ABSTRACT

Title of Thesis: **Toxicity of Catanionic Vesicles to Biological Cells**

Veena V. Rao, Master of Science, 2013

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This thesis investigates "catanionic" vesicles, which are nanoscale containers that are spontaneously formed by mixing cationic and anionic surfactants. These structures are easy to prepare and indefinitely stable. In comparison, unilamellar liposomes based on phospholipids are cumbersome to prepare, requiring multiple steps and intense shear (extrusion or sonication); moreover, they have limited stability, especially when stored at room temperature. Despite the many advantages, catanionic vesicles are not frequently used in the pharmaceutical industry because of concerns over their cytotoxicity. In this thesis, we systematically explore the cytotoxicity (on mammalian cell lines) of a range of catanionic vesicles formed by mixing various commercially available cationic and anionic surfactants. We examine how cytotoxicity is influenced by the surfactant tail length, the nature of the surfactant tail (saturated vs. unsaturated) and the net charge on the vesicles; as a control, we also study liposomes from phospholipids. A live/dead assay was our primary tool for assessing cytotoxicity. Our results reveal several systematic trends and we have found that certain vesicles based on unsaturated cationic surfactants are relatively nontoxic and biocompatible. These results could potentially lead to new classes of catanionic vesicles that could be safely utilized for biomedical applications.

Toxicity of Catanionic Vesicles to Biological Cells

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Dedication

This thesis is dedicated to my family for their immense love, support and encouragement throughout my career and personal life decisions.

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Chapter 1: INTRODUCTION & OVERVIEW

Researchers have been investigating nanocontainers for biomedical applications such as the targeted delivery of drugs, proteins or other biomolecules.[1] One commonly used nanocontainer is a hollow spherical structure called a vesicle. Vesicles prepared from lipids are called liposomes and these are the ones that are commonly used in real applications.[2] The typical structure of a liposome is shown in Figure 1.1. As shown in this figure, vesicles can encapsulate hydrophilic as well as hydrophobic drugs in their core and in their membrane respectively.[1] The membrane is referred to as a bilayer because it has two layers of lipid molecules sandwiched together, and this bilayer structure closely resembles the structure of a cell membrane.[3]

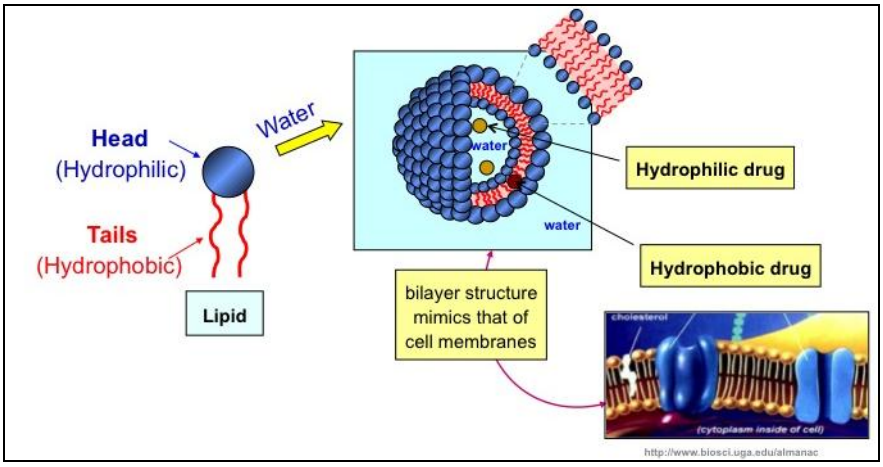

Figure 1.1. Schematic of a vesicle made from lipids (liposomes). Lipids are shown as molecules with a spherical head (hydrophilic) in blue and two hydrophobic tails in red. These are arranged in a bilayer that envelops the vesicle. Hydrophilic drugs can be stored in the interior of the vesicle while hydrophobic drugs can be stored in the membrane.

Liposomes, however, have several disadvantages that make it difficult to readily utilize them in biomedicine. Specifically, preparation of liposomes involves a laborious extrusion process and these structures do not remain stable for more than a couple of days at room temperature.[4] Scientists have been in search of an alternative vesicle formulation for several decades. One such formulation, described first by Kaler et al., is a class of surfactant vesicles formed by spontaneous mixing of cationic and anionic surfactants in water.[5-8] These are commonly referred to as catanionic surfactant vesicles.[5-8] A schematic showing the formation of such vesicles is given in Figure 1.2.

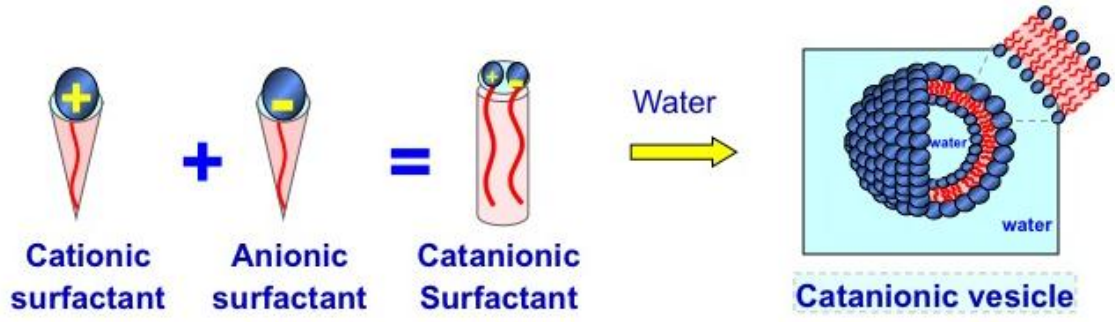

Figure 1.2. Spontaneous formation of catanionic surfactant vesicles in water (for details see Chapter 2). The cationic surfactant has a positively charged spherical head and a single hydrophobic tail. The anionic surfactant has a negatively charged spherical head a single tail. When combined, the oppositely charged heads bind to give a net molecule that has a geometry similar to that of a lipid. In turn, these molecules self-assemble to form vesicles.

There are certain aspects of catanionic vesicles that make them more attractive than the conventionally used liposomal formulations.[5-8] The vesicles are very easily formed by mixing cationic and anionic surfactants together in water. Vesicle formation is spontaneous and an external input of energy (shear, extrusion, sonication etc.) is not necessary. Catanionic vesicles also remain stable for years, in comparison to lipid

vesicles which are often stable only for a few days. Besides, the surfactants that form these vesicles are usually much cheaper than lipids. Despite these advantages, catanionic vesicles are not currently used in biomedical applications. The reason for this is that the surfactants (and in turn the vesicles) are believed to be quite toxic to biological cells.[8-10] However, to our knowledge, there have been no studies to-date that have systematically studied the toxicity of these vesicles against cells.

The main motivation of this thesis is therefore to conduct a systematic study on the cytotoxicity of catanionic vesicles and thereby attempt to bridge the gap in literature. We have studied how cytotoxicity is influenced by the surfactant tail length, the nature of the surfactant tail (saturated vs. unsaturated), the nature of the surfactant headgroup, and the net charge on the vesicles. As a comparison, we have also examined the cytotoxicity of liposomes prepared from conventional lipids. Our main assay tool is the live/dead assay and our studies are mainly conducted with an epithelial breast cancer cell line called MCF-7. As will be shown in Chapter 3, through our studies, we have identified certain catanionic vesicle formulations that are of relatively low toxicity. We have advanced a couple of hypotheses for this lower toxicity. This result is worthy of further exploration and may pave the way for the adoption of catanionic vesicles in biomedicine.

Chapter 2: BACKGROUND

In this chapter, we describe the structure of surfactants, formation of vesicles from surfactants and lipids, and some of the common techniques used to analyze cytotoxicity of nanostructures. We also briefly describe the characterization techniques used in studying the vesicles.

2.1. SURFACTANTS

The term surfactant is an abbreviation for surface-active agent.[11] Surfactants are amphiphilic in nature, i.e. they contain a water-loving or hydrophilic head and a single hydrophobic tail (see Figure 2.1). Surfactants are classified into the following categories based on the type of head group:

a) Anionic: The head group bears a negative charge. Examples: sodium dodecyl benzene sulfonate (SDBS), sodium bis(2-ethylhexyl)sulfosuccinate (AOT).
b) Cationic: The head group bears a positive charge. Example: Cetyl trimethyl ammonium bromide (CTAB).
c) Zwitterionic: The head group has both cationic and anionic parts. Example: Erucyl dimethyl amidopropyl betaine (EDAB).
d) Nonionic: The head group does not bear any ionic charge. Example: Tween 80.

2.2. SELF-ASSEMBLY OF SURFACTANTS

Surfactants can self-assemble into structures like spherical or cylindrical micelles, vesicles and lamellar phases.[12] Self-assembly refers to a process of spontaneous

organization, and it is governed by thermodynamics, i.e., it occurs because the system minimizes its Gibbs free energy in the process. The first surfactant aggregate that self-assembles in water is a micelle, which starts to form at the Critical Micellar Concentration (CMC).[13-15] The driving force for self-assembly is the gain in entropy of water molecules when surfactant hydrophobes are buried in a micelle; this aspect is referred to as the hydrophobic effect.[13]

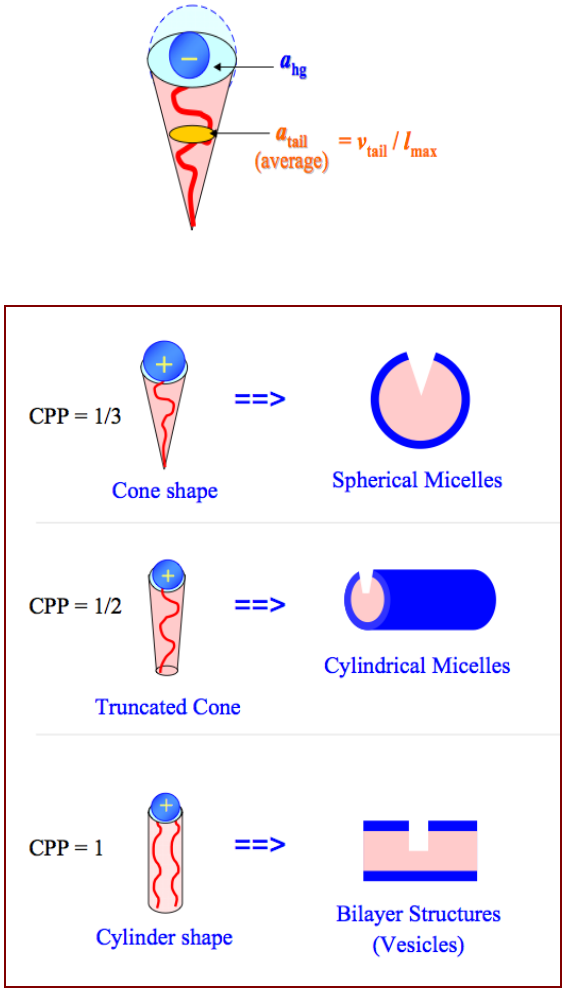

Figure 2.1. Schematics showing the connection between the self-assembled structures formed by amphiphiles in water with the geometry of the amphiphiles, as quantified by the critical packing parameter (CPP).[16,17] The amphiphiles are depicted with hydrophilic heads, shown in blue and hydrophobic tails, shown in red.

