LIPOSOMES FOR ENCAPSULATING DRUGS AND COMPLEXING DNA

by

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ABSTRACT

LIPOSOMES FOR ENCAPSULATING DRUGS AND COMPLEXING DNA

Surface-active substances, amphiphiles, show the ability of self-organization in solvents, forming self-assemblies with a large variety of morphologically different structures. One of these structures is liposomes, formed by phospholipid molecules. In this structure the hydrophobic chains of the lipids form a bilayer and the polar head groups of the lipids are oriented towards the outer and inner (core) regions. Liposomes can be prepared by thin film method. In order to control their size and lamellarity, traditional methods, extrusion and sonication, are used. Surface charge of the system can be adjusted by changing the amount of different types of phospholipids used. Liposomes are similar to cell membranes thus, they are very promising systems for drug delivery and gene delivery. These systems can easily fuse with the cell membranes, releasing their encapsulated materials into the cell, mimicking drug-delivery systems as well as acting as a vector carrying the genetic material in gene delivery. In this study, unilamellar vesicles have been prepared using synthetic phospholipids, zwitterionic DMPC and anionic DMPG mixtures. To increase the stability, cholesterol has been added to the phospholipid mixtures during the preparation of the liposomes. For the initial encapsulation studies, hydrophobic Vitamin-E and hydrophilic Cytochrome-C have been chosen. The same liposome system has also been used in encapsulating the Green Fluorescent Protein (GFP) encoding DNA fragments and transferring them to the target cells obtaining high transfection efficiency with low toxicity in the presence or absence of cationic mediator, divalent cation Ca^{2+}. The slightly anionic liposomes have been compared with the non-liposomal cationic transfecting agent with respect to transfection efficiency and toxicity in the vertebrate's retina cells, MIO-1 Müller cells.

ÖZET

İLAÇ KAPSÜLLENMESİNDE VE DNA TÜMLEŞİKLERİNDE LİPOZOM KULLANIMI

Amfifilik nitelikteki yüzey-aktif maddeler, çözücüler içerisinde kendi kendilerine kümeleşerek, morfolojik açıdan farklı yapılar oluşturabilirler. Bu yapılardan biri de lipozomlardır ve fosfolipidlerden oluşurlar. Bu yapıda, lipidlerin hidrofobik zincirleri çift katmanlı yapıyı oluştururken, polar baş grupları da lipozom dışındaki ve çekirdekteki sulu bölgeye doğru yönelirler. Lipozomlar ince film yöntemi ile hazırlanabilirler. Büyüklüklerinin ve lamellar yapılarının kontrolü için, geleneksel yöntemler olan ekstrüzyon ve sonikasyon kullanılmaktadır. Bu sistemlerin yüzey yükleri, lipidlerin miktarı değiştirilerek ayarlanabilir. Lipozomlar hücre membranlarına benzer yapılar olduklarından, ilaç kapsüllemeleri ve gen taşımacılığında umut vaadedici sistemlerdir. Hücre membranı ile kolaylıkla kaynaşabildiklerinden, kapsülledikleri malzemeyi hücre içine serbest bırakarak ilaç taşıma sistemlerini taklit edebilirler ve gen taşımacılığında, genetik materyali taşıyıcı vektör olarak kullanılabilirler. Bu çalışmada, zwitterion DMPC ve anyonik DMPG sentetik fosfolipidleri kullanılarak ünilameler lipozomlar hazırlanmıştır. Lipozomların hazırlanması sırasında, fosfolipid karışımına kolesterol eklenerek, sistemin durağanlığı arttırılmıştır. İlk kapsülleme çalışmaları için, hidrofobik Vitamin-E ve hidrofilik Sitokrom-C seçilmiştir. Aynı lipozom sistemi, DNA fragmanlarını kodlayan Yeşil Floresan Proteininin kapsüllenmesi ve sonrasında hedef hücrelere yüksek transfeksiyon verimlilik ve düşük toksiklik değerleriyle taşınmasını amaçlayan, katyonik iki değerlikli Ca^{2+} varlığında ve yokluğunda yapılan, gen taşımacılığı deneylerinde de kullanılmıştır. Az oranda anyonik karakter gösteren lipozomlar, katyonik transfeksiyon ajanları ile omurgalıların retina hücreleri olan MIO-M1 Müller hücrelerinde, transfeksiyon verimliliği ve toksiklik açısından karşılaştırılmıştır.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii
ABSTRACT v
ÖZET vi
LIST OF FIGURES xi
LIST OF TABLES xix
LIST OF SYMBOLS xxi
LIST OF ACRONYMS/ABBREVIATIONS xxii
1. GENERAL INTRODUCTION 1
2. SURFACTANTS AND THEIR AGGREGATIONS 7
2.1. Surfactants 7
2.2. Self-Assemblies of Surfactants and Molecular Packing Parameter 9
2.2.1. Micelles and Reverse Micelles 12
2.2.2. Vesicles 13
2.2.3. Types of Vesicles 15
2.2.3.1. Unilamellar Vesicles (ULV) 15
2.2.3.2. Multilamellar Vesicles (MLV). 15
2.2.3.3. Monolayer Vesicles 15
2.2.4. Techniques in Vesicle Characterization 16
2.2.4.1. Cryogenic Transmission Electron Microscopy (cryo-TEM)... 16
2.2.4.2. Dynamic Light Scattering (DLS) 17
2.2.5. Dynamics of Formation and Stability of Vesicles 19
3. PHOSPHOLIPIDS AND LIPOSOMES 23
3.1. General Information about Lipids 23
3.2. Triglycerides 24
3.3. Steroids 25
3.4. Phospholipids 28
3.5. Glycerophospholipids or Phosphoacyl-glycerols 30
3.5.1. Phosphatidylcholine (PC) 31
3.5.2. Phosphatidylglycerol (PG) 32
3.5.3. Phospholipids Technology and Applications 33
3.6. Liposomes 34

3.6.1. General Information about Liposomes 34
3.6.2. Forces between Lipid Molecules 35
3.6.3. Dynamics of Liposomes 36
3.6.4 Preparation of Liposomes 38
3.6.4.1. Mechanical Dispersion Method. 39
3.6.4.2. Solvent Dispersion Method. 40
3.6.4.3. Detergent Solubilization Method. 40
3.6.4.4. Processing of Lipids Hydrated by Physical Means. 42
3.6.5. Stability of Liposomes 44
3.5.6. Methods for Enhancement of Liposome Stability 46
3.5.7. Size Determination of Liposomes 48
3.5.8. Applications of Liposomes 48
3.5.9 Liposomal Drug Delivery Vehicles 49
3.5.10. Liposomes in Gene Therapy 52
3.5.11. Limitations of Liposome Technology 53
4. GENE DELIVERY 54
4.1. Methods for Delivering DNA to Target Tissues and Cells 54
4.1.1. Viral Vectors for DNA Delivery 54
4.1.2. Non-viral Methods for DNA Delivery 56
4.1.2.1. Naked DNA. 57
4.1.2.2. Cationic Lipids. 57
4.1.2.3. Cationic Polymers. 61
4.1.2.4. Anionic Liposomes. 62
4.1.2.5. Toxicity of Non-Viral Vectors. 63
5. AIM OF THE STUDY 65
6. MATERIALS AND METHODS 66
6.1. Materials 66
6.1.1. Phospholipids 66
6.1.2. Cholesterol 67
6.1.3. Encapsulated Materials 69
6.1.3.1. Cytochrome-C (cyt-C). 69
6.1.3.2. Vitamin-E (Vit-E, α-tocopherol). 70
6.1.4. Chloroform 71

6.1.5. Phosphate Buffer (PBS) Materials 71
6.1.6. Cholic Acid, Sodium Salt (Sodium Cholate) 71
6.1.7. High-performance Liquid Chromatography Analysis Materials 72
6.1.8. Transfecting Reagent 72
6.1.9. Dulbecco's Modified Eagle's Medium (DMEM) 72
6.1.10. Penicillin-Streptomycin 100X Solution 73
6.1.11. Fetal Bovine Serum (FBS) 73
6.1.12. Paraformaldehyde (PFA) Fixative 73
6.1.13. GFP 74
6.1.14. 4'6-Diamidine-2'-Phenylindole Dihydrochloride (DAPI) 74
6.1.15. Rotary Evaporator 75
6.1.16. Water Bath 76
6.1.17. Vortex Mixer Fusion Whirlimixer 76
6.1.18. Liposome Down-Sizing Methods 76
6.1.19. Centrifuge and Filtration 77
6.1.20. Ultraviolet Visible (UV/VIS) Spectrophotometer 77
6.1.21. HPLC 77
6.1.22. Particle Size and Zeta Analyzer, DLS 78
6.1.23. The Neubauer Improved Haemocytometer 78
6.1.24. Centrifuge 78
6.1.25. Fluorescence Microscopy 78
6.2. Methods 78
6.2.1. Preparation of Liposomes 78
6.2.2. HPLC Analysis 80
6.2.3. Removal of Excess Cyt-C 80
6.2.4. Breakdown of Liposomes 80
6.2.5. Parameterized Model Number 3 (PM3) 81
6.2.6. MIO-M1 Cell Culture Maintenance 82
6.2.7. Transfection Experiments 83
7. RESULTS AND DISCUSSION 84
7.1. Liposomes for Drug Encapsulation Studies 84
7.1.1. Comparison of Preparation Methods In Terms of Liposome Size 84
7.1.2. Liposome Stability and Zeta Potential Analysis 84

