did not allow good resolution of the aggregates as the BAM resolution was at best several microns. Visualization of the morphology and topography of these complexes with nanometer resolution could provide aid in understanding the biophysical and biological properties of GAs and how they interact with biological membranes.

Herein we report a simple method to effectively capture and visualize the structures of the complexes formed between GAs and sterols. The complexation of the GA α-tomatine with three different sterols, cholesterol, coprostanol, and epicholesterol, alone or in combination with two different lipids DMPC and egg sphingomyelin (egg SM) were investigated (Figure 3.1). α-Tomatine is an important glycoalkaloid found in leaves, stems, and fruits of tomato plants. Green tomatoes can have up to 500 mg kg$^{-1}$ of tomatine. The tomatine content decreases to about 5 mg kg$^{-1}$ upon ripening of the fruit. α-Tomatine has a branched carbohydrate chain, consisting of two D-glucose, one D-galactose, and one D-xylose unit, attached to the steroidal skeleton, at the 3-hydroxyl position. The prefix α – suggests an intact oligosaccharide unit while the prefixes β$\text{I}$, β$\text{II}$, γ- and δ- are used upon hydrolysis of one (either xylose or glucose from the branch), two or three saccharide units, respectively.
The mixture of α-tomatine and sterols or α-tomatine, sterols, and lipids were spread as monolayers on a Langmuir trough containing Milli-Q water as subphase. The spread monolayers were then compressed to a surface pressure of 15 mN m$^{-1}$ after allowing the spreading solvent to evaporate for 10 min and then transferred onto the freshly cleaved mica at the transfer rate of 4 mm min$^{-1}$ surface using the Langmuir-Blodgett transfer technique. The transferred layers were then analyzed using AFM operated in tapping mode.

Further, as a comparison, the effect of α-tomatine on membrane integrity was observed under physiological conditions using a supported lipid bilayer composed of DMPC: Chol in the molar ratio 7:3. Rapid disruption of the membrane was observed within 5 min of injection of a 10 μM solution of α-tomatine above the supported lipid
bilayer using an AFM liquid cell. Figure 3.2 shows the synopsis of the procedure used in this project.

Figure 3.2 Various experimental procedures, Π-A isotherm, transfer of monolayer formed at the air-water interface, preparation of SLB and AFM study in air and fluid medium

3.2 Results and Discussion

3.2.1 Langmuir-Blodgett transfer experiments

3.2.1.1 Sterol: α-tomatine interaction

During initial investigations, deposited layers composed of cholesterol and α-tomatine mixed at different molar ratios were transferred onto the mica. AFM images of the transferred layers showed the presence of aggregated structures (Figure 3.3 and Figure 3.4) suggesting the method was successful in capturing the complexes formed. These aggregates are composed of collections of 1:1 complexes between α-tomatine and cholesterol. The transferred monolayer of cholesterol alone was uniform with RMS roughness (Sq) of 0.040 ± 0.003 nm whereas, there was an increase in roughness, Sq =
0.132 ± 0.003 nm, for the transferred layers composed of an equimolar mixture of cholesterol and α-tomatine. The AFM image also showed the presence of porous defects. The deposited layer consisting of cholesterol and α-tomatine at three different molar ratios, 1:2, 2:1, and 4:1, were then analyzed. AFM images showed the presence of dark (lower height) and bright (higher height) regions with height differences of nearly 0.8 nm. The bright clusters were separated further apart and appeared more straw-like with the increment in moles of cholesterol. It is well established that α-tomatine forms a 1:1 complex with cholesterol. The stoichiometry of complexation with cholesterol has been confirmed by precipitation experiment\textsuperscript{18}. Therefore, the fraction of components forming complexes in the case of the mixtures of cholesterol and α-tomatine in 1:2 and 2:1 molar ratio should be the same. The excess cholesterol left after complexation in the 2:1 cholesterol – tomatine mixture might help retain the complex formed. Likewise, in the case of 1:2 molar ratio, the excess molecules after complexation would be α-tomatine which might be lost in the subphase which could explain the large porous defects observed AFM image (Figure 3.3). While the fraction of components forming complexes in the case of the 4:1 mixture is less which might have resulted in the separation of aggregates into more clearly identifiable straw-like structures dispersed in the matrix of the excess of cholesterol left after complexation.
Figure 3.3 AFM topography image of Cholesterol (A) and Cholesterol: Tomatine deposits at different molar ratios 1:1 (B), 1:2 (C) 2:1 (D) and 4:1 (E) transferred at 15 mN m\(^{-1}\). A comparison of height distribution in C, D, and E is shown in F. The green box 500 nm x 500 nm was selected for roughness comparison.

It is important to note that spread solutions of \(\alpha\)-tomatine alone prepared in 9:1 chloroform – ethanol mixture did not result in the formation of monolayer indicated by the very minimal rise in surface pressure during the compression. The mixture of cholesterol and tomatine did however resulted in the rise of surface pressure. Several previous studies have demonstrated that non-amphiphilic molecules that normally do not form a stable monolayer at the air-water interface were able to form easily transferable stable monolayers upon mixing with fatty acids\(^{100-103}\). It was inferred that at low concentration the non-amphiphilic molecules were accommodated in the excess fatty acid matrix and at high concentration, non-amphiphilic molecules were likely squeezed out of the air-water interface and remained sandwiched between the fatty acid chain and on the surface of the monolayer. The conclusion was made based on the pressure–area isotherm, which showed an initial increase and then a decrease of average area per molecule with an
increase in the mole fraction of non-amphiphilic molecules. Based on the nature of pressure-area isotherms and the AFM images observed we assume a similar mechanism might be occurring in the mixed monolayer of tomatine and cholesterol.

The isotherm for the cholesterol - tomatine mixtures with a low fraction of cholesterol (cholesterol: tomatine at 1:2 and 1:1) resembled that of cholesterol with a sharp rise in slope but had high collapse pressure above 50 mN m⁻¹ (Figure 3.4). The nature of the isotherms for the mixtures with a higher fraction of cholesterol (cholesterol: tomatine at 2:1 and 4:1) were similar with less steep slopes. Similar observations were made previously where the spreading and compression of tomatine at the air-water interface exhibited a minimal rise in surface pressure and did not show any transitory features under BAM⁹⁷. The interaction between tomatine and cholesterol was effective at retaining tomatine at the interface and the retention was more pronounced at higher cholesterol concentrations.