The type of self-assembled structure formed by surfactants can be explained by a geometrical term called the Critical Packing Parameter (CPP).[16,17] The CPP is the ratio between the cross-sectional area of the hydrophobic tail and that of the head group, as shown in Figure 2.1. Thus, CPP is given by the following formula:

$$\mathrm{CPP} = \frac{a_{tail}}{a_{hg}} = \frac{V_{tail}}{l_{\max} \cdot a_{hg}} \tag{2.1}$$

where V_{tail} is the volume of the hydrophobic tail, $l_{\max}$ is the maximum extended length of the hydrophobic tail, and a_{hg} is the effective cross-sectional area per head group. As shown in Figure 2.1, micelles correspond to a CPP ~ 1/3 whereas vesicles and bilayer structures correspond to a CPP ~ 1.

2.3. VESICLES AND LIPOSOMES

Vesicles are self-assembled containers formed in water by lipids or surfactants.[6,18] The shell of the vesicle is a bilayer (*ca*. 2-5 nm in thickness) of the amphiphilic molecules, with the hydrophilic heads on both sides of the bilayer and thereby exposed to water, while the hydrophobic tails inside the bilayer are shielded from water. A vesicle can be considered to form by the folding of amphiphilic bilayers. Vesicles with a single bilayer are called unilamellar vesicles (ULVs), while vesicles with several concentric bilayers are called multilamellar vesicles (MLVs).[4] A British hematologist, Alec Bangham was the first to report in 1961 that synthetic vesicles could be formed in water using lipids.[19-21] Lipids are two-tailed surfactants, as shown in the bottom panel of Figure 2.1, and thus tend to have a net cylindrical structure (CPP ~ 1). Thus, lipids have a tendency to spontaneously form bilayers.[4] Vesicles formed from lipids are called

liposomes. The tendency for bilayers to fold is driven by a desire to minimize contact of the hydrophobes with water at the bilayer ends. However, the preferred equilibrium state for lipids is as a lamellar phase (long bilayers) and therefore, liposomes often have limited stability.[4,18]

Lipids fall into many categories. The most common type of lipids are the phospholipids, which have a phosphate moiety in their headgroup (e.g., the phosphatidylcholines or PC lipids). Since they have two hydrophobic tails, lipids are not soluble in water.[4,18] Thus, to prepare liposomes, one requires the use of an organic solvent and also some input of energy.[18] First, the lipid is dissolved in an organic solvent such as chloroform. Thereafter, the solvent is removed by evaporation to yield a dry lipid film. This film is then hydrated by adding water and the mixture is either sonicated or extruded through a filter of given pore size.[4] During the latter, long bilayer sheets are chopped into smaller fragments, which assemble into unilamellar liposomes. However, liposomes formed this way are kinetically trapped structures and hence quite unstable.[18]

2.4. CATANIONIC VESICLES

Mixtures of single-tailed cationic and anionic surfactants can also form vesicles.[5,6,8] The formation of such "catanionic" vesicles can be understood via the CPP concept (Figure 1.2). In this case, each individual surfactant molecule resembles a cone because of the electrostatic repulsion from its headgroup.[16] When mixed together, however, the cationic and anionic headgroups mutually mitigate their repulsive electrostatic effects, leading to a significant reduction in headgroup area.[16] The

combination of these molecules thus resembles a cylinder (CPP ~ 1), and consequently leads to vesicle structures.[16,17] Interestingly, catanionic vesicles tend to spontaneously form when the two individual surfactants are mixed (shear is not necessary in this process).[6] Moreover, the vesicles are indefinitely stable, which suggests that they may actually be equilibrium structures.[6]

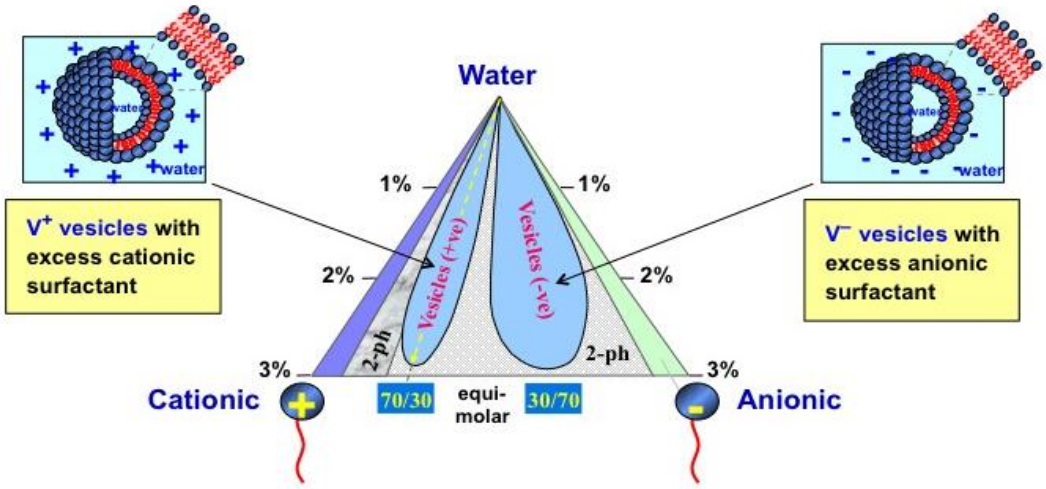

Figure 2.2. Typical ternary phase diagram for mixtures of a cationic and an anionic surfactant in water at room temperature.[22] The lobes in light blue correspond to vesicles.

Formation of catanionic vesicles in mixtures of two surfactants is usually shown on a ternary phase diagram, an example of which is given in Figure 2.2.[22] Here, this diagram corresponds to a constant temperature (room temperature) and the apices represent pure components. Note that both micelle and vesicle phases occur at different locations of this diagram. Vesicles are formed along two lobes. The V^+ lobe on the left corresponds to an excess of cationic surfactant, and thus the vesicles have a net cationic charge in this lobe (a typical weight ratio of cationic/anionic = 70/30 runs along the center of this lobe). In comparison, the V^- lobe lobe on the right corresponds to an excess of anionic surfactant, and thus the vesicles have a net anionic charge in this lobe (a

typical ratio of cationic/anionic = 30/70 runs along the center of this lobe). In our studies, we have mostly chosen to work with surfactant ratios of 30/70 and 70/30 and an overall surfactant concentration of 1 wt%.

2.5. CHARACTERIZATION TECHNIQUE I. DLS

Dynamic Light Scattering (DLS) is a common technique used to characterize the sizes of particles in solution. The experimental setup of DLS consists of a laser source that is focused through a lens on a sample, and the scattered light from the sample is recorded by means of a detector that is typically placed at a 90° angle to the source. Since this technique is very sensitive to the scattering of particles, it is very important that the detector and the sample cell be kept dust free at all times. Also, it is recommended to wipe the sample vials with acetone to remove any fingerprints and dust before placing them into the sample cell.

DLS probes the Brownian motion of particles in the fluid. In a DLS experiment, the fluctuating intensity of light scattered from the sample is recorded. The fluctuations are then correlated to yield the intensity autocorrelation function vs. correlation time.[23] From this function, the translational diffusion coefficient of the particle D is obtained, which in turn can be related to particle size by the Stokes-Einstein equation[24]:

$$D = \frac{k_B T}{6\pi\eta R_h} \tag{2.2}$$

where k_B is the Boltzmann constant, T the absolute temperature and η the viscosity of the solvent. The size obtained from DLS is the hydrodynamic radius R_h, which is the bare particle size along with any solvation layer.

2.6. CHARACTERIZATION TECHNIQUE II. SANS

Small-angle neutron scattering (SANS) is an invaluable probe of the nanostructure in soft materials. The intensity of scattered radiation from a structured fluid is a function of the size, shape, and interactions of the particles present. In SANS, the contrast between the solvent and particles is achieved by switching from hydrogen to deuterium, e.g., using D_2O instead of H_2O. SANS requires a nuclear reactor to generate neutrons and we have one of the premier SANS facilities nearby at the National Institute of Standards and Technology (NIST) in Gaithersburg, MD. SANS data is collected and placed on an absolute scale using calibration standards. It is analyzed as a plot of scattered intensity I vs. wave vector q, which is given by:[25]

$$q = \frac{4\pi}{\lambda}\sin\left(\frac{\theta}{2}\right) \tag{2.3}$$

Here, λ is the wavelength of the incident radiation and θ is the scattering angle. q can be considered an inverse length scale, with high q pertaining to small scales, and vice versa.