7.1.3. Turbidity Measurements of Liposomes 87
7.1.4. Breakdown of Liposomes 89
7.1.5. Encapsulation of Hydrophilic Cyt-C and Hydrophobic Vit-E in Liposomes 90
7.1.6. Calculations of Available Volume for Encapsulation 92
7.2. Liposomes for Gene Delivery Studies 93
7.2.1. Determining the Type of Liposome as a Gene Delivery Vector 93
7.2.2. Effect of Liposome Volume on Transfection Efficiency 94
7.2.3. Effect of Incubation Time on Transfection Efficiency 95
7.2.4. Effect of Liposome Size on Transfection Efficiency 97
7.2.5. Effect of Cholesterol on the Transfection Efficiency 97
7.2.6. Effect of Liposome Composition on the Transfection Efficiency 98
7.2.7. Effect of DNA Amount on the Transfection Efficiency 99
7.3. Complexing DNA-Liposome Systems with Divalent Cation Ca^{2+} 99
7.3.1. Determining the Complexation Sequence of Anionic Liposome, DNA and Divalent Cation, Ca^{2+} 99
7.3.2. Determination of the Optimum DNA (SIK2-GFP) Amount in the Presence of Ca^{2+} 100
7.3.3. Determination of the Optimum Liposome Volume in the Presence of Ca^{2+} 103
7.3.4. Effect of Liposome Composition on the Transfection Efficiency in the Presence of Ca^{2+} 104
7.3.5. The Liposome Effect on the Transfection Efficiency in the Presence of Ca^{2+} 108
7.3.6. The Effect of Changing the Cell Type on the Transfection Efficiency in the Presence of Ca^{2+} 109
8. CONCLUSION 110
9. FUTURE WORK SUGGESTIONS 113
APPENDIX A: DLS ANALYSIS RESULTS 114
APPENDIX B: FLUORECENCE MICROSCOPY ANALYSIS IMAGES 121
REFERENCES 136

LIST OF FIGURES

Figure 1.1. Schematic representation of a liposome. 1

Figure 2.1. Schematic representation of surfactant molecules and their arrangements on an air-water interface. 7

Figure 2.2. The general structures of non-ionic, anionic, cationic, and zwitterionic surfactants. 9

Figure 2.3. A schematic drawing of a surfactant molecule, showing the curvature packing parameter. 10

Figure 2.4. Packing parameters and their related lamellar and non-lamellar aggregates formed by the self-assembly of surfactants in water. 11

Figure 2.5. Schematic representation of micelle formation. 12

Figure 2.6. Schematic representation of reverse/inverse micelle. 13

Figure 2.7. Schematic view of a vesicle. 14

Figure 2.8. Schematic representation of some possible vesicular structures. 14

Figure 2.9. Schematic representation of a monolayer. 16

Figure 2.10. (a) Electron micrograph of a thin section of phospholipid vesicles, (b) diagram of vesicle in cross-section. 16

Figure 2.11. Cryo-TEM image of SOS/CTAB/H_2O. 17

Figure 3.1. Lipid classification. 23

Figure 3.2. Example of an unsaturated fat triglyceride containing glycerol (left part) and palmitic acid, oleic acid, alpha-linolenic acid (right part from top to bottom) with a chemical formula $C_{55}H_{98}O_6$. 24

Figure 3.3. Chemical structure of the most abundant steroid, cholesterol. 26

Figure 3.4. Position occupied by cholesterol in the membrane bilayer. 26

Figure 3.5. Different packing adaptations: (a) space requirement of head groups and tails is the same, (b) head group requires more space than tails, (c) tilted arrangement of tails allowing tighter packing. 28

Figure 3.6. Components of phospholipids. 28

Figure 3.7. Schematic representation of a phospholipid molecule. 29

Figure 3.8. Chemical structure of a glycerophospholipids molecule. 30

Figure 3.9. Chemical structure of a phosphotidic acid molecule. 31

Figure 3.10. Chemical structure of PC. 32

Figure 3.11. General structure of PG. 33

Figure 3.13. Schematic diagram of lipids incorporated into a bilayer membrane to form a liposome. 34

Figure 3.14. Differences in gel and liquid crystalline phases in phospholipid bilayers. .. 36

Figure 3.15. Typical differential scanning calorimetry (DSC) diagram of phospholipid bilayers undergoing gel-to-liquid crystal phase transition under controlled heating. 37

Figure 3.18. Classic DLVO model. 44

Figure 3.19. Electrical double layer. 45

Figure 3.20. Liposome-cell interactions: liposomal adsorption (upper left), uptake of liposomes by endocytosis (middle left), fusion (middle right), and the exchange of lipids (lower left). 50

Figure 3.21. Schematic representation of a drug loaded liposome. 51

Figure 4.1. Schematic representation of viral gene therapy. 55

Figure 6.1. Structure of DMPC. 66

Figure 6.2. Three-dimensional structure of DMPC. 66

Figure 6.3. Structure of DMPG. 67

Figure 6.4. Three-dimensional structure of DMPG. 67

Figure 6.5. Structure of cholesterol. 68

Figure 6.6. Three-dimensional structure of cholesterol. 68

Figure 6.7. Structure of cyt-C. 69

Figure 6.8. Three-dimensional structure of cytochrome c (green) with a heme molecule coordinating a central iron atom (orange). 70

Figure 6.9. Structure of Vit-E. 70

Figure 6.10. Structure of sodium cholate. 71

Figure 6.11. Structures of mobile phases of HPLC analysis, acetonitrile and methanol. . 72

Figure 6.12. Structure of PFA. 73

Figure 6.13. Schematic representation of GFP. 74

Figure 6.14. Structure of DAPI. 75

Figure 6.15. Three-dimensional structure of DAPI. 75

Figure 6.16. Avestin Lipofast mini-extruder. 76

Figure 6.17. Schematic representation of centrifugal filters. 77

Figure 7.1. An example to zeta potential measurement of DMPC: DMPC: cholesterol, 9:1:3, liposome. 86

Figure 7.2. UV/VIS graph for the turbidity measurements of six different liposomes prepared with approximately same lipid weights and the same method. 88

Figure 7.3. Breakdown of DMPC/DMPG liposomes upon addition of sodium cholate the bile salt at 350 nm. 89

Figure 7.4. Absorbance plot of cyt-C in liposome filtrates and of release cyt-C. 90

Figure 7.5. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated cholesterol containing liposome and SIK2-GFP vector (transfection efficiency approximately 5%). 94

Figure 7.6. Fluorescence microscopy images for incubation time 60 minutes (17% transfection efficiency). 96

Figure 7.7. Fluorescence microscopy images for 0.5 µg SIK2-GFP with 2 µL X-tremeGENE HP (positive experiment) (18% transfection efficiency). 101

Figure 7.8. The transfection efficiency percentage results while changing the SIK2-GFP amount and the incubation time at each Ca^{2+} concentration. 103

Figure 7.9. Transfection efficiency percentage results for 9:1:3 molar ratio DMPC:DMPG:cholesterol. 105

Figure 7.10. Transfection efficiency percentage results for 7:3:3 molar ratio DMPC:DMPG:cholesterol. 106

Figure 7.11. Transfection efficiency percentage results for 5:5:3 molar ratio DMPC:DMPG:cholesterol. 106

Figure 7.12. Transfection efficiency percentage results for 3:7:3 molar ratio DMPC:DMPG:cholesterol. 107

Figure 7.13. Transfection efficiency percentage results for 1:9:3 molar ratio DMPC:DMPG:cholesterol. 107

Figure A.1. DLS analysis of an empty, extruded liposome. 115

Figure A.2. DLS analysis of a sonicated liposome. 116

Figure A.3. DLS analysis of cholesterol containing liposome immediately after sonication. 117

Figure A.4. DLS analysis of cholesterol containing liposome after 1 week of sonication. 118

Figure A.5. DLS analysis of cholesterol containing liposome after 2 weeks of sonication. 119

Figure A.6. DLS analysis of cholesterol containing liposome after 3 weeks of sonication. 120

Figure B.1. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%). 121

Figure B.2. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%). 121

Figure B.3. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded cholesterol containing liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%). 122

Figure B.4. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded cholesterol containing liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%). 122

Figure B.5. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated cholesterol containing liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 5%). 123

Figure B.6. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%). 123

Figure B.7. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%). 124

Figure B.8. Fluorescence microscopy images for 20 minutes incubation time (5% transfection efficiency). 124

Figure B.9. Fluorescence microscopy images for 30 minutes incubation time (12% transfection efficiency). 124

Figure B.10. Fluorescence microscopy images for 2 μg SIK2-GFP with 20 mM Ca^{2+} at 90 minutes incubation time (15% transfection efficiency). 125

Figure B.11. Fluorescence microscopy images for 4 μg SIK2-GFP with 20 mM Ca^{2+} at 90 minutes incubation time (15% transfection efficiency). 125

Figure B.12. Fluorescence microscopy images for 2 μg SIK2-GFP with 30 mM Ca^{2+} at 90 minutes incubation time (30% transfection efficiency). 126

Figure B.13. Fluorescence microscopy images for 4 μg SIK2-GFP with 30 mM Ca^{2+} at 90 minutes incubation time (33% transfection efficiency). 126

Figure B.14. Fluorescence microscopy images for 2 μg SIK2-GFP with 40 mM Ca^{2+} at 90 minutes incubation time (26% transfection efficiency). 127