![Figure 3.4](image-url)

Figure 3.4 Pressure – area isotherms for the binary mixtures of cholesterol and α-tomatine and cholesterol (A), and ternary mixtures of cholesterol, α-tomatine, DMPC (B) and cholesterol, α-tomatine, egg SM (C) at different molar ratios.

A further effect of variation in sterols structure on the morphology of the complex formed was studied using two different sterols coprostanol and epicholesterol.
Coprostanol differs from cholesterol in having a non-planar ring system and saturation of double bond while epicholesterol has an inversion of stereochemistry from 3β-OH to 3α-OH. The ratio of sterols to α-tomatine was kept at 2:1. The structure of the aggregates observed for mixed monolayers of coprostanol and α-tomatine were shorter and more granular in comparison to those seen in the cholesterol - α-tomatine mixture observed at the same molar ratio (Figure 3.5). In contrast, no aggregated structures were visualized for 1:2 α-tomatine – epicholesterol mixtures. The lack of observation of aggregates upon combining α-tomatine and epicholesterol is consistent with the earlier report of a lack of aggregate formation seen by Brewster angle microscopy when α-tomatine interacted with spread monolayers containing epicholesterol. The stereochemistry of the 3-OH group is clearly crucial for complex formation.
Figure 3.5. AFM topography image and height distribution analysis of cholesterol: tomatine (A, B) and coprostanol: tomatine deposits at molar ratio 2:1 transferred at 15 mN m⁻¹.

### 3.2.1.2 DMPC + sterol: α-tomatine interaction

The effect of lipids on the interaction between sterol and α-tomatine was then investigated as the most significant aspect of GAs is their interaction with both natural and model membranes. Two different lipids, DMPC and egg SM was chosen for the study. DMPC forms a stable monolayer and remains only in liquid - expanded (LE) phase throughout compression, which is apparent from the lack of plateaus in its pressure-area
isotherm (Figure 3.5). The mixture of cholesterol and DMPC is known to co-exist in an immiscible liquid phase below a certain temperature-dependent critical pressure in a monolayer at the air-water interface. To avoid the transferring of phase-separated domains and obtain a uniform liquid-expanded phase, the transfer experiments were done at 15 mN m⁻¹, a surface pressure well above the critical pressure. AFM analysis showed clear evidence of phase-separated domains of cholesterol and DMPC when transferred at a lower surface pressure (Figure 3.6). The domains were more pronounced when the cholesterol content in the binary mixture was increased from 30 mol% to 50 mol%. The domains appeared to fuse and form a uniform liquid phase when the monolayers were compressed to a surface pressure of 15 mN m⁻¹. Uniformity of the transferred monolayers was desired for any changes observed in the uniform monolayer seen upon the incorporation of α-tomatine can then be attributed to the added α-tomatine.

Figure 3.6 Surface pressure -area isotherm for cholesterol, DMPC and their mixtures at different molar ratios (A) and cholesterol, egg SM and their mixtures at different molar ratios (B)
Figure 3.7 AFM topography images of monolayers composed of DMPC: Cholesterol 7:3 transferred at 9 mN m\(^{-1}\) (A) and 15 mN m\(^{-1}\) (B). DMPC: Cholesterol 1:1 at 9 mN/m (C) and 15 mN m\(^{-1}\) (D)

For the ternary mixture of DMPC, cholesterol, and tomatine, three different compositions were studied. The structures observed in the mixture composed of 7:1:2 DMPC: cholesterol: tomatine were different in appearance compared to structures observed in DMPC: cholesterol: tomatine mixture of composition 7:1.5:1.5 and 7:2:1 (Figure 3.7). The structures in 7:1:2 mixtures seemed to be formed of straw-like strands bundled together. While the straw-like strands in the 7:1.5:1.5 and 7:2:1 mixtures were seen to be further apart.
Figure 3.8. AFM topography images and height distribution analysis of the deposits composed of a ternary mixture of DMPC: Cholesterol: Tomatine at 7:1:2 (A, D), 7:1.5:1.5 (B, E), and 7:2:1 (C, F) transferred at 15mN m\(^{-1}\).

Interestingly, no aggregates were observed for the transferred monolayers in which cholesterol was replaced by coprostanol for two different compositions studied, DMPC: coprostanol: tomatine of molar ratios 7:2:1 and 7:1:2. The transferring of the monolayer was done at 15 mN m\(^{-1}\) surface pressure. The result indicates the presence of DMPC affects the interaction between α-tomatine and coprostanol. Keukens et al. previously have reported complete loss of membrane disruption when cholesterol was replaced by coprostanol in large unilamellar vesicles composed of egg yolk phosphatidylcholine, egg yolk phosphatidic acid, and sterol in the molar ratio of 6.5:0.5:3 as indicated by lack of 6-Carboxyfluorescein leakage from the vesicles\(^1\).
3.2.1.3 SM cholesterol α-tomatine interaction

Both SM and DMPC have phosphatidylcholine as a head group but unlike DMPC, SM has a sphingosine base as the backbone and the fatty acid is amide linked. SM acts as both hydrogen bond donor and acceptor and SM is known to interact with cholesterol resulting in the formation of lipid rafts on the biological membrane. The addition of α-tomatine to egg SM and cholesterol mixture would mean α-tomatine would need to compete with SM to interact with cholesterol and the result of such competition on the structure should be an interesting observation.

The AFM image of the monolayer of egg SM transferred at 15 mN m\(^{-1}\) presence of small and large circular domains was observed (Figure 3.8). Smaller domains ranged from 180 to 400 nm in size while the larger domains were around 5 μm. The average height of such domains was 0.4 ± 0.1 nm. Further, at higher surface pressure (30 mN m\(^{-1}\)), the egg SM monolayer exhibited uniform distribution of thin ribbon-like ligaments, with an average width of 57 nm and an average inter ligament distance of 55 nm (Figure 3.10). Similar phase-separated domains were reported by Weeraman et al.\(^{103}\) The reported height of the domains was 0.4 to 0.7 nm which is similar to the height observed in the present study. Slotte previously observed that for N-palmitoyl sphingomyelin (N-P-SM) and cholesterol mixtures the lateral boundary between LE and liquid condensed (LC) phase dissipated with increased surface pressure or cholesterol concentration\(^{105}\). The surface pressure required for the merger of the LC phase ranged from 3 mN m\(^{-1}\) to 12 mM m\(^{-1}\) depending upon the cholesterol concentration. For cholesterol concentration of 35 mol\%, the mergers of the LC phase were observed at 12 mN m\(^{-1}\). AFM topographic images of SPM: cholesterol monolayer at 7:3 molar ratio transferred at 15 mN m\(^{-1}\) revealed such merging of LC domains (Figure 3.10). In the topography image of SPM: cholesterol monolayer at 8.5:1.5 molar ratio, two morphologically different LC domains
were observed. The large circular domains protruded 0.5 ± 0.3 nm above the ribbon like domains that were 0.6 ± 0.3 nm above the surface. A similar observation was reported by Slotte for N-P-SM and cholesterol mixture composed of 20 mol% cholesterol. It is important to note that Slotte used the epifluorescence microscopy for the visualization of lateral monolayer domains and thus scale of observations is at least 50-fold lower in magnification in comparison to the present study.