The SANS intensity $I(q)$ from a structured fluid is a function of particle size and shape, given by the form factor $P(q)$, and the interactions between the particles given by $S(q)$, the structure factor.[26] When the particles are in dilute solution or are non-interacting, the structure factor $S(q) \rightarrow 1$ and the SANS intensity $I(q)$ can then be modeled purely in terms of the form factor $P(q)$. Form factors for different particle geometries are known and these can be fit to the data to obtain structural information about the particles. An alternate method of analysis that requires no prior knowledge about the scatterers is the Indirect Fourier Transform (IFT) method, and here a Fourier

transformation is done on the scattering intensity $I(q)$ to give the pair distance distribution function $p(r)$ in real space. $I(q)$ and $p(r)$ are related by the following equation:[26]

$$I(q) = 4\pi \int_0^\infty p(r) \frac{\sin(qr)}{qr} dr \tag{2.4}$$

The $p(r)$ function provides structural information about the scatterers in the sample. In particular, the largest dimension of the scattering entities can be estimated. Typical $p(r)$ functions for spherical micelles, cylindrical micelles, and unilamellar vesicles are known. Note that this IFT analysis is valid only for non-interacting scatterers.

2.7. LIVE-DEAD ASSAY

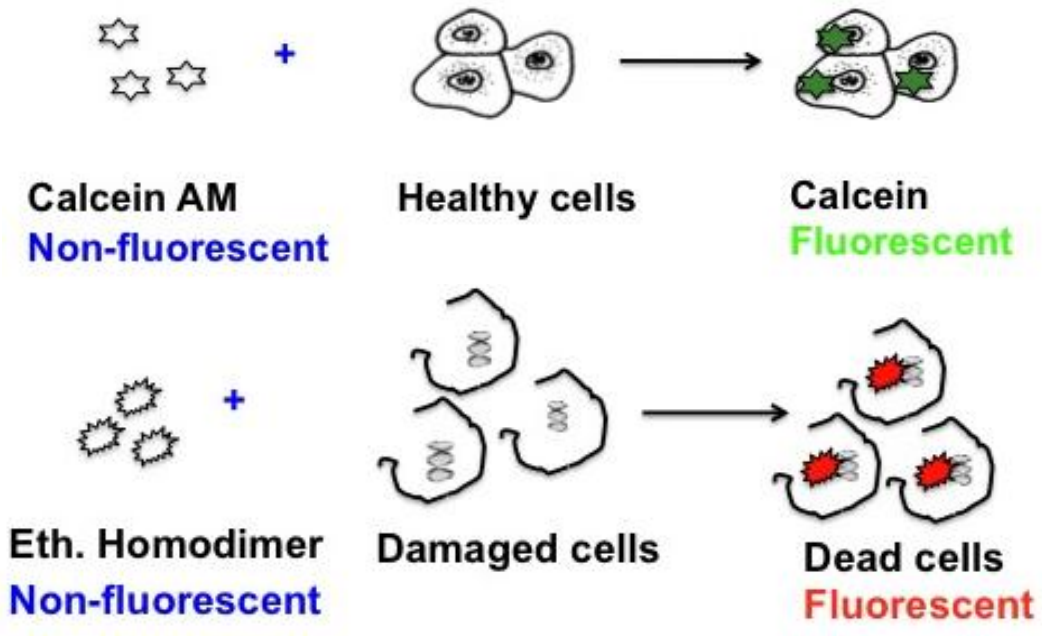

Figure 2.3. Schematic representation of a live/dead assay.

The live-dead assay[27] is a quick and useful technique for determining the viability of cells after exposure to a test sample. The assay is based on plasma membrane integrity

and cell esterase activity. A simplified mechanism is shown in Figure 2.3. Initially, the cells are stained with two fluorescent dyes, calcein AM (acetomethoxy ester) and ethidium homodimer-1 (Ethd-1). Calcein AM, which is non-fluorescent initially, enters both live and dead cells. However, this dye becomes fluorescent only after it undergoes hydrolysis to calcein. Such hydrolysis can be done by intracellular esterases, which are present and active only in live cells. Thus live cells show a bright green fluorescence due to calcein. On the other hand, the Ethd-1 dye can enter only cells with damaged membranes, whereupon this dye intercalates within DNA, and undergoes 40-fold enhancement in its fluorescence. Thus, dead cells show a red fluorescence due to Ethd-1. In short, green fluorescence is indicative of live cells while red fluorescecnce is indicative of dead cells. The fraction of viable cells can be quantified from the fluorescence images using the following formula:

$$\text{Fraction of viable cells (\%)} = \left(\frac{\text{Average number of live cells}}{\text{Average number of initial cells}} \right) \times 100 \qquad (2.5)$$

2.8. MTT ASSAY

Cell viability can also be assessed quantitatively using an assay based on the MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide).[28,29] In this method, viability of cells is measured based on their metabolic activity. Viable cells use nicotinamide adenine dinucleotide (NADH), a coenzyme that participates in redox reactions in the Krebs cycle to produce ATP.[30] The reduction of the tetrazolium MTT dye depends on the activity of this enzyme. Thus viable cells are capable of converting the yellow tetrazolium dye to insoluble purple formazan crystals. These crystals dissolve in

dimethyl sulfoxide (DMSO) to form a colored solution (see Figure 2.4). Thus the dye color remains yellow if there is no metabolic activity, whereas it turns purple if there is such activity. A spectrophotometer (plate reader) is used to read the absorbance at 540 nm corresponding to the purple dye and thus to quantify the cell viability.[31] The following formula is used to calculate the fraction of viable cells in this method:

$$\text{Fraction of viable cells (\%)} = \left(\frac{A_{sample} - A_{background}}{A_{control} - A_{background}} \right) \times 100 \qquad (2.6)$$

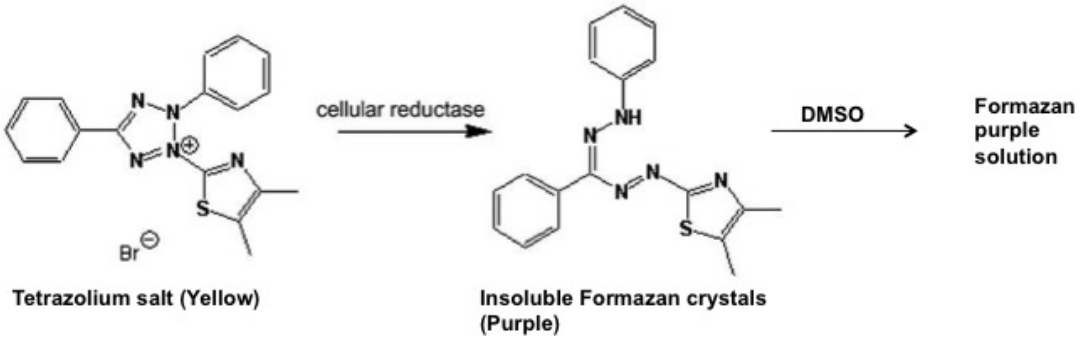

Figure 2.4. Schematic representation of the MTT assay.

Chapter 3: CYTOTOXICITY OF CATANIONIC VESICLES

3.1. INTRODUCTION

In this chapter, we conduct systematic studies on the toxicity of catanionic surfactant vesicles to biological cells. Catanionic vesicles were discovered by Kaler et al. about 25 years ago.[6] Since that time, there have been few investigations on the cytotoxicity of these vesicles, and none of these studies have systematically explored a range of vesicles. A brief description of previous studies is given below.

Risuleo et al.[9] studied catanionic vesicles of cetyl trimethylammonium bromide (CTAB)/sodium dodecyl sulfate (SDS) and studied their toxicity to mammalian cell lines, 3T6 and HeLa cells. The authors reported that CTAB/SDS vesicles are toxic at high dosage rates and cause cell death by apoptosis. A similar report was also published by Kuo et al.[32] who studied CTAB/SDS vesicles on murine macrophages and found similar results as the previous study. Blanzat et al.[33] investigated catanionic vesicles on human epithelioid bone marrow cells. They found that cationic surfactants having sugar moieties in their head group are relatively less toxic. A recent study by Vlachy and co-workers reported that catanionic vesicles from CTAB/sodium dodeconate and CTAB/SDS were very toxic to HeLa cells.[10] Additionally, Das et al.[8] studied vesicles formed by mixing an amino acid-based cationic and SDS on NIH3T3 fibroblast cells. Their investigation suggests that a minute modification in the head group of the cationic surfactant can reduce the toxicity of the vesicles. While these previous studies have investigated

cytotoxicity, the scope of their investigations has typically been restricted to one or two vesicle formulations. As such, these studies have not compared a broad range of catanionic vesicles, nor have comparisons been made to liposomes.

Our approach is as follows. We prepare a series of catanionic surfactant vesicles from commercially available cationic and anionic surfactants. The vesicles are characterized by dynamic light scattering (DLS) and small angle neutron scattering (SANS).[25] Cells from an epithelial breast cancer cell line called MCF-7 are cultured and these cells are contacted with the vesicles. Our main assay tool is the live/dead assay.[27] We thereby study how cytotoxicity is influenced by the surfactant tail length, the nature of the surfactant tail (saturated vs. unsaturated), the nature of the surfactant headgroup, and the net charge on the vesicles. As a comparison (control), we also examine the cytotoxicity of liposomes prepared from egg-phosphatidylcholine (egg-PC). A key result from our studies is the identification of certain catanionic vesicle formulations that are of relatively low toxicity. Hypotheses for the lower toxicity of these vesicles are discussed at the end of this Chapter.

3.2. EXPERIMENTAL SECTION

Surfactants and Lipids. The phospholipid egg-phosphatidylcholine (egg-PC) was purchased from Avanti Polar Lipids Inc. EHAC (ETHOQUAD® E/12-75) and OHAC (ETHOQUAD® O/12 PG) were received as gifts from Akzo Nobel. Both these products are supplied by the manufacturer as solutions of 75% surfactant in isopropyl alcohol (IPA). The IPA was removed by placing the samples in a lyophilizer at room temperature. The surfactants were then dried to a constant weight and were stored in a desiccator. Surfactants, cetyl trimethylammonium tosylate (CTAT), octyl trimethyl ammonium bromide (OTAB), cetyl pyridinium chloride (CPyCl), dioctyl sodium sulfosuccinate (AOT), stearyl dimethyl benzyl ammonium chloride (SDBAC) were purchased from Aldrich while sodium dodecyl benzene sulfonate (SDBS) was purchased from TCI. Surfactants were used as received without any further purification. Dulbecco's Phosphate Buffer Saline (DPBS) with calcium and magnesium (1X, 0.1 μm sterile filtered) was purchased from Thermo Scientific. Vesicle and liposome samples were prepared in DPBS in all experiments.