Figure B.15. Fluorescence microscopy images for 4 μg SIK2-GFP with 40 mM Ca^{2+} at 90 minutes incubation time (25% transfection efficiency). 127

Figure B.16. Fluorescence microscopy images for 2 μg SIK2-GFP with 10 mM Ca^{2+} at 180 minutes incubation time (2% transfection efficiency). 128

Figure B.17. Fluorescence microscopy images for 4 μg SIK2-GFP with 10 mM Ca^{2+} at 180 minutes incubation time (2% transfection efficiency). 128

Figure B.18. Fluorescence microscopy images for 2 μg SIK2-GFP with 20 mM Ca^{2+} at 180 minutes incubation time (26% transfection efficiency). 129

Figure B.19. Fluorescence microscopy images for 4 μg SIK2-GFP with 20 mM Ca^{2+} at 180 minutes incubation time (33% transfection efficiency). 129

Figure B.20. Fluorescence microscopy images for 2 μg SIK2-GFP with 30 mM Ca^{2+} at 180 minutes incubation time (14% transfection efficiency). 130

Figure B.21. Fluorescence microscopy images for 4 μg SIK2-GFP with 30 mM Ca^{2+} at 180 minutes incubation time (15% transfection efficiency). 130

Figure B.22. Fluorescence microscopy images for 0.5 μg SIK2-GFP with 2 μL X-tremeGENE HP (positive control) (20% transfection efficiency). 131

Figure B.23. Fluorescence microscopy image of experiment 1 in Table 7.7. 131

Figure B.24. Fluorescence microscopy image of experiment 2 in Table 7.7 132

Figure B.25. Fluorescence microscopy image of experiment 3 in Table 7.7. 132

Figure B.26. Fluorescence microscopy image of experiment 4 in Table 7.7. 132

Figure B.27. Fluorescence microscopy image of experiment 5 in Table 7.7. 133

Figure B.28. Fluorescence microscopy image for 4 μg SIK2-GFP, 2 μL liposome with 20 mM Ca^{2+} at 90 minutes incubation time (1.8% transfection efficiency). 133

Figure B.29. Fluorescence microscopy image for 4 μg SIK2-GFP, 5 μL liposome with 20 mM Ca^{2+} at 90 minutes incubation time (7.5% transfection efficiency). 133

Figure B.30. Fluorescence microscopy image for 4 μg SIK2-GFP, 8 μL liposome with 20 mM Ca^{2+} at 90 minutes incubation time (11% transfection efficiency). 134

Figure B.31. Fluorescence microscopy image for 4 μg SIK2-GFP, 2 μL liposome with 30 mM Ca^{2+} at 90 minutes incubation time (22.7% transfection efficiency). ... 134

Figure B.32. Fluorescence microscopy image for 4 μg SIK2-GFP, 5 μL liposome with 30 mM Ca^{2+} at 90 minutes incubation time (17.4% transfection efficiency). ... 134

Figure B.33. Fluorescence microscopy image for 4 μg SIK2-GFP, 8 μL liposome with 30 mM Ca^{2+} at 90 minutes incubation time (30% transfection efficiency). 135

Figure B.34. Fluorescence microscopy images for 0.5 µg SIK2-GFP with 2 µL X-tremeGENE HP (positive control) (11% transfection efficiency). 135

LIST OF TABLES

Table 2.1. Stability behavior of the colloidal dispersion according to the zeta potential values. 19

Table 3.1. Preparation methods for liposomes. 41

Table 3.2. Applications of liposomes in the sciences. 49

Table 4.1. Comparison of viral- and non-viral gene delivery systems. 58

Table 7.1. DLS analysis results to determine the stability of cholesterol containing sonicated liposome. 85

Table 7.2. Liposome size measurements (× = not present, √ = present). 85

Table 7.3. Turbidity comparison for liposome solutions. 88

Table 7.4. Encapsulated amounts of cyt-C and Vit-E in 10 mL solutions. 91

Table 7.5. Summary of transfection efficiency results for the different types of liposomes with a volume of 5 μL. 93

Table 7.6. Summary of transfection efficiency results for the effect of incubation time (0.5 μg SIK2-GFP and 5 μL liposome). 96

Table 7.7. Transfection efficiency results with 2 and 4 μg of DNA at 90 minutes incubation time for different Ca^{2+} concentrations. 101

Table 7.8. Transfection efficiency results with 2 and 4 μg of SIK2-GFP at 180 minutes incubation time for different Ca^{2+} concentrations. 102

Table 7.9. Control Experiments. 103

Table 7.10. Transfection efficiency percentages when changing the liposome volume in the presence Ca^{2+} with 4 μg SIK2-GFP and 90 minutes incubation time. ... 104

Table 7.11. Turbidity and size value of two liposomes, Liposome 1 and Liposome 2. .. 108

Table 7.12. Transfection efficiency percentages of the two liposomes on different batches of MIO-M1 cells. 108

LIST OF SYMBOLS

A_{lipid}	Calculated head group area of the lipid
A_{TOT}	Total surface area of a spherical liposome
d_H	Hydrodynamic diameter
l	Length of the phospholipid including the head group
M_{lipid}	Number of moles of lipids per liposome
N_{AV}	Avogadro's number
N_{lipid}	The number of lipids on the exterior of the liposome
r	Hydrodynamic radius of the liposome
r_{inner}	Internal radius of the liposome
T_c	Phase transition temperature
t_s	Sonication time
V_{TOT}	Total volume of the liposome
V_{inner}	Internal volume of the liposome
ΔG	Gibbs Free Energy
λ_{max}	Maximum absorption wavelength
Λ	Characteristic decay length
ζ	Zeta potential

LIST OF ACRONYMS/ABBREVIATIONS

AAV	Adeno-associated Virus
CAC	Critical aggregation concentration
CFTR	Cystic fibrosis transmembrane conductance regulator
CMC	Critical micelle concentration
Cryo-TEM	Cryogenic transmission electron microscopy
CTAB	Cetyltrimethylammonium bromide
Cyt-C	Cytochrome-C protein
DAPI	4’6-Diamidine-2’-Phenylindole Dihydrochloride
DC-chol	3β-[N-(Dimethylaminoethane) carbamoyl] cholesterol
DDAB	Didecyldimethylammonium bromide
DEAE	Diethylamino ether
DHPC	Dihexanoylphosphatidylcholine
DLPG	Sodium dilauroylphosphatidylglycerol
DLS	Dynamic light scattering
DLVO	Derjaguin-Landau-Verwey-Overbeek theory
DMEM	Dulbecco’s modified eagle’s medium
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DMPG	1,2-dimyristoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)]
DNA	Deoxyribonucleic Acid
DOPE	Dioleoylphosphatidylethanolamine

DOPG	1,2-dioleoyl-sn-glycero-3-[phosphor-rac-(1-glycerol)] (sodium salt)
DPPC	Dipalmitoyl phosphatidylcholine
DSC	Differential Scanning Calorimetry
EMEM	Eagle's minimal essential medium
FBS	Fetal Bovine Serum
GFP	Green fluorescent reporter protein
GUV	Giant Unilamellar Vesicle
HEK	Human embryonic kidney cells
HPLC	High-performance liquid chromatography
IUV	Intermediate-size unilamellar vesicle
KH_2PO_4	Potassium Dihydrogen Phosphate
LBST	Laser beam gene transduction
LD	Liquid-disordered
LO	Liquid-ordered
LUV	Large Unilamellar Vesicle
LUVETs	Large Unilamellar Liposomes by Extrusion Techniques
MLV	Multilamellar Vesicle
MPS	Mononuclear phagocytic system
NaOH	Sodium Hydroxide
NDDO	Neglect of Differential Diatomic Overlap
PALA	N-(Phosphonoacetyl)-L-aspartate
PBS	Phosphate buffer solution

PC	Phosphatidylcholines
PEG	Polyethylene glycol
PEI	Polyethyleneimine
PFA	Paraformaldehyde
PG	Phosphatidyl glycerol
PI	Phosphatidylinositol
PINC	Protective interactive non-condensing polymers
PLL	Poly-L-Lysine
PM3	Parameterized model number 3
QLS	Quasielastic light scattering
RES	Reticular endothelial system
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SIK2	A serine/threonine kinase protein
siRNA	Small interference ribonucleic acid
SO	Solid-ordered
SOS	Sodium Octyl Sulfate
SUV	Small Unilamellar Vesicle
TAG	Triacylglyceride
TCP	Tocopherol
TI	Therapeutic index
UV	Ultraviolet Light

UV/VIS	Ultraviolet Visible Spectrophotometer
Vit-E	Vitamin E (α-tocopherol)

1. GENERAL INTRODUCTION

Lipid vesicles (Figure 1.1) were first prepared in the 1960s by Bangham and co-workers [1]. Since most vesicles are composed of concentric lipid bilayers, they are generally referred to as multilamellar vesicles (MLVs). Because the multilamellar character is often a limitation, efforts to produce more homogenous preparations have resulted in the formation of small unilamellar vesicles (SUVs). Unilamellar vesicles, also known as liposomes, are single bilayered systems usually with dimensions in the range of 25 nm to 100 nm. However, they are only kinetically stable, kinetically trapped systems [2-6], and their formation requires energy input. Large unilamellar vesicles (LUVs), with diameters ranging from 100 nm to 1 μm, were first prepared in the mid-1970s [7]. The physical properties of lipid vesicles depend on how, and under what conditions, they are prepared. They may fuse with each other (or with membranes [8]) or they can even aggregate [9, 10]. Vesicles can also be formed spontaneously by mixing solutions of anionic and cationic surfactants, with either one in excess. The curvature of the mixed surfactant bilayers controls size and shape of the vesicles. Extensive work has been carried out on preparation of vesicles using synthetic surfactants, such as SOS/CTAB and similar mixtures [11-13].