Figure 3.9. AFM topography images and height distribution analysis of the deposits composed SM (A, D), SM: cholesterol at 8.5:1.5 (B, E), and SM: Cholesterol: Tomatine at 6:2:2 (C, F) transferred at 15mN m⁻¹.

For the ternary mixture of SM, cholesterol, and tomatine, no sign of aggregation formed between cholesterol and tomatine was observed initially for two different compositions studied, SM: cholesterol: tomatine molar ratios of 7:2:1 and 7:1:2. Further,
the monolayer transferred showed the presence of phase-separated domains and the nature of the domains were comparable to the domains observed in binary mixture of SM and cholesterol. The topography image of the ternary mixture of the ratio 7:2:1 was like the binary mixture of the ratio 7:3 and the ternary mixture of ratio 7:1:2 was comparable to a binary mixture of 8.5:1.5 (Figure 3.9 and Figure 3.10). The result indicated for the compositions taken the interaction between cholesterol and SM was strong and prevented tomatine from interacting with cholesterol. Upon increasing the fraction of tomatine and decreasing the fraction of SM for the ternary mixture (SM: cholesterol: tomatine 6:2:2) the aggregation of the cholesterol tomatine complex was visible in the transferred monolayer. The interesting observation was the absence of the circular domain as seen in the SM and cholesterol binary mixture or the ternary mixture of SM, cholesterol, and tomatine with the previous composition (Figures 3.9 and 3.10). The lack of these circular domains suggests that these domains might have been composed of SM and cholesterol and the addition of tomatine replaced SM from such domains. SM and cholesterol are well-known to form such domains in biological membranes, called lipid rafts, that are involved in important biological functions such as protein trafficking and signal transduction.
3.2.2 Experiment on supported lipid bilayer

AFM has an advantage over other imaging techniques such as transmission electron microscopy and scanning electron microscopy as images can be captured under physiological conditions. To visualize the effect of GA and sterol complexation on the integrity of a membrane an in-vitro experiment was designed. SLB composed of DMPC and cholesterol in a 7:3 molar ratio was used as a model membrane system. The details
about vesicle preparation supported bilayer formation, and imaging conditions are explained in the experimental section.

The bilayer prepared although did not show complete coverage of the mica surface. However, the circular patches of the bilayer of size 1 to 2 μm were large enough to run the experiment (Figure 3.11). To confirm the observed patches were of lipid + cholesterol bilayer, a force curve was generated on the selected area of the bilayer. A single breakthrough force ($F_b$) of average value $5.76 \pm 0.03$ nN (n=130) was found. The representative force-distance curve and distribution of force curve obtained after analyzing 130 such force curves are shown in (Figure 3.11). The height of the bilayer was about 5 nm. After confirming the formation of SLB, 1 mL of 10 μM α-tomatine solution prepared in PBS with a final concentration of 1.8% DMSO and 0.4% ethanol was injected through tubing attached to a micro-volume cantilever holder ensuring complete replacement of imaging buffer with α-tomatine containing solution. After injection, the sample was allowed to equilibrate for 5 min and AFM images were captured continuously. Complete disruption of the bilayer was observed within 5 min of injection of the α-tomatine. A small section of $2 \times 2$ μm$^2$ was selected and imaged at the end of 60 min, a 3D representation of the section is shown in (Figure 3.12) for easy visualization. Straw-like structures like the ones observed in monolayer experiments could be seen confirming similarity between the structures formed in the spread monolayers and those formed upon the interaction of α-tomatine with the supported bilayer. A control experiment was conducted with just the buffer used for preparing α-tomatine to ensure the observation was not due to the buffer solution. No morphological and mechanical changes were observed for the initial 60 min confirming that the disruption observed was due to the presence of α-tomatine.
Figure 3.11. AFM topography image (A), height profile (B), and typical force-distance curve (C) and breakthrough force (F_b) distribution for supported lipid bilayer composed of DMPC: Cholesterol 7:3. The average F_b of 5.76 ± 0.03 nN was obtained from the Gaussian fit.
3.3 Conclusions

L-B transfer of the monolayer composed of sterol and α-tomatine or sterol, lipid, and α-tomatine in different molar ratios deposited at the air-water interface was done for the visualization of complexes formed between the GA α-tomatine and sterols using AFM. The method reported here allowed us to visualize the complexes formed at high resolution than the previously reported methods using BAM. The observed structures were morphologically similar to the structures observed using other high-resolution imaging techniques such as SEM and TEM. AFM method described here has advantages over these methods as it requires minimal sample preparation and can be operated under ambient conditions of pressure and temperature.
Further, AFM provides a unique ability to acquire high-resolution images under a liquid environment. This capability was utilized to monitor the effect of α-tomatine on lipid bilayer composed of DMPC and cholesterol in a 7:3 ratio. Disruption of the bilayer was observed within 5 min of α-tomatine introduction. The method provides encouraging results to expand the studies using other membrane-active molecules in real-time. Furthermore, a similar experimental setup could be utilized to carry out studies in live cells instead of model membrane systems. GAs are known to show anti-cancer activity. The effect of GAs on the morphology and nanomechanical properties of various cancerous cells could be further investigated.
CHAPTER 4 SYNTHESIS, CHARACTERIZATION, AND BIOCOMPATIBILITY STUDY OF MESOPOROUS SILICA NANOPARTICLES

4.1 Introduction

Mesoporous silica nanoparticles (MSNs) are one of the widely utilized materials for drug delivery and cancer therapy. MSNs because of their high specific surface area and large pore volume can encapsulate a variety of therapeutic agents in large amounts. The fabrication of MSNs is cost-effective, scalable, and simple. Control over various parameters such as shape, size, and pore diameter are well established. MSNs also provide an opportunity for surface functionalization and modification which helps in designing a DDS with increased blood circulation time, specificity to cancer cells, and ability of controlled drug release. Silica is approved by FDA as a Generally Recognized as Safe (GRAS) substance and is widely used in cosmetic products and as a food additive. All these properties make MSN promising as a candidate for a drug-delivering agent.