Preparation of Liposomes. Liposomes were prepared using the extrusion method to obtain uniform unilamellar structures. In this process, egg-PC lipids received from the manufacturer were dissolved in chloroform. Dried cakes of lipids were hydrated and moderately stirred. Lipids were then freeze-thawed five times in liquid nitrogen and were passed through two double-stacked polycarbonate membrane filters (100 nm pore size) using a Lipex pressurized extrusion system. The liposomes formed after this process were unilamellar structures with an average diameter around 100 nm.[4]

Preparation of Surfactant Vesicles. Surfactants vesicles were prepared by simple mixing.[6] Cationic and anionic surfactants were mixed in 70/30 and 30/70 weight ratios (typically at a total surfactant conccentration of 1 wt%) and were left to equilibrate overnight at room temperature. Vesicles were prepared in DPBS for all cell viability experiments to maintain osmotic balance and a biological pH ~ 7.4. Sample preparation for SANS experiments was done in D_2O instead of DPBS.

Dynamic Light Scattering (DLS). DLS was used to characterize the sizes of vesicles in solution.[23] Vesicle samples were studied at 25°C using a Photocor-FC light scattering instrument with a 5 mW laser light source at 633 nm with a scattering angle of 90°. A logarithmic correlator was used to measure the autocorrelation function, from which the diffusion coefficient was estimated. The hydrodynamic size of the vesicles was obtained from the diffusion coefficient via the Stokes-Einstein relationship (Chapter 2).[24]

Small-Angle Neutron Scattering (SANS). SANS measurements were made on the NG-7 (30 m) beamline at the National Institute of Standards and Technology (NIST) in Gaithersburg, MD. Three sample-detector distances were used to probe a wide range of wave vectors from 0.004 to 0.4 $Å^{-1}$. Samples were studies in 2 mm quartz cells at 25°C. The scattering spectra were studies and placed on an absolute scale using calibration standards provided by NIST. The data are analyzed as plots of the radially averaged intensity I vs. wave vector q. Analysis of SANS data by the Indirect Fourier Transform (IFT) method was performed using the commercial PCG software.[22,25]

Cell Culture. Cells from an epithelial breast cancer cell line (MCF-7) were purchased from ATCC. MCF-7 cell culture reagents: Dulbecco's Modified Eagle Medium (DMEM) containing high glucose, fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from ThermoScientific. Live/Dead® assay kit for mammalian cells was purchased from Invitrogen. Dimethyl Sulfoxide (DMSO) was purchased from Sigma Aldrich. All chemical reagents and materials were used as received without further purification. The growth media for MCF-7 cells was completed by adding 5 µL/mL of penicillin or streptomycin and 10% FBS to DMEM and this was stored at 4°C. The media were thawed at 37°C before use with cells. MCF-7 cells were cultured in T75 Corning flasks and incubated at 37°C with 5% CO_2. The cells were subcultured every 3-5 days by trypsinization with 0.25%/0.02% trypsin/EDTA. For cell viability experiments, cells were cultured until they were confluent.[34] They were then trypsinized and re-suspended in the T75 flasks. One third of the cells were plated back in a fresh T75 flask with cell media to maintain the cell line. The remaining two-thirds of the cells were centrifuged to form a pellet and re-suspended in about 1 mL of growth media. The cell density for each experiment was determined using a c-chip hemocytometer and then seeded in 96-well plates. These plates with seeded cells were incubated for 18-24 h before the assay. Vesicle samples were then added to the adhered cells and were incubated for 4 h before further assays were performed.

Live/Dead Assay. In this assay, first the Live/Dead reagents received from Invitrogen were removed from the freezer and thawed in a water bath at 37°C.[27,34] A stock solution of the reagents was prepared by mixing 5 µL of supplied 4 mM live reagent (Calcein-

AM) and 20 µL of supplied 2 mM dead reagent (Ethidium homodimer-1) in 10 mL of sterile, tissue culture-grade DPBS. Aqueous stock solutions were used within a day to avoid hydrolysis of Calcein-AM. In this assay, the cells were first seeded 18-24 h before adding the test samples, allowing the cells to completely adhere to the surface of the plate. Vesicle samples were then incubated with the adhered cells for 4 h on the following day. After 4 h, everything but 50 µL was aspirated from each well of the 96-well plate. 30 µL of Live/Dead stock solution was then added to each well and incubated for 15 min at room temperature. The live and dead cells were then imaged under an Olympus TH4-100 fluorescence microscope. Live cells were imaged at an excitation of 495 nm and the emitted light from the sample was detected by setting the band pass filter at 505-554 nm. Similarly, for imaging dead cells, excitation was done at 556 nm and the emitted light was detected by setting the band pass filter at 568-700 nm. Both live and dead cell images were taken within 15 min, to avoid false positive readings due to hydrolysis of Calcein-AM.

3.3. RESULTS AND DISCUSSION

AOT	Dioctyl Sodium sulfosuccinate
EHAC	Erucyl bis(hydroxyethyl) methyl ammonium chloride
CTAT	Cetyl trimethyl ammonium tosylate
CTAB	Cetyl trimethyl ammonium bromide
CTAC	Cetyl trimethyl ammonium chloride
CPyCl	Cetyl Pyridium chloride
SDBAC	Stearyl dimethyl benzyl ammonium chloride
SDBS	Sodium dodecyl benzene sulfonate
OTAB	Octyl trimethyl ammonium bromide
OHAC	Oleyl bis(hydroxyethyl) methyl ammonium chloride

Table 3.1. Abbreviations of the surfactants used in this study.

3.3.1. CHARACTERIZATION OF VESICLES:

We prepared and studied a variety of catanionic vesicle formulations.[6] The abbreviations of the surfactants are given in Table 3.1 while Table 3.2 lists the various formulations, indicating both the cationic and the anionic component in a given pair. Typically, vesicles were prepared at 1 wt% total surfactant with a cationic/anionic weight ratio of 30/70 (thus the vesicles were typically anionic). All vesicles were characterized for their hydrodynamic radius R_h using DLS. The mean R_h values for the different vesicles are presented in Table 3.2. Note that in preparing these vesicles, high shear (sonication or extrusion) was not employed and thus the sizes reflect the vesicles formed spontaneously by self-assembly.[4] Most of the vesicles had mean radii around 60 nm. In

the case of the liposomes, they were prepared at a lipid concentration of 1 wt% by extrusion through filters with a pore size of 100 nm. The mean radius (R_h) of the liposomes was typically around 50 nm, consistent with the above pore size.

Cationic surfactant	**Tail length**	**Anionic surfactant**	**Tail length**	**Mean radius by DLS (nm)**
CTAT	C_{16}-saturated	SDBS	C_{12}-saturated	62
CTAB	C_{16}-saturated	SDBS	C_{12}-saturated	60
CPyCl	C_{16}-saturated	SDBS	C_{12}-saturated	69
CTAC	C_{16}-saturated	SDBS	C_{12}-saturated	65
OTAB	C_{8}-saturated	SDBS	C_{12}-saturated	60
SDBAC	C_{18}-saturated	SDBS	C_{12}-saturated	60
OHAC	C_{18}-unsaturated	SDBS	C_{12}-saturated	68
EHAC	C_{22}-unsaturated	SDBS	C_{12}-saturated	68
CTAT	C_{16}-saturated	AOT	C_{16}- saturated	96
CTAB	C_{16}-saturated	AOT	C_{16}- saturated	95
CPyCl	C_{16}-saturated	AOT	C_{16}- saturated	97
CTAC	C_{16}-saturated	AOT	C_{16}- saturated	92
OTAB	C_{8}-saturated	AOT	C_{16}- saturated	90
SDBAC	C_{18}-saturated	AOT	C_{16}- saturated	100
OHAC	C_{18}-unsaturated	AOT	C_{16}- saturated	109
EHAC	C_{22}-unsaturated	AOT	C_{16}- saturated	103

Table 3.2. The various catanionic vesicle formulations prepared in this study and their mean radii, as measured by DLS. All formulations correspond to 1 wt% total surfactant at a weight ratio of 30/70 (cationic/anionic).

Vesicle and liposome samples were stored at room temperature. The R_h of catanionic vesicle samples was periodically checked over several months and was found to remain constant. This indicates that catanionic vesicles remained very stable over long time scales. On the other hand, the R_h of lipsomes increased appreciably over a period of few days, and the solution turned from bluish to cloudy. This suggests that unilamellar liposomes transform into multilamellar structures within a few days. In other words, our data confirm the much improved stability with time of catanionic vesicles over liposomes.

Catanionic vesicle formation in mixtures of surfactants has been confirmed in the literature through a variety of techniques including SANS and cryo-transmission electron microscopy (cryo-TEM). The most popular formulation of catanionic vesicles is the CTAT/SDBS system, which has been studied by many authors. In our study, we have investigated a few vesicle formulations that have not been described in the literature so far, including EHAC/SDBS and OHAC/SDBS. To confirm vesicle formation in these new systems, we used SANS. Figure 3.1 shows SANS spectra for the intensity I vs. wave vector q. Data are shown for CTAT/SDBS, EHAC/SDBS, and OHAC/SDBS samples, each of which was prepared in D_2O at a weight ratio of 30/70 and at a total surfactant concentration of 1 wt%. In all cases, the data show a q^{-2} decay in the intensity at low to moderate q, which is reflective of scattering from vesicle bilayers. Thus, the SANS data confirms vesicle formation in all the three systems.