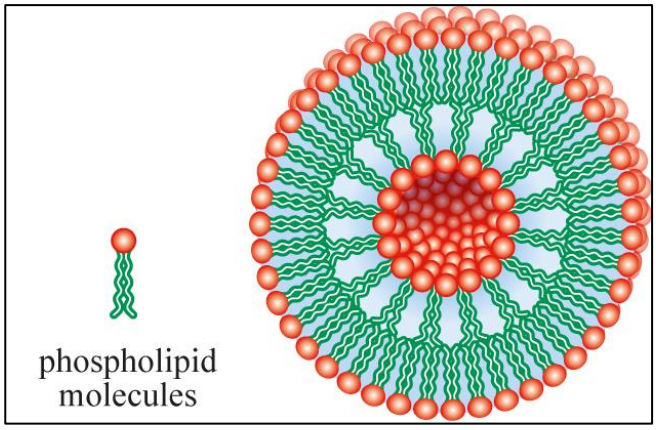


Figure 1.1. Schematic representation of a liposome [14].

Since liposomes are metastable structures, their preparations require “energetic” methods such as sonication and extrusion from dilute lamellar dispersions. Sonication has been known to break down large MLVs into SUVs in the 5-50 nm size range [15]. Maulucci *et al*. [16] have studied particle size distribution in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) vesicle solution undergoing sonication, and have concluded that the lipid film, upon sonication, forms large and floppy unilamellar vesicles which then progressively reduce their size down to saturation values when increasing sonication time, t_s. At shorter t_s, the total energy transferred by sonication is not sufficient to reduce multilamellar vesicles and/or giant aggregates to their optimal thermodynamical size. If t_s is too long, the vesicles are damaged by the generated free radicals. The most common preparation method of unilamellar vesicles is extrusion through polycarbonate filters, which results in reasonably stable dispersions [17]. Unilamellar dispersions exhibiting a narrow size distribution, with an average diameter close to the pore size of the filters used to extrude them, have been obtained by MacDonald *et al*. [18]. Hunter and Frisken have reported that as the extrusion pressure is increased, the flow rate of vesicles through the pores increases and the size of the extruded vesicles decrease [19].

Although liposomes are being used in drug formulations, their entrapment efficacies and fundamentals have not been extensively studied. Lapinski *et al.* compared liposomes prepared by sonication and extrusion, and have shown that the method of preparation “does not affect the molecular scale organization of these systems” [20]. Oliger *et al*., however, have investigated the preparation and properties of vesicles formed from phospholipid analogues of N-(Phosphonoacetyl)-L-aspartate (PALA) both by sonication, and extrusion, and they concluded that sonicated and extruded dispersions of di*Cn*-PALA have quite different properties, with the latter ones presenting more of the characteristics usually associated with closer vesicular structures [21].

Fan *et al.* investigated the effect of different preparation methods on the physicochemical properties of liposomes, containing salidroside [22]. They have shown that the salidroside liposomes prepared by the melting method had higher encapsulating efficiency and physicochemical stability. Salidroside loading efficiency highly influenced the encapsulating efficiency, and preparation method had a great effect on the leakage of

salidroside from liposomes. Similarly, leakage properties of phosphatidylserine vesicles have been found to differ depending on the preparation method [23].

Today, liposomes are a very useful model, reagent and tool in various scientific disciplines, including mathematics and theoretical physics (topology of two-dimensional surfaces floating in a three-dimensional continuum), biophysics (properties of cell membranes and channels), chemistry (catalysis, energy conversion, photosynthesis), colloid science (stability, thermodynamics of finite systems), biochemistry (function of membrane proteins) and biology (excretion, cell function, trafficking and signaling, gene delivery and function) [24]. On the applied side, several products rely on their colloidal, chemical, microencapsulating and surface properties; these products range from drug-dosage forms (antifungals, anticancer agents, vaccines) to diagnostics and various uses in the food industry. However, it seems that drug-delivery applications are now the most widely investigated area of their practical applications [24].

Because of the amphiphilic character of phospholipids and their organization in closed structures, liposomes can encapsulate (i) hydrophobic molecules in the bilayer membrane, (ii) hydrophilic compounds in the aqueous internal cavity, and (iii) amphiphilic substances. These special properties have generated numerous applications of lipid liposomes as models for biological membranes. They can be readily used in membrane-mimetic chemistry, as well as in mimicking drug-delivery systems *in vivo* applications, in which the encapsulated “drugs” can be carried to the target, and released upon breaking down of the vesicles into micelles. The use of drug loading liposomes *in vivo* requires strict control of liposome characteristics, such as particle size, stability, encapsulation rates, and the determination of the leakage kinetics of the entrapped substances [25].

The formation of stable, monodisperse, and unilamellar vesicles based on biocompatible components is prerequisite for drug delivery applications. Amongst the surfactant systems identified for such studies are included phospholipid-based surfactants such as lecithin-bile salt mixtures and mixtures of sodium dimyristoylphosphatidylglycerol (DMPG) and sodium dilauroylphosphatidylglycerol (DLPG); solutions of dimyristoylphosphatidylcholine (DMPC) or

dihexanoylphosphatidylcholine (DHPC) after doping with calcium ions; solutions of sodium bis-(2-ethylhexyl) phosphate or oleyldimethylamine oxide after pH changes; certain cationic/anionic mixtures above their respective gel-to-liquid crystalline phase transition temperature; mixtures between tetradecyltrimethylammonium hydroxide and certain fatty acids; diblock copolymers and copolymers mixed with surfactants; dimeric surfactants containing sugar-based head groups; and single-chain bolaamphiphiles (amphiphilic molecules that have hydrophilic groups at both ends of a sufficiently long hydrophobic hydrocarbon chain) mixed with single-tail surfactants [26].

As a promising strategy for the treatment of many inherited and acquired diseases, gene therapy which is defined as the genetic modification of cells for therapeutic benefit of an entire organism has attracted many researchers during the past several decades [27, 28]. The aim for gene therapy is to deliver healthy exogenous drugs such as plasmid DNA, RNA and single-strand oligonucleotides to replace a missing gene that otherwise have a normal makeup so as to cure genetic diseases, such as cystic fibrosis, malignant melanoma, and gaucher's disease [29, 30]. Based on these factors, reliable and efficient vectors delivering exogenous genes into target cells are urgently awaited. Successful gene therapy necessitates targeted transfection of plasmids containing transgenes of interest into cells. Owing to the limited ability of naked DNA to enter cells and the susceptibility of DNA to enzymatic degradation, DNA transfection has largely been achieved using viral and non-viral delivery vectors. Such delivery systems can protect the DNA from degradation and have been able to successfully transfer it both *in vitro* as well as *in vivo*. Viral delivery systems, involving the development of replication-deficient virus, used on a broad scale include retrovirus, adenovirus, adeno-associated virus, herpes simplex virus and lentivirus. In general, viral protocols provide higher transfection efficiency, although viral vectors might provoke carcinogenesis and repeated administration induces an immune response that abolishes the transgenic expression. Shortcomings of non-viral methods include low efficiency and transient expression, but transient expression can be used to treat acute or chronic diseases by repeated administration.

Non-viral vectors have become more popular due to their advantages over viral vectors in recent years. They are safe and cheap, easy to manufacture in larger scale, and they can also deliver large pieces of DNA [31]. The materials used in non-viral methods

have less potential toxicity concerns and common non-viral delivery methods for gene therapy include direct injection of purified plasmid, gene gun and liposome. Among these techniques, liposomal vehicles are a well-known drug delivery system with the advantages of simplicity in preparation, safety in human use and versatility in compositions with a diverse range of morphologies, size, and release characteristics. Liposomes can be used to deliver different types of genetic material, such as plasmid containing a therapeutic gene and oligonucleotides for antisense and ribozyme, and they are the most commonly used non-viral vector in current clinical trials.

Since their introduction as potential delivery systems in 1987 [32], DNA-cationic lipid complexes, also known as cationic lipoplexes, have been used in numerous research protocols for DNA delivery in a range of cell types and are currently being investigated in several gene therapy clinical trials [33]. However, although cationic liposomes are relatively efficient in delivering DNA into cells, they can be inactivated in the presence of serum, and there have been reports of instability upon storage [34]. In addition, cytotoxicity of cationic liposomes remains a concern irrespective of the preferred route of DNA transfer, and such effects have been shown to occur both *in vitro* and *in vivo*. Under *in vitro* and *ex vivo* conditions, massive cell death caused by toxic delivery systems can significantly reduce their overall potential for DNA delivery. It is evident that there is a need for efficient and well-tolerated delivery systems to exploit the benefits of gene medicine.