The project aimed to synthesize biocompatible MSNs to employ them for the safe delivery of tomatine in the future. To this end, we synthesize amino-functionalized MSNs (MSN-NH$_2$) that were further coated with polydopamine and polyethylene glycol. The hemolytic. The hemolytic activity of the nanoparticles (NP) was tested. PD and PEG-coated MSN showed no sign of hemolysis and were considered safe for intravenous injection.

Further, the phagocytosis of MSN at different stages of functionalization and surface modification was studied. The effect of protein corona formation in phagocytosis was also evaluated. All tested MSN showed increased phagocytosis under corona forming conditions with exception of PEGylated MSN. The result suggested the synthesized nanoparticle will have prolonged blood circulation time increasing the probability of reaching the cancer site. The uptake of nanoparticles by HepG2 cancer cells was
visualized by confocal LSM. Moreover, the toxicity of NP after the final coating of PEG was tested against HepG2 cells using MTT cell viability assay. The NP did not show signs of a reduction in cell viability up to 300 µg mL⁻¹ concentrations. The result suggested we were able to successfully design a DDS which were non-hemolytic, showed a reduction in phagocytosis and were non-toxic to the cells.

4.2 Result and Discussion

4.2.1 Synthesis and Characterization of MSNs

The average size of MSNs was 144 ± 1.5 nm based on TEM measurements. The powder X-ray diffraction pattern of MSN (Figure 4.1) suggested a well-ordered hexagonal arrangement of the pores with a lattice parameter of 4.4 nm (a₀ = 2d₁₀₀/√3). The hexagonal pore arrangement was apparent from the TEM micrograph. The pore diameter of the nanoparticles from BJH analysis was calculated to be 3.7 ± 0.1 nm (Figure 4.2). SEM images showed the spherical morphology of the MSNs (Figure 4.3). The synthesized MSNs were functionalized with the amine group after the removal of CTAB. Post modification MSNs designed for drug loading with various functional groups such as carboxyl and amino is a common practice that helps in capturing the drug molecules. The successful functionalization of the amine group was confirmed by SEM-EDX measurements (Figure 4.4).
Figure 4.1 Powder X-ray diffraction pattern of MSNs

Figure 4.2 BET (left) and BJH (right) analysis of MSNs along with the results obtained
Further, a decrease in surface area and pore volume of MSN-NH$_2$ was observed in comparison to MSN complimented the findings of the IR study. Type IV BET isotherms characteristic for mesoporous materials were observed for both MSNs and MSN-NH$_2$ (Figure 4.3). The specific surface area and pore volume of MSNs were 1106 ± 1.1 m$^2$ g$^{-1}$ and 1.21 ± 0.05 cm$^3$ g$^{-1}$ respectively which decreased to 721 ± 3 m$^2$ g$^{-1}$ and 0.69 ± 0.05 cm$^3$ g$^{-1}$ after amino functionalization. TGA analysis of MSN-NH$_2$ indicated a 7.3% increase in the content of organic matter compared to MSNs (Figure 4.4). The weight % obtained from TGA was in good agreement with the atomic weight percentage obtained from SEM-EDX analysis (Table 1).
Figure 4.4 SEM-EDX analysis showing the presence of C and N atoms after amino functionalization of MSNs.

<table>
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<tr>
<th>Element</th>
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<th>Atomic % Error</th>
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<td>0.4</td>
<td>2.2</td>
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<tr>
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<td>0.1</td>
<td>42.4</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Table 1. Result of SEM-EDX analysis summarizing atomic and weight percentage of different elements present in MSN-NH$_2$
The MSN-NH$_2$ was coated with PD following amine functionalization. Dopamine is known to form a PD coating on almost any surface by self-polymerization$^{111}$. At pH near 8.5, the catechol group of dopamine first oxidizes to quinone, which further reacts with other catechol or quinone to form PD (Scheme figure 4.1). PD-modified NPs have been extensively used in nanomedicine as drug carriers because of their biocompatibility, high photothermal conversion capacity, biodegradability, and their ability to act as pH-sensitive gatekeeper$^{112}$. Further, PD provides a convenient platform for secondary modifications. Nucleophiles such as thiols and amine can be immobilized on the PDA layer through the Michael addition or a Schiff base reaction. The coating of NP with the PD layer was confirmed by TEM. The particle size distribution analysis indicated about 10 nm thick layer of PD was coated on the surface of MSN-NH$_2$@PD (Figure 4.5).
Figure 4.5 TGA of MSNs at different stages of functionalization and surface coating.

Figure 4.7 Mechanism for polymerization of dopamine to polydopamine (top). The polymerization process is visible as the color of the solution gets darker after the formation of polydopamine is its structure correct as drawn with no double bond in the five-
membered ring? The mechanism for PEGylation (bottom). PEG with amine group can be immobilized on the PDA layer through the Michael addition or a Schiff base reaction.

Finally, the NPs were coated with PEG molecules. PEGylation of the NP is shown to reduce phagocytosis and increase blood circulation time. The average size of NP after PEG coating was $160 \pm 1.9$ nm with about $16$ nm of polymer coating (Figure 4.5). TGA analysis was done to calculate the percentage of polymer coated on the surface of the NPs (Figure 4.4). The total weight % of the polymer after two steps of polymer coating was calculated to be $9.6 \pm 1.3$.

![Figure 4.8 TEM micrographs of MSN (A), MSN-NH$_2$ (B), MSN-NH$_2$@PD (C), MSN-NH$_2$@PD/PEG (D). The size distribution of the NP based on >100 measurements MSN (A'), MSN-NH$_2$ (B'), MSN-NH$_2$@PD (C'), MSN-NH$_2$@PD/PEG (D').]
4.2.2 Hemolysis assay

The biocompatibility of nanomaterials expected to interact with biological systems is extremely important. For a DDS designed to be injected intravenously, it is therefore required that the system be non-hemolytic. Assessment of the hemocompatibility of synthesized MSN at different stages of functionalization and surface modification was carried out using human RBC. A significant reduction in hemolysis was observed upon PD and PEG modification of NP (Figure 4.6). Both MSN-NH$_2$@PD and MSN-NH$_2$@PD/PEG were < 2% hemolytic at the highest experimental concentration of 800 μg mL$^{-1}$. Hence, it could be inferred that the designed DDS could be used for the safe delivery of drugs intravenously.