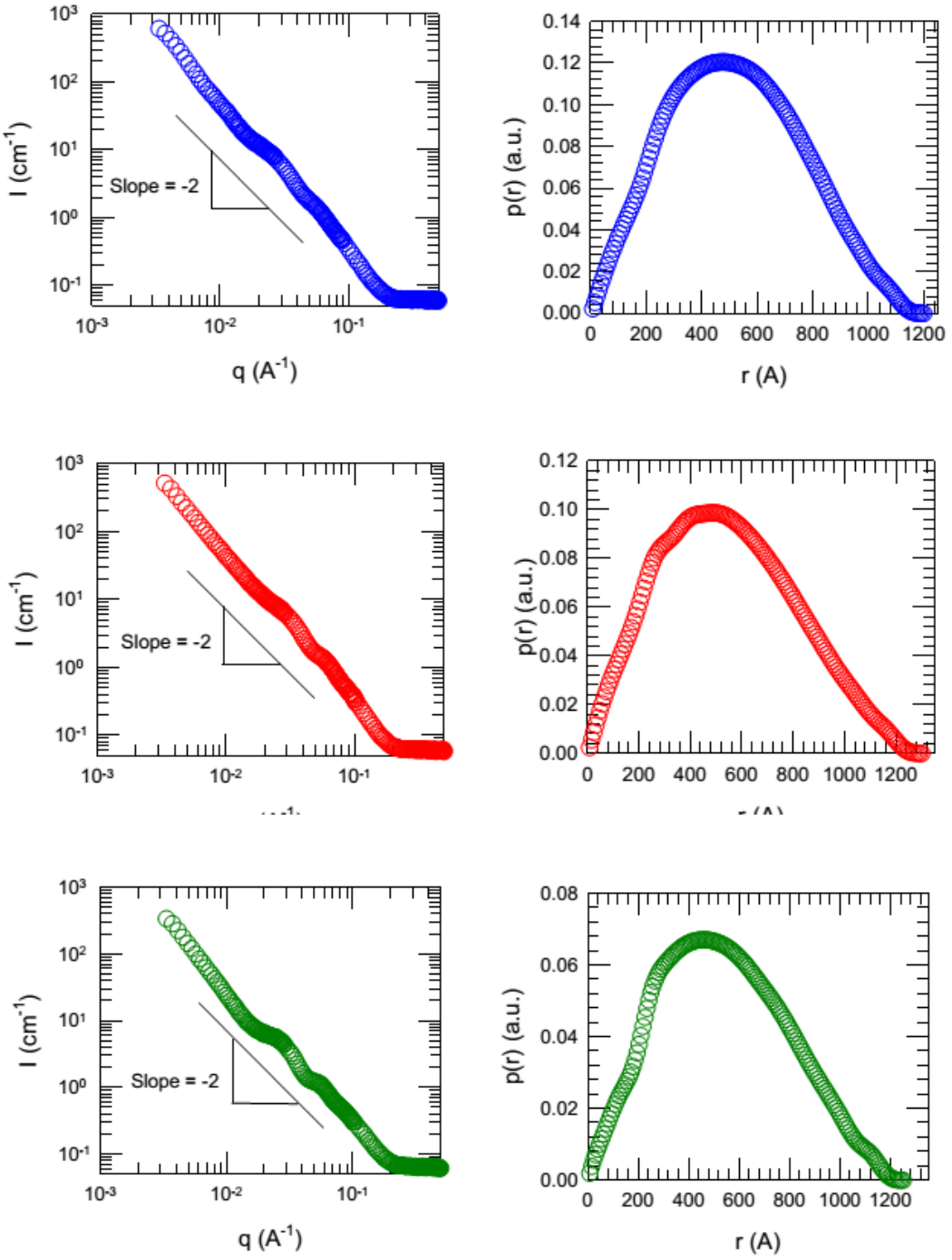

Figure 3.1. SANS data for three catanionic vesicle formulations. All samples contain 1 wt% total surfactant at a weight ratio of 30/70 (cationic/anionic). (Top) CTAT/SDBS (Middle) OHAC/SDBS (Bottom) EHAC/SDBS. In each case, the left panel shows $I(q)$ data while the right panel shows the pair distance distribution function $p(r)$ from IFT analysis.

SANS data were further analyzed by the IFT method method, which requires no *a priori* assumptions on the nature of the scatterers.[22,25] In the IFT method, a Fourier transformation is done on the scattering intensity $I(q)$ to give the pair distance distribution function $p(r)$ in real space (see eq 2.4 in Section 2.6). For each of the vesicle samples in Figure 3.1, the corresponding $p(r)$ plots are also included. The plots have the shape expected for vesicles, with a broad peak. The point of intersection of the $p(r)$ curve with the x-axis gives an estimate for the mean diameter of the vesicles. The corresponding values of vesicle radius are given in Table 3.3 and the values match well with the radii measured by DLS from Table 3.2. In addition, Table 3.3 also shows estimates for the average thickness t of the bilayers present in each vesicle. This can be obtained by analyzing I at high q using the Guinier approximation for the form factor in this range: [25]

$$q^2 \cdot I(q) \sim t^2 \exp(-q^2 R_t^2) \tag{3.1}$$

Here, R_t is the radius of gyration for the thickness. This equation shows that a semilog plot of $\ln(Iq^2)$ vs. q^2 will be a straight line with a slope equal to R_t^2 (such a plot is called a cross-sectional Guinier plot). The thickness t is related to R_t by the equation below: [25]

$$t = \frac{R_t}{\sqrt{12}} \tag{3.2}$$

Cationic surfactant	Anionic surfactant	Mean radius from SANS (nm)	Bilayer thickness (nm)
CTAT	SDBS	60	2.7
OHAC	SDBS	65	2.4
EHAC	SDBS	65	2.8

Table 3.3. Parameters obtained by modeling the SANS data in Figure 3.1 for the three catanionic vesicle formulations. All samples contain 1 wt% total surfactant at a weight ratio of 30/70 (cationic/anionic). The mean radii were obtained by IFT modeling. The bilayer thickness was obtained from Guinier plots using eq 3.1 and 3.2.

3.3.2. BIOCOMPATIBILITY OF VESICLES:

Liposomes and surfactant vesicles were incubated with MCF-7 cells in 96-well plates for 4 h. Typically, 3×10^6 cells/well were used and typically 50 µL of test solution was added to each well. First, we show results for a sample of 1 wt% egg-PC liposomes (Figure 3.2). The results are presented as images of green fluorescence on the left panel (indicating live cells) and red fluorescence on the right panel (indicating dead cells). We note substantial green fluorescence in the left panel and negligible red fluorescence on the right panel. This indicates that most cells are alive and very few cells are dead. We infer that liposomes are benign to mammalian cells, which is as expected.

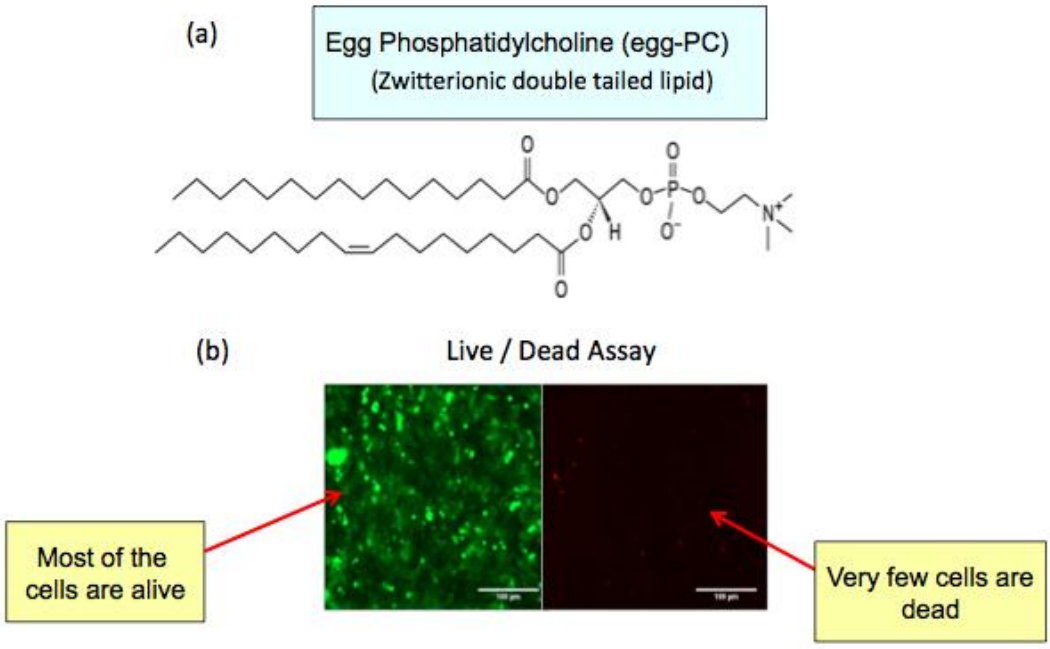

Figure 3.2. (a) Chemical structure of egg-PC lipid. (b) Live/dead assay of the corresponding liposomes with MCF-7 cells. The left panel is the extent of green fluorescence, which is indicative of live cells, while the right panel is the extent of red fluorescence, which is indicative of dead cells. The results show that most MCF-7 cells remain viable (live) when contacted with the liposomes.