A few studies [35, 36] that use anionic liposomal DNA delivery vectors as an alternative to cationic liposomes have been reported in recent years. Anionic liposomes have been previously used as models to stimulate the cell surface to study non-receptor-mediated transport of DNA across cells [37]. For DNA delivery purposes, however, anionic liposomal formulations have predominantly been used to transfer oligonucleotides into hippocampal neurons [36] and bacterial cells [35]. However, these investigations are limited owing to (i) inefficient entrapment of DNA molecules within anionic liposomes [36], and (ii) lack of toxicity data [35]. Lack of further development of these systems may be attributed to the inefficient association between anionic lipids and DNA molecules resulting from the repulsive electrostatic interaction between these negatively charged species.

In this study, the DMPC and DMPG containing liposome formulations were optimized in terms of their preparation methods as well as their encapsulation efficiency of hydrophilic and hydrophobic drugs, with or without cholesterol. This synthetic phospholipid containing liposome system could be prepared either with extrusion or sonication, since the same stability upon duration was obtained for both methods. On the other hand, extrusion method was determined to be advantageous compared to sonication in terms of the encapsulation of hydrophilic material. For the case of hydrophobic material encapsulation, both methods are applicable. The same liposome systems were also tested, with or without the divalent cation, Ca^{2+} for encapsulating DNA vectors in terms of transfection efficiency and toxicity. For the gene delivery part, the sonicated cholesterol containing liposomes managed to transfer the transgenes into MIO-M1 cells more efficiently. The transfection efficiency was further increased by the complexation manner of the divalent cation Ca^{2+}, with the negatively charged DNA and the cell membrane. The transfection efficiency results were compared with the cationic non-liposomal transfecting reagent, X-tremeGENE HP (Roche). Despite the negative moiety coming from DNA, liposome, and cell membrane, promising transfection efficiency values were obtained with anionic liposome formulation.

2. SURFACTANTS AND THEIR AGGREGATIONS

2.1. Surfactants

The word *amphiphile*, first introduced by Hartley [38], comes from the combination of two Greek words, the prefix *amphis* meaning both and the root *philia* expressing affinity, love and friendship. An amphiphilic substance exhibits double affinity, which can be defined from the physicochemical point of view as a polar-nonpolar duality. A typical amphiphilic molecule consists of two parts: a polar group and an essential nonpolar group. The polar group contents heteroatoms such as oxygen, sulfur, nitrogen, and phosphorus, included in functional groups. Examples are alcohol, thiol, ether, ester, acid, sulfate, sulfonate, phosphate, amine, and amide. The nonpolar part is in general a hydrocarbon chain of the alkyl or alkylbenzene type, sometimes with halogen atoms and even a few nonionized oxygen atoms.

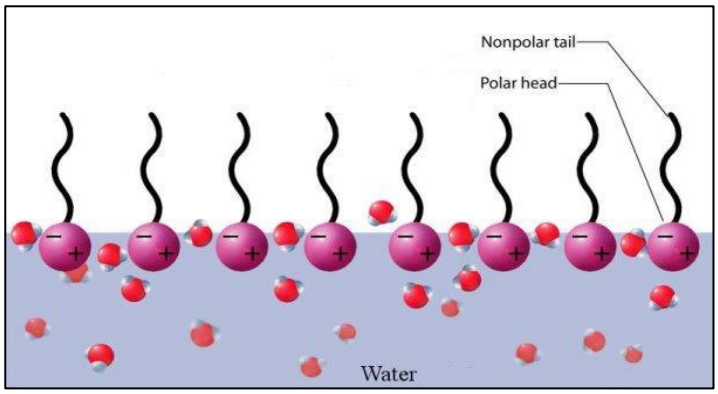


Figure 2.1. Schematic representation of surfactant molecules and their arrangements on an air-water interface [39].

Many biological compounds, most notably phospholipids making up the living cell walls, as well as many drugs are amphiphilic. The term surface-active-agent (surfactant) is used somewhat interchangeably with amphiphile, although surfactant is usually implied to mean a man-made substance, as opposed to a biological lipid. The hydrophilic part in surfactant molecule is referred as the head group, which is covalently bonded to one hydrophobic moiety, generally a single or double alkyl chain also called the *tail*, as shown

in the Figure 2.1. They are sparingly soluble in both nonpolar organic solvents and polar media such as water.

Surfactants are classified according to the ionic charge on their head group. Ionic surfactants may be of two types: anionics, having a negatively charged head group, and cationics having a positively charged head group. A typical example is sodium dodecyl sulfate (SDS). In cationic surfactants, the head group is often based around a quaternary ammonium ion- hence the common name of "quat". A typical example of a cationic surfactant is the "quat" didecyldimethylammonium bromide (often abbreviated as DDAB). Cationics based on amines are also common. If the head group bears both a negative and a positive charge, it is called zwitterionic. The positive charge is usually associated with an ammonium group and the negative charge is often a carboxylate. Amphoteric surfactants can be cationic, zwitterionic or anionic, depending on the pH. They pass from cationic to anionic form on increasing pH, with the zwitterionic form only being stable in a certain pH range. Surfactant molecules with no charge on the head group are termed as nonionic surfactants [40]. The hydrophilic part of nonionic surfactants is usually a polyether chain, and more rarely a polyhydroxyl chain. The tail part, alkyl chain, of the surfactant molecule may be a hydrocarbon, a perfluorocarbon, or mixed hydrocarbon/perfluorocarbon group it may also be partly aromatic [41, 42]. The general structures of surfactants are given in Figure 2.2.

The dual character of surfactants is responsible for their unique behavior in the presence of water. In a system made up of water and a small amount of surfactant solubilized in the water, alkyl chain/water contacts are energetically unfavorable when compared with the water/water contacts [43-45]. In order to avoid such contacts, some surfactants tend to locate at the air/water interface, with the head group in water and the tail in the air side of the interface, thereby forming an adsorbed layer of surfactant. The adsorption of surfactants at the air/solution interface reduces the surface tension of water. Similarly, surfactants adsorb on the solid walls of the flask containing the solution. The structure of an adsorbed layer of surfactant on a solid surface depends on the nature of the surface and the surfactant [46, 47].

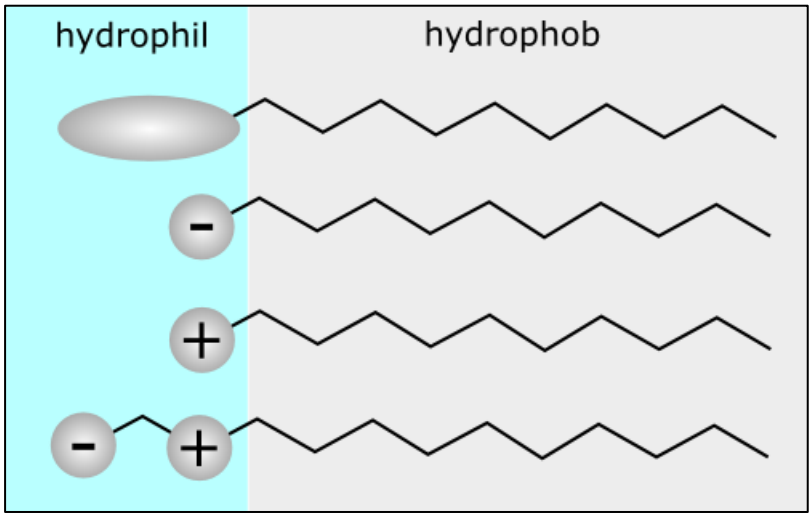


Figure 2.2. The general structures of non-ionic, anionic, cationic, and zwitterionic surfactants [48].

2.2. Self-Assemblies of Surfactants and Molecular Packing Parameter

Surfactants show the ability of self-organization in the solvents, forming self-assemblies with a large variety of morphologically different structures. The driving force in the association of surfactant molecules is the tendency of the hydrophobic part to minimize the contact with water. This is the so-called hydrophobic effect.

The self-assembly of surfactants in solutions has been widely investigated both experimentally and theoretically. Numerous practical applications take advantage of the resulting multimolecular aggregates. The structure of these aggregates influences the properties of surfactant solutions, such as: their solubilization capacity for hydrophobic substances, their viscous and viscoelastic properties; the performance of surfactants in various applications. To select molecules that would yield desired structures it is necessary to know how the molecular structure of the surfactant controls the shape and size of the resulting aggregate.

Tanford [49] and Israelachvili, Mitchell and Ninham [50] pioneered two of the most important ideas to answer the above question. Tanford proposed the concept of two opposite forces; (i) the attractive force caused by hydrophobic attraction of the

hydrocarbon chain units in the hydrocarbon-water interface, and (ii) the repulsive force between the neighboring head groups due to the hydrophilic, steric and ionic repulsion, to formulate a quantitative expression for the standard free energy change on aggregation. Using this free energy expression and the geometrical relations for aggregates, he was able to explain why surfactant molecules aggregate in aqueous solutions, why they grow, and why they do not keep growing but are finite in size, and why they assume a given shape.

Israelachvili, Mitchell, and Ninham proposed the molecular packing parameter concept which has been widely cited in the chemistry, physics, and biology literature. This concept is based on the geometrical considerations, makes it possible to assume the shape and size of the aggregates at equilibrium. Shapes of the spontaneously formed aggregates can be predicted with considerable certainty using three nominal geometric parameters of the surfactant molecule; optimal polar head surface area (*a*), tail volume (*V*), and critical chain length of the hydrocarbon tail (*l*) (Figure 2.3).

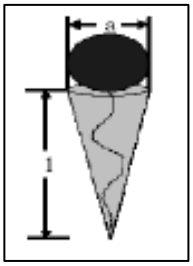


Figure 2.3. A schematic drawing of a surfactant molecule, showing the curvature packing parameter [50].