![Figure 4.9 Hemolytic activity of NPs at different stages of synthesis and surface modifications.](image)

4.2.3 Phagocytosis of NP

The clearance of nanomaterials designed for therapeutic and/or diagnostic purposes by phagocytes is a major problem in nanomedicine. Adsorption of protein on the surface of NP upon contact with biofluids results in the formation of protein corona.
which triggers phagocytes and initiates phagocytosis of NP. The amount of protein adsorbed, and the conformation of adsorbed proteins depends upon the physicochemical properties of nanoparticles such as size, geometry, surface functionality, and surface charge. An ideal DDS should be less prone to phagocytosis and have increased blood circulation time.

The influence of functionalization and surface modification in the phagocytosis of NP was evaluated using human THP-1 macrophages. No significant recognition of NP by THP-1 macrophages was observed in non-corona forming conditions with exception of MSN-NH₂ (Figure 4.7). Positively charged NP are reported to be taken up by macrophages at a higher degree compared to negative or neutral NP. It has been hypothesized that macrophages bear negatively charged sialic acid on their surface which binds to positively charged NP. Although our preliminary observation supports the hypothesis, not much information is available on DDS based on MSNs and requires more investigation which is beyond scope of the present study. On the other hand, a larger number of NP were present on the surface of THP-1 macrophages under the corona forming conditions except for MSN-NH₂@PD/PEG (Figure 4.7) indicating a reduction in phagocytosis of NP upon PEGylation.
Figure 4.10 Phagocytosis of NP by THP-1 macrophages Phalloidin-iFluor 555 was used to label actin filaments shown in red, DAPI was used to stain nuclei shown in blue, and NPs were labeled with fluorescein shown in green.

4.2.4 Cellular internalization and toxicity evaluation of MSN-NH$_2$@PD/PEG

The cellular uptake of MSN-NH$_2$@PD/PEG by HepG2 cells was visualized by confocal LSM using fluorescence-labeled NP after 3 h of incubation. The internalization of NP was apparent (Figure 4.8). Further, an increase in actin filament polymerization was observed in the cells treated with NP compared to untreated cells suggesting actin-dependent endocytosis. An increase in cellular uptake of MSNs upon PEGylation through actin-dependent pathway has been previously reported$^{117}$. Similarly, bundling of actin filament upon exposure to magnetic nanoparticles has also been observed$^{118}$. The cytotoxicity of MSN-NH$_2$@PD/PEG was tested using an MTT cell proliferation assay. The percentage of viable cells was greater than 95% for all concentrations except at 300 μg mL$^{-1}$, the highest concentration tested, at which only 65% of cells were viable. Based on the result of the cell viability assay it could be concluded that MSN-NH$_2$@PD/PEG are non-toxic to the cells at concentrations of <300 μg mL$^{-1}$. 

Figure 4.11 Visualization of cellular internalization of MSN-NH2@PD/PEG by HepG2 cells. Actin filaments were labeled with Phalloidin-iFluor 555 shown in red, the nucleus is labeled with DAPI shown in blue, and MSNs were fluorescein-labeled shown in green.

4.3 Conclusions

MSNs with a pore diameter of 3.7 nm, pore volume of 1.21 cm$^3$ g$^{-1}$, and specific surface area of 1106 m$^2$ g$^{-1}$ were synthesized. The average diameter of NP was 144 nm. The MSNs as-synthesized were hemolytic. The NPs were further amino-functionalized and coated with PD and PEG. A significant reduction in hemolysis of RBCs upon polymer coating was observed. The reduction in hemolysis would allow the application of such porous material for intravenous drug delivery. Further, it was observed that upon PEGylation the nanoparticles were not recognized by macrophages of the immune system. The reduction in phagocytosis of NPs designed for drug delivery is a desired property that increases the circulation time of the NPs in the blood increasing their chance of reaching the cancer location. The average size of NPs upon PEG coating was 160 nm,
which is in the range suitable for the EPR effect (enhanced permeability and retention) for accessing cancer cells through leaky vasculature.
CHAPTER 5 MESOPOROUS SILICA NANOPARTICLES FOR LOADING AND SAFE DELIVERY OF A-TOMATINE FOR EFFECTIVE TREATMENT OF HEPG2 CANCER CELL IN VITRO

5.1 Introduction

Plant-based natural products have been widely used by mankind since ancient times until the present day\textsuperscript{119}. Nearly 25\% of pharmaceutical compounds available today are derived from natural products. Several synthetic drugs available or under development draw their inspiration from natural products. Various computational studies are being conducted to simplify complex natural product structures in small fragments with potential therapeutic application\textsuperscript{120}. While studies are being conducted to develop synthetic therapeutic drugs based on natural products the use of plant extracts is declining\textsuperscript{121}. Although natural products have advantages over synthetic chemicals such as low side effects and costs, their lack of bioavailability, poor solubility, low stability, lack of target-specific delivery, and poor absorption in the body resulted in the loss of interest. To this extent, the application of recent drug delivery systems and strategies can solve most of these problems.

Herein we report the synthesis of DDS consisting of tomatine-loaded MSNs coated with PD and PEG. α-Tomatine a GA found in tomato plants, especially in green fruits, leaves, and stems were used as a representative natural product. Glycoalkaloids such as α-tomatine (the designation alpha refers to the compound with the fully intact tetrasaccharide) are known to bind strongly to 3β-hydroxy sterols such as cholesterol and perturb membrane structure in a manner that can result in the leakage of cellular contents. The developed drug delivery system showed a remarkable decrease in hemolytic activity
when compared to free tomatine. The effectiveness of the developed system was tested against human HepG2 cells. A significant reduction in viability of cells was observed in the concentration range of 1.6 to 12.7 μg mL⁻¹. The effectiveness was comparable to free tomatine at the same concentration range. The result from apoptosis/necrosis assay indicated a sign of late apoptosis which was further confirmed by an increase in cell viability upon the addition of 1 μM of poly caspase inhibitor, QVD-OPh along with the tomatine loaded MSN (MSN-NH₂-Tom@PD/PEG).

A significant reduction in hemolysis was observed for MSN-NH₂-Tom@PD/PEG in comparison to free tomatine. MSN-NH₂-Tom@PD/PEG showed < 5% hemolysis at the highest experimental concentration of 10 μg mL⁻¹. Further, the phagocytosis of MSN at different stages of functionalization and surface modification was studied. The result of the research is intended to encourage the application of DDS based on nanomaterials in the field of natural product therapeutics.