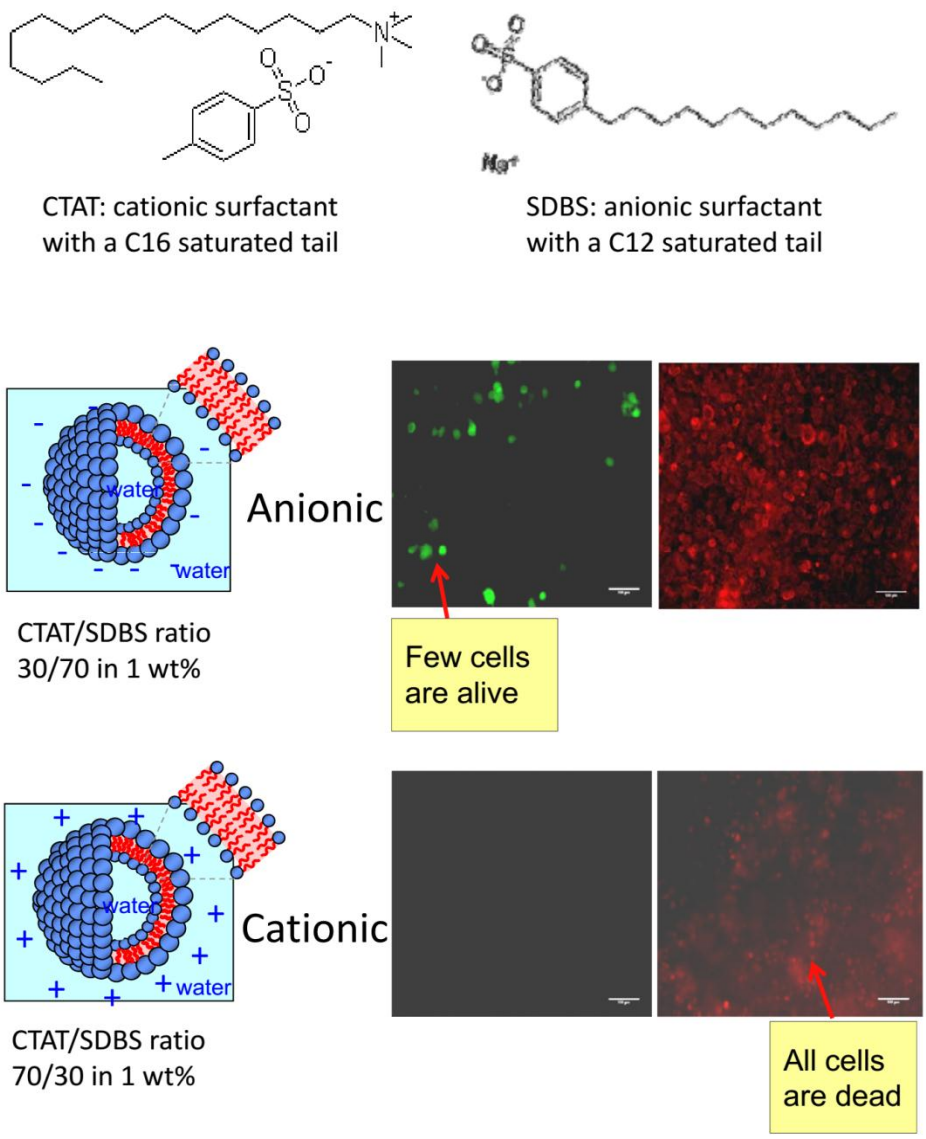

Figure 3.3. (Top) Chemical structures of the surfactants CTAT and SDBS. Live/dead assay results with MCF-7 cells for: (Middle): 30/70 CTAT/SDBS vesicles (anionic). and (Bottom) 70/30 CTAT/SDBS vesicles (cationic). The left panel is the live image (green indicates viable cells) and the right panel is the dead image (red indicates cell death).

Next we show results for CTAT/SDBS vesicles, which are the most studied type of catanionic vesicles. CTAT is a cationic surfactant with a saturated C_{16} tail and SDBS is an anionic surfactant with a saturated C_{12} tail. Cationic vesicles are formed at a weight ratio of 70/30 CTAT/SDBS (i.e., excess of CTAT), while anionic vesicles are formed at a

weight ratio of 30/70 CTAT/SDBS (i.e., excess of SDBS). Results from live/dead assays on the two above formulations are shown in Figure 3.3. We note that the cationic vesicles are extremely toxic (no viable cells are found). The anionic vesicles are also very toxic, as shown by the substantial red fluorescence. However, a few live cells do remain, as indicated by the green spots on the left panel. Thus, CTAT/SDBS vesicles are generally toxic; additionally, cationic vesicles are much more toxic than anionic vesicles. This result has been noted in the literature.[10] For the rest of our studies, we only worked with anionic vesicles, which were formed at a 30/70 ratio of cationic/anionic surfactant.

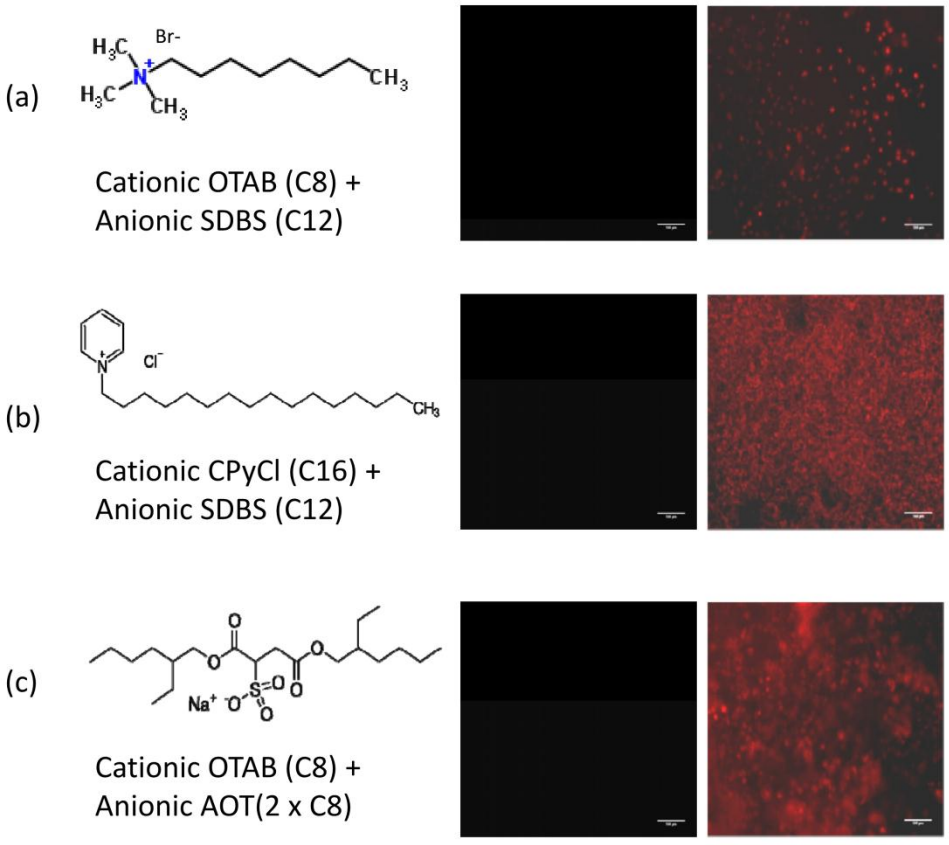

Figure 3.4. Live/dead assay results with MCF-7 cells for: (a): 30/70 OTAB/SDBS vesicles; (b) 30/70 CPyCl/SDBS vesicles; (c) 30/70 OTAB/AOT vesicles. The structures of OTAB, CPyCl, and AOT are also shown. In all cases, the left panel is the live image (green implies viable cells) and the right panel is the dead image (red implies cell death).

We then varied the tail length of the cationic surfactant in the catanionic vesicle, while maintaining the same anionic surfactant SDBS (which has a saturated C_{12} tail, as noted above). Specifically, we investigated OTAB, a cationic with a saturated C_8 tail, and CPyCl, a cationic with a saturated C_{16} tail. Results for catanionic vesicles at a 30/70 ratio of OTAB/SDBS and CPyCl/SDBS are shown in Figure 3.4a and 3.4b respectively (both have a net anionic character due to an excess of the anionic surfactant). In both cases, no viable cells were indicated in the live image whereas the dead image showed substantial red fluorescence. Thus, varying the tail length of the cationic surfactant had no significant effect on cytotoxicity. Note that the head group in CPyCl is pyridinium whereas it is trimethylammonium in OTAB as well as in the previously studied CTAT. However, both CTAT/SDBS vesicles (Figure 3.3) and CPyCl/SDBS vesicles (Figure 3.4b) were extremely toxic and thus the two headgroups are indistinguishable in the context of cytotoxicity.

We also experimented with a different anionic surfactant AOT, which has two saturated and branched C8 tails and a sulfosuccinate head group. Note that although AOT has two tails, it does not form vesicles on its own. However, mixtures of the cationic surfactant OTAB and AOT at a 30/70 ratio formed catanionic vesicles. To our knowledge, this is the first report of this type of catanionic vesicles. The results from a live/dead assay with these vesicles are shown in Figure 3.4c. Once again, we find that these vesicles are toxic to cells, with substantial red fluorescence in the dead image and no green fluorescence from viable cells in the live image.