The value of the molecular packing parameter, *P*, is calculated as the ratio of the tail volume (*V*) to the product of the optimal polar head surface area (*a*) and critical chain length of the hydrocarbon tail(*l*), according to the following equation

$$P = {}^{V}\!/_{al} \tag{2.1}$$

Surfactants characterized by values of $P < 1/3$ give rise to spherical/spheroidal micelles. Surfactants with $1/3 < P < 1/2$ tend to form elongated micelles that are named as

cylindrical micelles and the ones with $1/2 < P < 1$ form disklike micelles, named as flexible bilayers or vesicles, can be viewed as the precursors of surfactant bilayers and of lamellar phases. Last, surfactants with $P > 1$ form reverse micelles. Figure 2.4 represents schematically the shapes of the surfactant molecule and of the surfactant self-assembly for different values of the packing parameter, *P*.

Although the molecular packing parameter, *P* can only be considered to be crude and approximate model for predicting self-assembly, it provides valuable insight into how changes of molecular structure affect the shape of the formed aggregate.

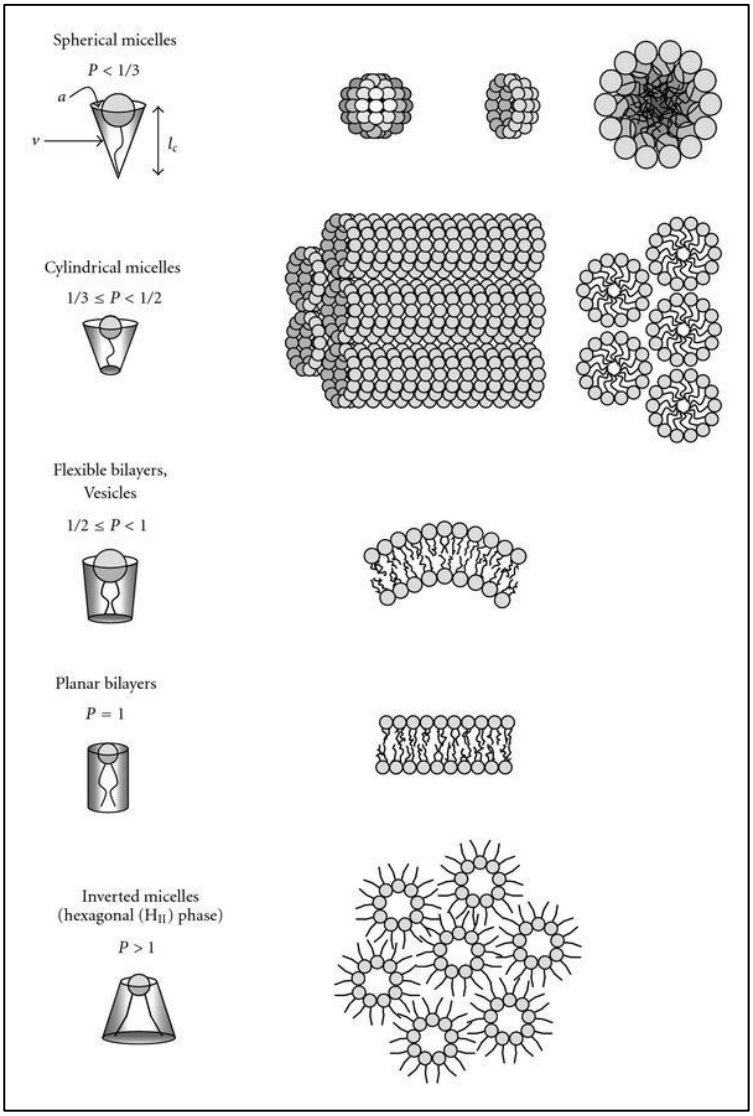


Figure 2.4. Packing parameters and their related lamellar and non-lamellar aggregates formed by the self-assembly of surfactants in water [51].

2.2.1. Micelles and Reverse Micelles

Micelles are the simplest form of self-assembled structures. As the surfactant concentration in the solution is increased, the amount of surfactant adsorbed at the air/solution interface increases up to the point where the interface becomes saturated by adsorbed surfactant. The free energy of the system increases with the concentration, due to the increasing number of unfavorable alkyl chain/water contacts. At the *critical micelle concentration* (CMC), the surfactant starts to self-associate into *micelles*, driven by the hydrophobic interaction of the alkyl chains, in order to prevent a further increase of free energy [38, 41, 42]. A typical micelle (Figure 2.5) in an aqueous solution forms an aggregate with the hydrophilic head groups in contact with the surrounding solvent segregating the alkyl chain groups in the oily core, center of the micelle [38, 52]. The formation of micelles is spontaneous and reversible [38, 42, 53]. Micelles are thermodynamically stable aggregates that are in chemical equilibrium with the single surfactant molecules.

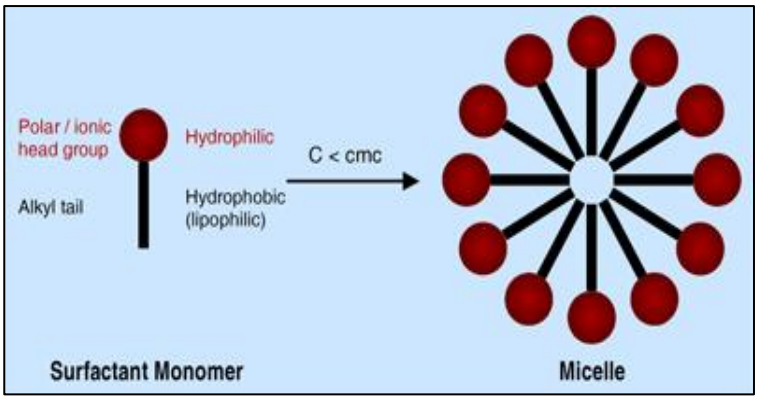


Figure 2.5. Schematic representation of micelle formation [54].

Reverse/inverse micelles are water-in-oil droplets stabilized by a surfactant. Some surfactants, like sodium diethylhexylsulfosuccinate also known as AOT, are soluble in oil [40]. In a non-polar solvent, the exposure of the hydrophilic head groups of these kinds of surfactant molecules to the surrounding solvent is energetically unfavorable. In this case the hydrophilic groups are segregated in the micelle core and the hydrophobic tails extend away the polar center which result in the formation of reverse/inverse micelles (Figure

2.6). These kinds of micelles are also thermodynamically stable which are formed spontaneously [55].

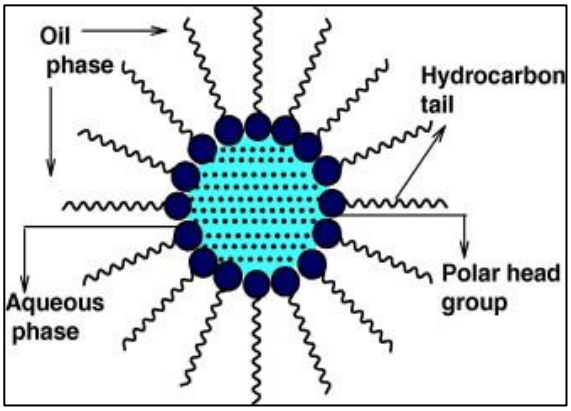


Figure 2.6. Schematic representation of reverse/inverse micelle [56].

2.2.2. Vesicles

Vesicles are enclosed structures, composed of flexible bilayers of amphiphilic molecules, dispersed into and filled with liquid solvent forming an inner compartment separated from the external phase (Figure 2.7). Due to the flexibility of the membrane, vesicles can deform in time, assuming, in general, a spherical shape in average but can form other shapes. These aggregates can vary quite a lot in size (from few nanometers to several micrometers) and shape, being unilamellar (a single bilayer), or multilamellar (shell of bilayers) (Figure 2.8). In contrast to micelles, vesicles may not be thermodynamically stable [57]. These objects, however, are often reported in the literature with different names, depending on the type of the starting amphiphile involved. Specifically, vesicle is the common name used when the amphiphile is a short chain surfactant [58]. In the case of phospholipids and block co-polymers, these aggregates are called, respectively, liposomes and polymerosomes [1, 59]. Even though differently named, in different branches of science and for different applications, these systems can be considered very similar from a physical standpoint regardless of the nature and type of the amphiphile.

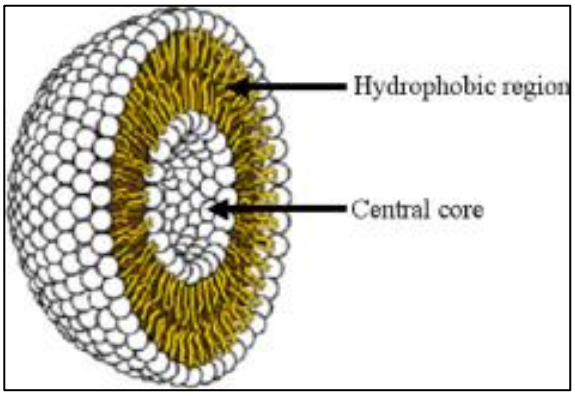


Figure 2.7. Schematic view of a vesicle [60].

The first experimental observation of vesicles was made in the context of biological research and it dates back to the middle of 1960s [1]. Since then, vesicular structures have been identified in systems studied in several different branches of science and they have been successfully prepared to formulate many commercial products and applied to many industrial processes. Today, surfactant vesicles are present in a number of industrial formulations, like laundry detergents and personal care products [61].