5.2 Results and Discussions

5.2.1 Loading of Tomatine in MSNs

Various steps involved during synthesis, functionalization loading, and surface modification of the DDS are shown in Figure 5.1. The amount of tomatine loaded inside MSN-NH₂ was estimated using TGA (Figure 5.2). Percentage weight change between 100 °C and 650 °C was used to calculate the weight % of tomatine inside NP. Initially, a loading of 8.0 % was observed. The observed loading was a significant improvement compared to unfunctionalized MSNs in which no loading was detected. Although molecules tend to adsorb inside the pores of MSN, an increase in loading upon amino functionalization has been reported. Increased loading of curcumin upon amino functionalization of different types of MSN has been reported previously¹². In another study, a similar result was obtained in which the functionalization of MSN with amino groups resulted in increased
loading of betulinic acid due to the formation of weak intermolecular interaction between the amine group and polar groups of betulinic acid. A loading of 4.2 % was achieved after the final coating of MSN with PEG molecules. A loss of 3.8 % which accounts for about 47.5 % of initially loaded tomatine occurred during polymer coating of the NP.

Figure 5.1 Scheme representing various steps involved in the designing of DDS.
The specific surface area of NP decreased to 136±10 m²g⁻¹ after tomatine loading. The nitrogen adsorption-desorption isotherm resembled that of type II (Fig. 2) corresponding to non-porous materials indicating blocking of the pores by adsorbed tomatine. Further, a decrease in pore volume from 0.69±0.05 cm³ g⁻¹ to 0.24±0.02 cm³ g⁻¹ confirmed that the pores of NP were occupied by tomatine. The appearance of peaks near 1450 cm⁻¹ corresponding to C – H bending of the methyl group, between 1390 cm⁻¹ and 1315 cm⁻¹ corresponding to O–H bending of alcohol, at 1630 cm⁻¹ corresponding to N–H bending of amine group along with C–H stretching between 2973 cm⁻¹ and 2973 cm⁻¹ in the IR spectrum which resembled the peaks on the IR spectrum of tomatine (Figure 5.2) also confirmed the loading of tomatine in MSN-NH₂.
5.2.2 Reduction in Hemolysis due to Tomatine

A significant reduction in the hemolytic activity of tomatine was observed upon its entrapment inside the MSN-NH₂. MSN-NH₂–Tom@PD/PEG showed hemolysis of 4.6 % at the concentration of 10 μg mL⁻¹ which was more than 20-fold less than free tomatine at the same concentration (Figure 5.3). The observed result indicated MSN-NH₂–Tom@PD/PEG were hemocompatible and could safely be injected. Free tomatine showed > 5 % hemolysis up to 2 μg mL⁻¹ and hemolysis of 2.2 % were observed at 1 μg mL⁻¹, a concentration at which no significant cellular toxicity was observed (vide infra). Hence, it could be inferred that the designed DDS could be used for the safe delivery of tomatine at concentrations effective to treat cancer cells.

Figure 5.4 Comparison of hemolytic activity of free tomatine and MSN-NH₂–Tom@PD/PEG. Data are shown as Mean ± SEM of two independent experiments performed in triplicate (n=6).
5.2.3 Cell Viability Assay

Dose-dependent cytotoxicity was observed based on the results from the MTS assay (Figure 5.4). Further, the effectiveness of the MSN-NH$_2$-Tom@PD/PEG was compared with the free tomatine. The results indicated no significant difference between the two except at 12.7 μg mL$^{-1}$, the highest concentration tested, at which MSN-NH$_2$-Tom@PD/PEG was more effective than free tomatine. The IC$_{50}$ of tomatine was calculated to be 1.8±0.4 μg mL$^{-1}$ (Figure 5.5). The cytotoxicity of MSN-NH$_2$@PD/PEG was tested as a control. The percentage of viable cells was greater than 95% for all concentrations except at 300 μg mL$^{-1}$ at which only 65% of cells were viable. The 300 μg mL$^{-1}$ concentration of MSN-NH$_2$@PD/PEG is equivalent to 12.7 μg mL$^{-1}$ of tomatine in MSN-NH$_2$-Tom@PD/PEG (based on 4.2% loading). The IC$_{50}$ value of MSN-NH$_2$-Tom@PD/PEG was calculated to be 1.62 ± 0.04 μg mL$^{-1}$ (Figure 5.5). From the result of the MTS assay, it could be concluded that the effectiveness of MSN-NH$_2$-Tom@PD/PEG was comparable to free tomatine and the concentration ranging between 1.6 μg mL$^{-1}$ and 6.3 μg mL$^{-1}$ of tomatine which corresponds to 37.5 μg mL$^{-1}$ to 150 μg mL$^{-1}$ of MSN-NH$_2$-Tom@PD/PEG would be a safe dose that can be used for effective treatment of HepG2 cells.
Figure 5.5. Visualization of cellular internalization of MSN-NH₂@PD/PEG by HepG2 cells. Actin filaments were labeled with Phalloidin-iFluor 555 shown in red, the nucleus is labeled with DAPI shown in blue, and MSNs were fluorescein-labeled shown in green (A). Cell viability assay performed using MTS assay (B). Data are shown as Mean ± SEM of two independent experiments performed in triplicate (n=6). The pairs with p < 0.05 are marked with *. 
5.2.4 Apoptosis/ Necrosis Assay and Effect of Caspase Inhibition in Cell Viability

The inhibitory effects of tomatine on the proliferation of various cancer cells have been reported and both apoptotic and necrotic pathways have been suggested as a possible mode of action\(^2\),\(^3\)\(^2\). Therefore, apoptosis/necrosis assay was performed to distinguish between the two purposed possibilities. The assay is based on the use of a phosphatidylserine (PS) sensor, apopxin green, that binds to PS exposed on the surface of apoptotic cells. The cells were co-stained with 7-aminoactinomycin D (7-ADD), a membrane-impermeable DNA labeling dye, and CytoCalcein Violet 450, cell cytoplasm labeling dye. 7-ADD impermeable to healthy cells can enter the necrotic cells with compromised membrane integrity and thus label the nucleus. CytoCalcein Violet 450 was used for labeling healthy cells. The cells were visualized using confocal LSM after 30 min of incubation. The results were interpreted according to the guidance provided by the manufacturer (Abcam Inc.). Briefly, cells positive to apopxin green were designated as
apoptotic, cells positive to 7-ADD as necrotic, cells positive to cytocalcein violet as healthy, and cells positive to both apopxin green and 7-ADD were labeled as late apoptotic cells.