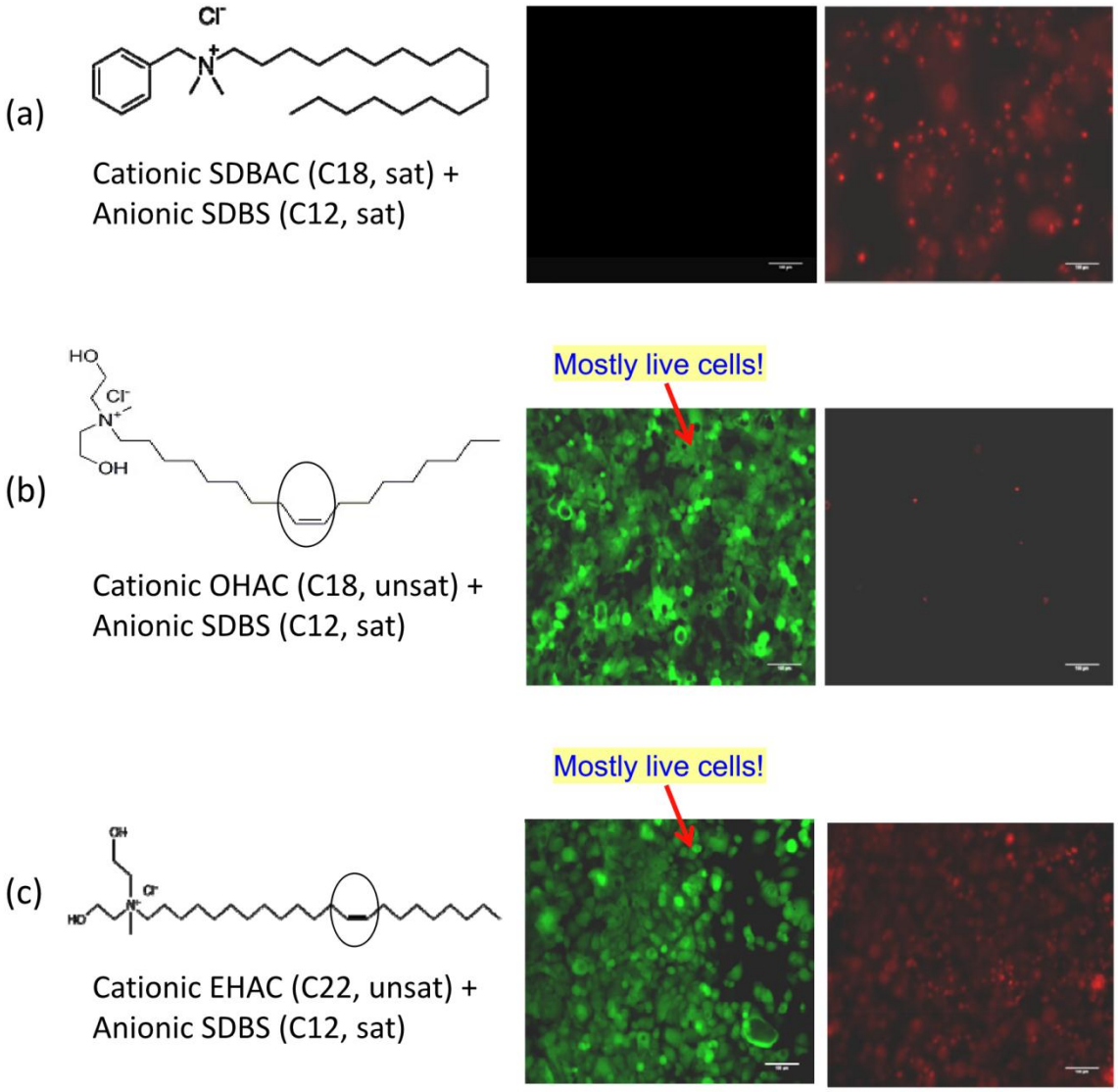

Figure 3.5. Live/dead assay results with MCF-7 cells for: (a): 30/70 SDBAC/SDBS vesicles; (b) 30/70 OHAC/SDBS vesicles; (c) 30/70 EHAC/SDBS vesicles. The structures of SDBAC, OHAC and EHAC are also shown. In all cases, the left panel is the live image (green implies viable cells) and the right panel is the dead image (red implies cell death).

Next we compared cationic surfactants with saturated and unsaturated tails. First, we worked with SDBAC, a cationic with a C_{18} saturated tail and a benzyl dimethylammonium headgroup. Live/dead assay results for vesicles of SDBAC/SDBS

(30/70) show that these were toxic to cells (Figure 3.5a), much like vesicles of CTAT/SDBS and CPyCl/SDBS. For comparison, we studied OHAC, a cationic with an oleyl (C_{18} unsaturated) tail and a bis(hydroxyethylmethyl)ammonium headgroup. Live/dead assay results for vesicles of OHAC/SDBS (30/70) are shown in Figure 3.5b. Surprisingly, the live image in this case shows substantial green fluorescence, indicating that the majority of cells are alive and viable. Moreover, the dead image shows only a small amount of red fluorescence. This shows that anionic OHAC/SDBS vesicles are relatively nontoxic to cells. To substantiate this result, we then studied EHAC, a cationic with an erucyl (C_{22} unsaturated) tail and the same bis(hydroxyethylmethyl)ammonium headgroup. Live/dead assay results for vesicles of EHAC/SDBS (30/70) are shown in Figure 3.5c. The results are mostly consistent with those for OHAC/SDBS in that we again see significant green fluorescence, indicating viable cells; however, there is more red fluorescence, indicative of some cell death. Nevertheless, Figure 3.5 shows that vesicles formulated from OHAC and EHAC are much less toxic than those made from other cationic surfactants.

Figure 3.8 provides a quantification of the images shown in Figures 3.2 to 3.5. The y-axis here is a measure of the fraction of viable MCF-7 cells, which was estimated using eq 2.5 in Chapter 2. For this, we counted the viable or live (green) cells that were visible in the live images from the above figures using the ‘cell counter’ on the ImageJ software. This was divided by the average number of initial cells, which was estimated by counting the cells in a control sample (contacted with cell growth media and DPBS). From the graph below, the fraction of viable cells was 90% in the case of liposomes,

about 80% in the case of OHAC/SDBS vesicles, and about 65% in the case of EHAC/SDBS vesicles. In comparison, no viable cells could be detected for many of the other catanionic vesicle formulations. Thus, the results show that among the catanionic vesicles, those of OHAC/SDBS and EHAC/SDBS are by far the most benign to mammalian cells.

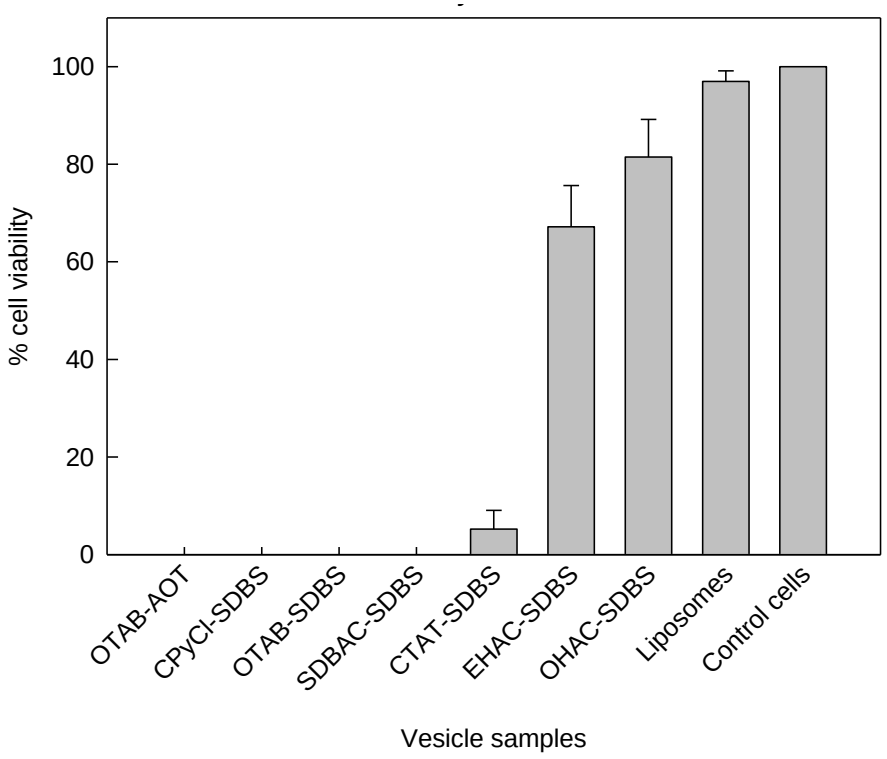

Figure 3.8. Fraction of viable MCF-7 cells that survive after 4 hrs exposure to different test samples. The fraction was determined by counting the cells in the fluorescent images corresponding to the live assay for each test sample, and this number was divided by the cells in the control sample. The control cells were exposed to cell growth media and DPBS.

Similarly, Figure 3.9 provides a quantification of the dead images shown in Figures 3.2 to 3.5. In the bar chart, the y-axis indicates the fraction of dead cells while x-axis shows the vesicle samples that were exposed for 4 hrs time period to MCF-7 cells. The same equation 2.5 was used to estimate the percentage dead cells in each sample. For

this, we counted the dead (red) cells that were visible in the dead images from the above figures using the 'cell counter' on the ImageJ software. This was divided by the average number of initial cells, which was estimated by counting the cells in a sample that indicated no live cells that is, 100% of the cells were dead. Most of the catanionic vesicles such as OTAB/AOT showed no live cells. From the graph below, the fraction of dead cells was 3% in the case of liposomes, about 18% in the case of OHAC/SDBS vesicles, about 33% in the case of EHAC/SDBS vesicles while 94.7% of cells were dead in case of CTAT/SDBS.

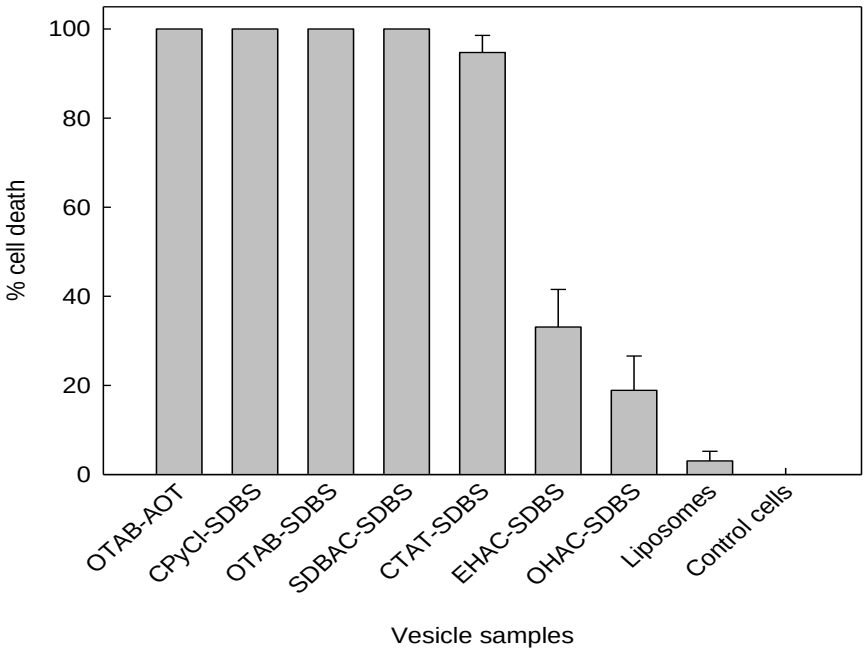

Figure 3.9. Fraction of dead MCF-7 cells that died after 4 hrs exposure to different test samples. The fraction was determined by counting the cells in the fluorescent images corresponding to the dead image for each test sample, and this number was divided by the cells in the control sample. The control cells were exposed to cell growth media and DPBS.