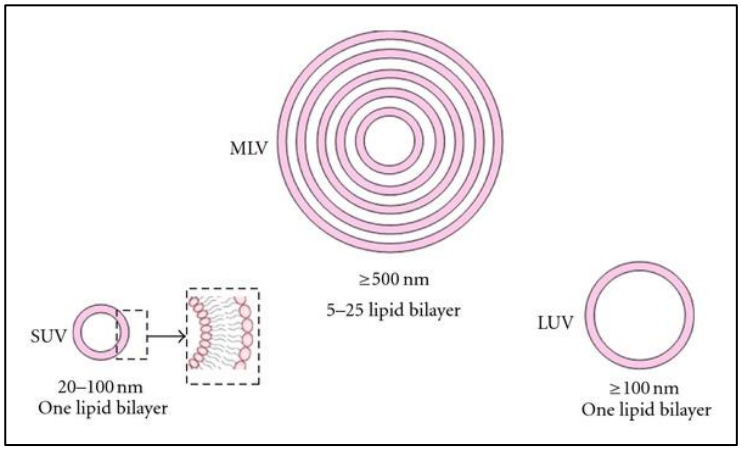


Figure 2.8. Schematic representation of some possible vesicular structures [62].

Phospholipids based liposomes have been identified in many biological systems, where they have the function of transporting nutrients into the cells [63]. Polymerosomes, produced from block co-polymers, are engineered to produce vesicles of the desired properties, to be used, for example, as active carrier vehicles (e.g. in pharmaceutical or

cosmetics industry), or encapsulation media, or even as micro-confined reactors to drive reactions selectively [62, 64].

2.2.3. Types of Vesicles

Vesicles are classified according to their size as follows:

2.2.3.1. Unilamellar Vesicles (ULV). These are vesicles with one single bilayer around the inner aqueous compartment. Here, one can distinguish between SUVs with diameter between 20 and 100 nm, LUVs with diameter in the order of magnitude of 1000 nm, intermediate-sized unilamellar vesicles, IUV with diameters of the order of magnitude of 100 nm, and giant unilamellar vesicles, GUV with diameter between 1 and 50 μm [65].

2.2.3.2. Multilamellar Vesicles (MLV). These are vesicles with more than one bilayer where one has additional bilayers added to the surface analogous to the many layers of skin on an onion [65]. Aggregate structures such as spherical and rod-like micelles, vesicles, lamellar phases, and precipitates have all been observed depending on the concentrations of the two surfactants in the solutions. As a general tendency, ULVs are more likely to be observed for dilute systems, while MLVs are frequently found in more concentrated surfactant systems.

2.2.3.3. Monolayer Vesicles. These kinds of vesicles with hydrophilic head groups at both ends of the hydrophobic chain result in a membrane consisting of a single molecular layer.

Typically, for bilayer-forming amphiphilic systems, there exists a structural progression, from ULV to MLV and from MLV to planar bilayers, with the increasing concentration.

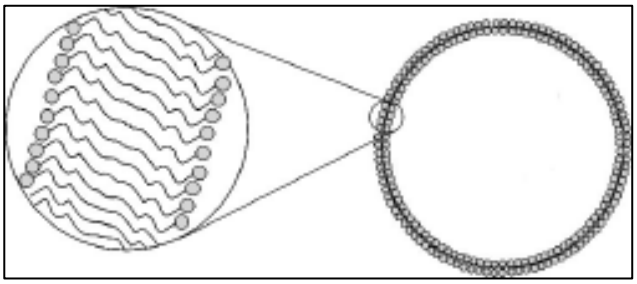


Figure 2.9. Schematic representation of a monolayer [63].

2.2.4. Techniques in Vesicle Characterization

Amphiphilic molecules undergo self-assembly and form various types of self-aggregated microstructures in different shape and size, and morphology. In general, multiple complementary methods are required to characterize the features of these self-assembled structures. Electroscopic methods are well suited for visualization of those amphiphilic structures in aqueous environment [66].

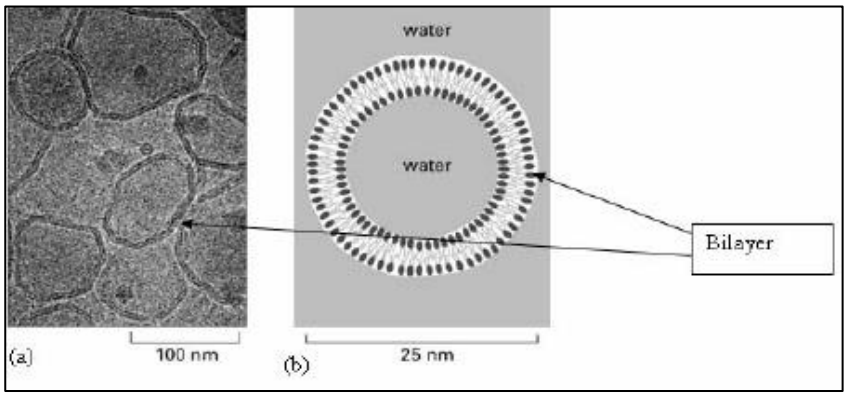


Figure 2.10. (a) Electron micrograph of a thin section of phospholipid vesicles, (b) diagram of vesicle in cross-section [66].

2.2.4.1. Cryogenic Transmission Electron Microscopy (cryo-TEM). Cryo-TEM is a method allowing the visualization of many fragile structures that form by self-assembly of amphiphiles (surfactants, lipids, or polymers) in aqueous environment. The distinctive feature of this method is that the objects are examined without dehydration. This is achieved by capturing the structures in a very thin aqueous film that is substantially turned

into glass at liquid nitrogen temperatures and examined using a microscope. This technique also allows the observation of specimens that have not been stained or fixed in any way, showing them in their native environment. Objects of size ranging from 5 to 500 nm are well suited for the method. This includes various emulsion particles, such as vesicles [67].

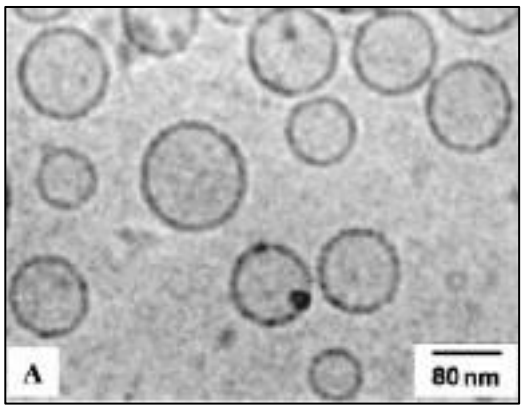


Figure 2.11. Cryo-TEM image of SOS/CTAB/H_2O [66].

2.2.4.2. Dynamic Light Scattering (DLS). DLS spectroscopy, also known as Quasielastic Light Scattering (QLS), is an optical method well-suited for the determination of diffusion coefficients of particles undergoing Brownian motion, random movement of particles suspended in a fluid (liquid or gas) resulting from their bombardment by the fast-moving atoms or molecules in the gas or liquid, in solution. Diffusion coefficients are determined by particle size, shape, and flexibility, as well as by their inter-particle interactions. All these parameters provide important information about the kinetics and structural transitions within system of particles in solution and can be studied by DLS. DLS is a useful, quantitative and non-destructive technique for studying structure and particle aggregations in colloidal systems and also rapid, sensitive, and non-invasive [68].

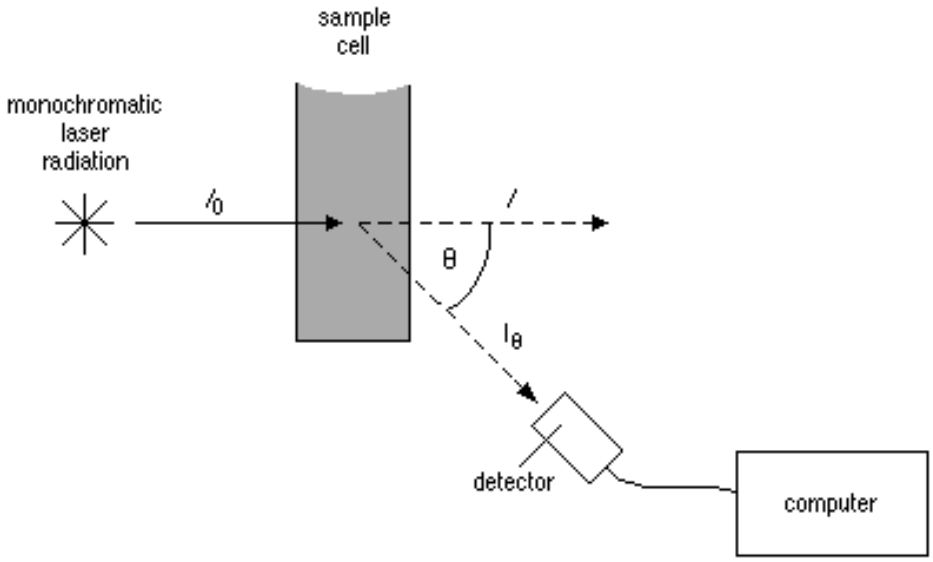


Figure 2.12. A simple diagram of a DLS instrument [69].