A significant increase in the number of late apoptotic cells was observed in the samples treated with MSN-NH$_2$-Tom@PD/PEG compared to the control group treated with MSN-NH$_2$@PD/PEG (Figure 5.6). The concentration of tomatine in MSN-NH$_2$-Tom@PD/PEG treated cells were 1.6 μg mL$^{-1}$ (based on 4.2% loading) and an equivalent amount of MSN-NH$_2$@PD/PEG (37.5 μg mL$^{-1}$) was used for the control experiment. The cells were incubated with MSN-NH$_2$-Tom@PD/PEG in the presence of a poly caspase inhibitor QVD-OPh, to validate the above interpretation and to evaluate the role of caspase in the observed apoptosis.

Caspases are a large family of evolutionarily conserved cysteine aspartates that recognize short-chain peptide molecules and uses a nucleophilic cysteine in their active site to cleave amino acid after an aspartate residue$^{124-126}$. So far there are 12 known caspases in humans$^{126}$. Caspases play an important role in apoptosis and a wide range of caspase inhibitors are available to ascertain the role of caspases in programmed cell death$^{124}$. QVD-OPh is one of such poly caspase inhibitors and is known for its ability to effectively inhibit all caspases except caspase 4 at 1 μM concentration$^{124}$. We observed that the addition of 1 μM QVD-OPh together with MSN-NH$_2$-Tom@PD/PEG resulted in a significant increase in the number of viable cells (Figure 5.6). The results suggested caspase-mediated apoptosis as a possible cause of observed cell death. The ability of tomatine to induce activation of caspase when used alone or in combination with chemotherapeutic agents such as paclitaxel has been reported previously$^{7,127}$ and is in corroboration with the present findings.

The effect of QVD-OPh in cell proliferation was further quantified by cell viability assay. We observed that the addition of 1 μM QVD-OPh together with MSN-NH$_2$-Tom@PD/PEG resulted in a significant increase in the percentage of viable cells at three
different concentrations (Figure 5.6). The highest difference in the percentage of viable cells was observed for 1.6 μg mL$^{-1}$ of tomatine, where the percentage of viable cells increased from 50.3% to 88.0% in the presence of QVD-OPh. It should be noted that, although the presence of QVD-OPh showed statistically significant improvement in cell viability at the other two concentrations, 3.2 μg mL$^{-1}$, and 6.3 μg mL$^{-1}$, it was not able to prevent cell death. Based on these observations it would be logical to conclude that the cells showed signs of apoptosis and caspases mediated apoptosis might be one plausible cause of cell death. Further investigations dedicated to understanding the molecular mechanism of cell death are required.
Figure 5.7. Apoptotic, necrotic, and healthy cells were visualized after treating HepG2 cells with 1.6 μg mL$^{-1}$ of tomatine (in MSN-NH$_2$-Tom@PD/PEG) in the presence or absence of caspase inhibitor, QVD-OPh (A). Cells treated with an equivalent concentration of NP without tomatine were used as control. Apoptotic cells were labeled with apopxin green shown in green, healthy cells were labeled with cytocalcein violet 450 shown in blue and necrotic cells were labeled with 7-ADD shown in red. The difference in cell viability upon addition of caspase inhibitor, QVD-OPh (B). Data are shown as Mean ± SEM of two independent experiments performed in triplicate (n=6). The pairs with p < 0.05 are marked with * (*p<0.03, **p<0.002, ***p<0.0002).

5.3 Conclusions

A DDS based on MSNs was developed for safe delivery of GA, α-tomatine, and its effectiveness against HepG2 cells was evaluated. A remarkable reduction in hemolysis was observed upon loading of tomatine inside MSNs modified with PD and PEG which assured safe intravenous delivery of tomatine at concentrations effective for the treatment of HepG2 cells. The effectiveness of the DDS was comparable to free tomatine but with the added benefit of reduction in hemolysis. Further, there are possibilities of decorating the DDS with ligand, specific to receptors overexpressed in cancer cells for targeted delivery of drugs.

Natural products provide an excellent alternative for the treatment of various diseases including cancer with the added benefit of low side effects, low cost, and easy availability which could be beneficial especially in the case of developing and underdeveloped countries with limited resources. GAs are one of such potential candidates with readily available plant sources such as tomatoes (α-tomatine), potatoes (α-chaconine and α-solanine), and eggplant (α-solamargine and α-solasonine) to name
some. To this extent, only a few studies with the use of nanomaterials for delivery of GA extracts for cancer treatment are reported\textsuperscript{128-129}. We hope that the present study provides aid and inspiration for the design and development of novel therapeutic nanodevices for the fruitful incorporation of natural products from plant extracts in cancer treatment.
CHAPTER 6 MISCELLANEOUS WORK

6.1 Application of AFM from Material Characterization to Studying Biological Samples

AFM has served as an important tool for studying properties of myriad samples ranging from research in material to biological sciences. AFM can be used for investigating various surface properties such as friction, adhesion forces, magnetic and electrostatic properties, Young modulus, and viscoelastic properties. The technique is useful for analyzing almost any kind of samples such as thin films or fibers, powders, polymers, adsorbed molecules under air, vacuum, or liquid medium. AFM requires minimal sample preparation and it requires relatively low cost for operation and maintenance when compared to other high-resolution imaging techniques such as SEM and TEM. The resolution of AFM is in atomic scale and is a non-destructive imaging technique.

The chapter discusses some of the AFM studies carried out to characterize the surface of a silicon wafer prepared as solid support for the attachment of a gold film free of an intermediate layer. Similarly, in AFM was utilized to study the morphology of the NPG surface and how the surface is changed after loading DOX.

Some of the preliminary results of the AFM study of RBC and HepG2 cells are also presented. The study aimed to investigate the morphological and mechanical changes that occur upon their interaction with α-tomatine. The hemolytic nature of α-Tomatine and its effectiveness against HepG2 cells was established during an earlier study and the goal of this project was to have visual evidence of such event. The protocol used for imaging is mentioned in Chapter 2. The present chapter discusses the areas to improve and modifications that could be done to improve the result for continuation of the project.
6.2 AFM to Characterize Surface of Silicon Wafer

The result presented below is the part of the project published in 2020 in the journal Sensors and Actuators A : Physical\textsuperscript{[131]}. The broad goal of the project was to develop a method to attach a film of gold on the surface of the silicon wafer without an intermediate adhesion layer between the two. The film of gold deposited on silicon substrate does not bind strongly which requires the presence of an intermediate adhesion layer. Traditionally a metallic (eg., Cr or Ti) or an organic (eg., silanes or polymer) adhesion layer is applied on the silicon substrate before the deposition of the gold film. However, for gold films deposited to design plasmonic–active sensors presence of such intermediate layer dampens the plasmonic response reducing their sensitivity. The presence of such an intermediate layer, therefore, is not desired.