In most of the other catanionic formulations, we found that 100% of the cells

were dead, while no dead cells were found in the control sample (cells in contact with cell media and DPBS). Thus, the results show that among the catanionic vesicles, those of OHAC/SDBS and EHAC/SDBS are by far the most benign to mammalian cells.

In all the experiments described previously, we had exposed MCF-7 cells to vesicle samples only for 4 hrs time period. However, since we found that OHAC/SDBS and EHAC/SDBS were benign to mammalian cells after 4 hrs, we incubated them for 24 hrs with MCF-7 cells and checked their toxicity. Fig. 3.10 shows the toxicity result of CTAT/SDBS, OHAC/SDBS and EHAC/SDBS to MCF-7 cells after 24 hrs of incubation. It can be clearly seen from the images that CTAT/SDBS killed most of the cells, while EHAC/SDBS and OHAC/SDBS were found to be least toxic to mammalian cells. Therefore, we can conclude from the result that EHAC/SDBS and OHAC/SDBS are the least toxic vesicle when incubated with MCF-7 cells, even after 24 hrs.

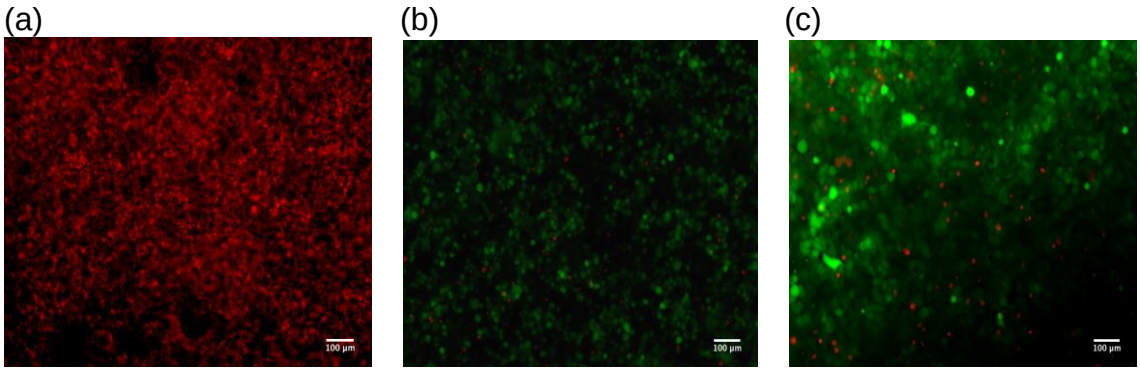

Figure 3.10. Live/dead assay results with MCF-7 cells for: (a): 30/70 CTAT/SDBS vesicles; (b) 30/70 EHAC/SDBS vesicles; (c) 30/70 OHAC/SDBS vesicles. In all cases, the image shows live/dead over-layed image after 24 hrs. In each image, green implies viable cells and red implies cell death.

3.3.3. DISCUSSION

The cell membrane (bilayer) in eukaryotic cells mainly consists of lipid molecules, cholesterol and membrane proteins.[3] The lipids are constantly moving along the lateral plane in the membrane. It is known that adjacent lipids switch positions at the rate of 10^7 times per second. Several functions of the membrane, such as membrane permeability and enzymatic protein activity, depend on the fluidity of the bilayer. This fluidity is largely aided by the fact that a large fraction of lipids have *cis*-unsaturations in one or both of their tails. A *cis*-unsaturation causes a kink at the location of the double bond and hence does not allow close packing of lipid molecules, as shown in Figure 3.9b. This in turn increases the fluidity of the membrane. If all the lipid tails were saturated, the membrane would be in a frozen and rigid state, as shown in Figure 3.9a.

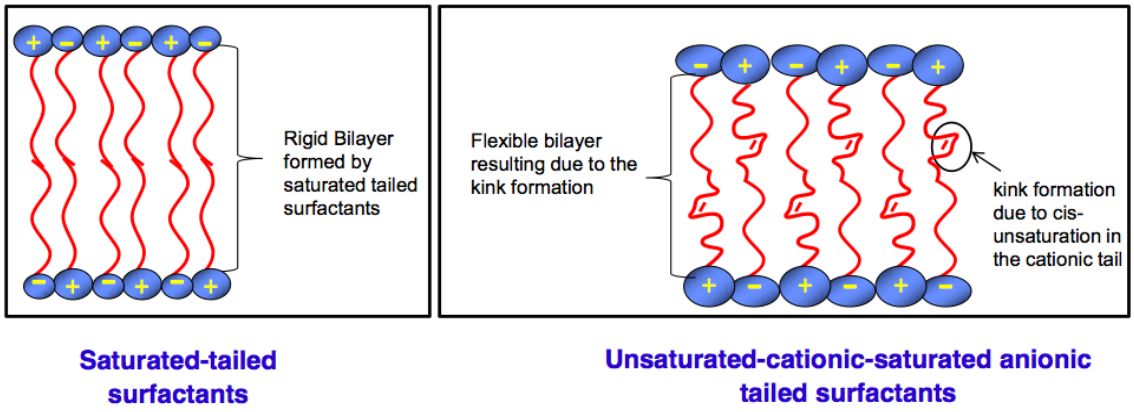

Figure 3.9. Comparison of bilayer dynamics in the case of catanionic vesicles where (a) both the cationic and anionic surfactant have saturated tails; and (b) the cationic surfactant has tails with a *cis* unsaturation (kink) whereas the anionic surfactant has a saturated tail. We hypothesize that the bilayer in (a) is quite rigid whereas the bilayer in (b) is more fluid due to looser packing of the tails.

We believe the lower toxicity of OHAC/SDBS and EHAC/SDBS is due to the

presence of *cis*-unsaturations in the tails of OHAC and EHAC. In forming catanionic vesicles, oppositely charged cationic and anionic surfactants (such as OHAC and SDBS) pair up and act like two-tailed lipids. We hypothesize that the bilayers in vesicles like OHAC/SDBS are more fluid than those in vesicles where both the surfactants have saturated tails. This is directly connected to the kinks in the OHAC and EHAC tails that arise due to the *cis* unsaturations. Furthermore, we speculate that when vesicles interact with cells, the fluidity of the vesicle bilayers is an important factor in the vesicle-cell interaction. In other words, fluid bilayers are postulated to cause less toxicity to cells. Currently, this hypothesis is speculative and further research is needed to substantiate it. Nevertheless, it could provide a starting point for future work.

3.4. CONCLUSIONS

In this chapter, we discussed that catanionic vesicles are generally toxic to cells. We compared several catanionic formulations by varying the tail lengths of cationic surfactant in the mixture, we could observe that shorter or longer saturated cationic tails did not contribute to the reduction of the overall toxicity of the catanionic vesicles. We then varied the cationic to anionic ratio in the mixture. It was clear for the catanionic vesicles studied in this thesis, that net negatively charged vesicles were less toxic than net positively charged vesicles. The anionic surfactant SDBS was less toxic than AOT. So substituting AOT instead of SDBS did not improve the toxicity of catanionic vesicles. The catanionic vesicles were then prepared with unsaturated cationic tailed surfactants. The catanionic vesicles with unsaturated tails seem to be less toxic as per the results shown by Live/Dead assay. Thus, catanionic vesicles with unsaturated cationic tails could be used potentially in biomedical applications.

Chapter 4: CONCLUSIONS & RECOMMENDATIONS

4.1. CONCLUSIONS

In this thesis, we have studied the biocompatibility of surfactant vesicles with mammalian cells by a relatively simple and visual screening tool, the live/dead assay. This assay allows us to scrutinize the toxicity of vesicles qualitatively. We found that most catanionic vesicles are toxic to mammalian cells. Vesicles that have an overall negative charge (anionic) are less toxic than positively charged vesicles (cationic). We have also found that certain cationic surfactants with a *cis*-unsaturation in their tail gave rise to catanionic vesicles that were relatively low in toxicity. We hypothesize that a moderate fraction of cis-unsaturated tails in the membrane is conducive to biocompatibility because it resembles the composition of lipid tails in natural cell membranes. Further studies are required to confirm the relatively benign nature of these vesicles. If confirmed, these vesicles could find use in several biomedical applications such as drug or biomolecule delivery, especially because of their advantages over liposomes, such as ease of preparation by simple mixing (no external input of energy), the commercial availability and low-cost of surfactants over lipids, and most importantly, their prolonged stability.

4.2. FUTURE DIRECTIONS

MTT Assay. In this thesis, we have analyzed biocompatibility of vesicles using the live/dead Assay. However, this assay is a rather qualitative one. It is recommended to

confirm these results using a more quantitative assay such as the MTT assay that was discussed in Chapter 2.

Anionic Surfactant with an Unsaturated Tail. In this thesis, we found that certain commercially available unsaturated cationic surfactants could reduce toxicity. Along similar lines, it would be worth trying an anionic surfactant with an unsaturated tail (e.g., an oleyl benzene sulfonate). Such surfactants are not commercially available, and might need to be synthesized.

Role of Head Groups In Toxicity. In addition to the unsaturated tail, another feature of OHAC and EHAC is that their head group is bis(hydroxyethylmethyl) ammonium rather than the trimethylammonium group found more commonly in cationic surfactants. It would be useful to compare the effects of the two headgroups while keeping the same unsaturated tail. An erucyl surfactant with a trimethylammonium head group is commercially available and this could be compared with EHAC in terms of its toxicity to mammalian cells.

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