The problem was tackled by roughening of silicon surface before deposition of the gold film. The surface was roughened by scratching and etching in 10 M NaOH for 20 min. The fabricated plasmon active nanostructured thin film of gold (PANTF-Au) was further used to study the interaction between lectin and glycoprotein as the proof-of-concept design for the point of care biosensor.

AFM was very useful in the characterization of the surface roughness and for understanding the reason for the stable attachment of gold film on the scratched-etched silicon surface (Figure 6.1). It was concluded that the formation of island-like features composed of a collection of granular structures in the scratched-etch silicon wafer resulted in improved stability of gold film attachment because of enhancement in nucleation sites for the gold atom to form gold films. AFM was used as complementary to SEM and provided additional information on surface roughness.
Figure 6.1 SEM images at low (A) and high (B) magnification and AFM topography image (C) of scratched silicon wafer the generated roughness profile is shown below C. SEM low (D) and high (E) magnification images and AFM topography image (F) of scratch-etched silicon wafer the generated roughness profile is shown below F.

6.3 AFM to Characterize NPG

The result presented below is the part of the project published in 2021 in the journal Nanomaterials¹⁵². The goal of the project was to develop an implantable drug delivery system. NPG millirods were fabricated by the chemical dealloying method. Thus, formed NPG millirods were loaded with DOX and were then encapsulated by poly (D, L-lactide-
co-glycolide) (PLGA, lactide: glycolide = 65:35, MW 40-75 kDa) containing rapamycin (RAPA) using the dip-coating method. The developed implantable DDS exhibited sustained co-release of DOX and RAPA. The devised DDS demonstrated that nanoporous gold-based implants have the potential to be used as a drug carrier for cancer treatment.

AFM was used to study the morphology of NPG and to compare the surface of NPG before and after loading of DOX (Figure 6.2). The AFM images of DOX-loaded NPG showed an increase in roughness. Further, height distribution analysis indicated an increase in height of 2.4 nm after DOX loading suggesting the presence of DOX molecule on NPG surface and may have been dimerized.
Figure 6.2 AFM topography images of NPG before (A) and after (B) DOX loading and their corresponding 3D view and height distribution profile on the right.

6.4 AFM Study of RBC and HepG2 Cells

The development of electron microscopy techniques has improved the resolution with which cellular features can be observed. The improvement is remarkable when compared to light microscopic techniques where the resolution is restricted due to the
optical diffraction limit. However, the preparation of biological samples is complicated and destructive, and the observation of live samples is not possible. To this end, AFM microscopy provides an opportunity to visualize biological samples in their native environment with atomic-scale resolution.

RBC is also known as erythrocytes play an important biological function of transporting oxygen from the lungs to the body tissue and carbon dioxide from tissues to the lungs. Healthy human RBCs are biconcave (discocyte) shaped with an approximate diameter of 8 µm and thickness of 2 µm. The morphology of RBC changes as a function of its environment, pathological condition, and age. It has been found that the RBC exists as stomatocytes (cup shape) form in hypotonic solution and as echinocytes in hypertonic solution (Figure 6.3). AFM was first used to image RBC by Butt et al. and Gould et al. in 1990. Since then AFM is used to image nanostructures on the surface of RBC and morphological and mechanical differences between healthy and pathological RBC samples.

Figure 6.3 3D confocal images of the canonical shapes of RBCs in different concentrations of NaCl. From left to right: 55, 80 (two stomatocytes), 154 (one discocyte), 200, 250, 300, and 350 mM NaCl (four echinocytes). Reproduced from reference.
RBC because of their soft membrane can deform when they come in contact with AFM probes. Fixation of RBC is therefore required for acquiring a high-resolution image. The immobilization of samples on an atomically flat solid support is essential for AFM imaging. For this poly-L-lysine is commonly used for coating the surface of glass slides. Poly-L-lysine provides a poly-cationic surface for the attachment of negatively charged glycocalyx coating of the cell surface.

The result of AFM images acquired is presented in Figure 6.4. The majority of RBC appeared as echinocytes while few discoytes were also observed. The morphological appearance of the discoyte cell, diameter, and thickness was in the normal range. One possible explanation for the presence of high echinocytes could be the use of a concentrated solution of glutaraldehyde during fixation of RBC, which might have increased in osmolality of PBS solution. Such effect of increased osmolality upon addition of glutaraldehyde in the morphology of RBC has been reported where the RBC fixed in 1% glutaraldehyde appeared flattened and had borders slightly spiky, resembling echinocytes\textsuperscript{141}. Therefore, the application of a low concentration of glutaraldehyde would be the next logical step for the optimization of the protocol. The overall goal of the project was to visualize morphological differences upon treatment of RBC with different GA and the establishment of an optimal protocol for getting a morphologically correct image of the control sample was the first step toward the goal.
Similarly, efforts were made to visualize HepG2 cancer cells and the effect of \( \alpha \)-tomatine treatment in the morphology using AFM. The results are presented in Figure 6.5. We could visualize the morphological difference between the untreated and treated cells. The tomatine-treated cells appeared spherical and the presence of blebbing on the cell surface could be visualized, these traits are typical for apoptotic cells and the results were complementary to the observation made in our previous studies. The results are promising although some improvement is required for the preparation of the sample. The presence of pits on the cell surface of the control cells suggests significant damage was done to the cells during sample preparation. One possible way of avoiding such damage is to grow the cells directly on sterile glass slides which can be mounted on the AFM pucks and would avoid trypsinization and centrifugation steps. The Bioscope Resolve instrument can
image samples in a petri dish, therefore, growth, treatment, and fixation of cells could be done on a cell culture dish and be imaged without the requirement of transferring it to the substrate. Further, the possibility of imaging on a petri dish allows the opportunity to image live cells and observe the effect of treatment in real-time.

Figure 6.5 AFM images of HepG2 cells before (left) and after (right) α-Tomatine treatment

6.5 Conclusions

AFM was utilized to characterize the surface of the silicon wafer and NPG. AFM was valuable for quantifying the surface properties as roughness and height distribution and the data generated helped confirm the results obtained by SEM.

Further, the potential application of AFM to study the effect of GAs on RBCs and HepG2 cells was explored. The preliminary results suggested upon optimization of sample preparing conditions the proposed experiments are feasible. Possible areas for improvement based on the literature survey and the instrumental capacity are suggested.
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