2.2.4.3. Zeta Potential Measurements. Zeta potential (ζ) is a scientific term for electrokinetic potential in colloidal systems. The liquid layer surrounding the particle exists in two parts; an inner region (Stern layer) where the ions are strongly bound and the outer (diffuse) region where they are less firmly associated (Figure 2.13).

Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to gravity), ions within the boundary move it. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is the zeta potential.

The significance of zeta potential is that its value can be related to the stability of colloidal dispersions. The zeta potential indicates the degree of repulsion between adjacent, similarly charged particles in dispersion. For molecules and particles that are small enough, a high zeta potential will confer stability, i.e., the solution or dispersion will resist aggregation. When the potential is low, attraction exceeds repulsion and the dispersion will break and flocculate. So, colloids with high zeta potential (negative or positive) are electrically stabilized while colloids with low zeta potentials tend to coagulate or flocculate as outlined in the Table 2.1.

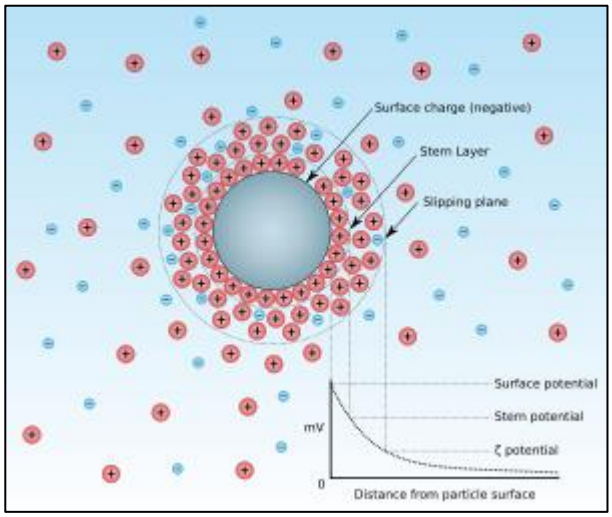


Figure 2.13. Diagram showing the ionic concentration and potential difference as a function of distance from the charged surface of a particle suspended in a dispersion medium [70].

Table 2.1. Stability behavior of the colloidal dispersion according to the zeta potential values [70].

Zeta Potential (mV)	Stability Behavior of the Colloid
from 0 to ±5	Rapid coagulation or flocculation
from ±10 to ±30	Incipient stability
from ±30 to ±40	Moderate stability
from ±40 to ±60	Good stability
more than ±61	Excellent stability

2.2.5. Dynamics of Formation and Stability of Vesicles

Amphiphilic systems display a various phase behavior and large diversity in aggregate structures. So far it has been concerned which surfactant systems form vesicles or, which vesicles are formed spontaneously. In general, the dynamics of the formation of amphiphilic structures have not been studied intensively. Accordingly, only little is known about the structural pathways and physico-chemical parameters that control vesicle formation [71]. On the other hand, kinetic pathways and intermediate structures involved in the dynamics of vesicle formation are important in colloidal science and receiving more

attention to understand how this process takes place. Once, a detailed mechanism of the formation process is understood then the formed vesicle structures will be controlled in order to be used in applications such as models for biological membranes, micro reactors for the preparation of inorganic nanoparticles, and vehicles for controlled release applications [72].

Aqueous vesicles are commonly observed with phospholipids and with their synthetic analogs, *i.e.*, two-chain surfactants having alkyl chains with 10 or more carbon atoms. Different methods are used for preparing vesicles [73]. For instance, turbid dispersions of appropriate sparingly water-soluble surfactants or phospholipids give rise upon sonication to clear and slightly bluish systems that contain vesicles. Vesicles may also be obtained by solubilizing an appropriate amphiphile in an organic solvent, depositing the solution on a glass plate, evaporating the organic solvent, and exposing the resulting film to water. Vesicle-forming surfactants can also be solubilized in an aqueous solution of a hydrotrope (a water-soluble compound that does not form micelles on its own but that is able to promote the solubilization of water-insoluble compounds in water) or of a micelle-forming surfactant. Dilution of this solution with water gives rise to vesicles. Sonication, extrusion through Millipore filters, and other methods have been used to transform large multilamellar vesicles into small unilamellar vesicles [73].

Vesicles prepared by the above methods can have long-term stability. Nevertheless, vesicles made up of a single surfactant or phospholipid are unstable and generally revert to liquid crystalline aggregates (lamellar phases, in most cases) after a time that can be quite long. Unilamellar vesicles obtained by mixing aqueous solutions of two surfactants of appropriate structure and of different electrical charge may be different. Such vesicles appear to form spontaneously and reproducibly and can have long-term stability [57]. It has been claimed that vesicles so prepared can be thermodynamically stable.

A surfactant monolayer takes up a curvature, whose value depends on the surfactant chemical structure and the interaction between surfactants. Considerations based on the bending energy of a surfactant monolayer lead readily to the conclusion that only flat bilayers (zero curvature) can be thermodynamically stable, and in turn, that vesicles (curved bilayers) formed from a single surfactant or phospholipid cannot be

thermodynamically stable [55]. In contrast, Safran *et al.* [74] showed that the free energy of a system of vesicles, made up from a mixture of two interacting surfactants forming monolayers with large bending constants, can be a minimum if the vesicle inner and outer monolayers have different compositions. The spontaneous curvatures of the two layers are then equal but have opposite signs. Systems containing such vesicles would be thermodynamically stable single-phase systems, like micellar solutions.

Vesicles can be transformed into micelles, and vice versa (Figure 2.14). In most instances the vesicle-to-micelle transition is induced by the addition of a micelle-forming surfactant [75] or a hydrotrope [76] to a vesicular system. The micelle-to-vesicle transition is often induced by mixing two oppositely charged surfactants [77] or removing a micelle-forming surfactant (or a hydrotrope) from a mixed micellar solution of this surfactant (or hydrotrope) and of a vesicle-forming surfactant. The transition is also be induced by a change of pH, temperature [78, 79] or ionic strength of a micellar solution or of a vesicular system, or by shearing the system [80].

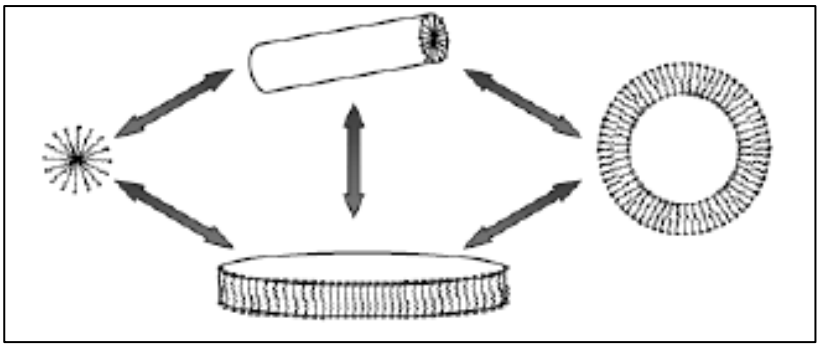


Figure 2.14. A schematic representation of possible pathways in the micelle-to-vesicle and vesicle-to-micelle transitions [81].

The intermediate structure [56, 77] between micelles and vesicles might be perforated vesicles, bilayer fragments, giant worm-like micelles, ring-like micelles, rod-like micelles, and disk-like micelles. These structures were visualized by cryo-TEM (Figure 2.15 and Figure 2.16).

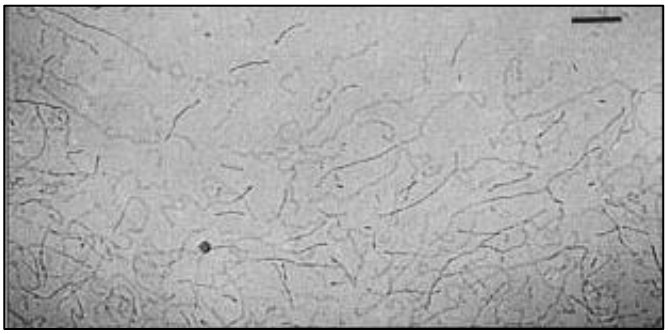


Figure 2.15. Aggregates visualized by cryo-TEM in the course of the vesicle-to-micelle transformation: coexistence of globular micelles, elongated micelles, and vesicles [82].

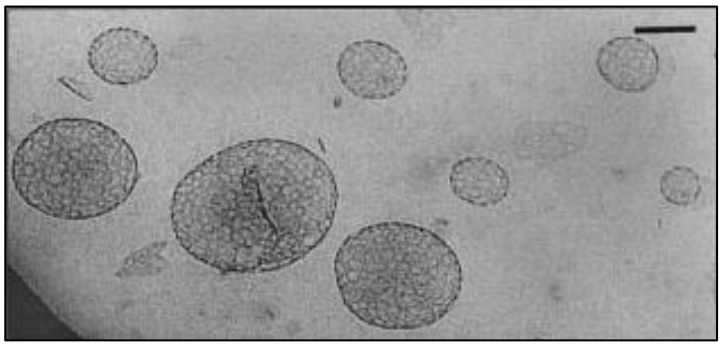


Figure 2.16. Aggregates visualized by cryo-TEM in the course of the vesicle-to-micelle transformation: perforated vesicles [82].

Vesicles are also dynamic objects. They exchange surfactant or phospholipid with the surrounding solution. These exchanges are much slower than in micellar solutions essentially because surfactants and lipids making up the vesicles are much more hydrophobic than those giving rise to micelles. Besides, since the number of surfactants making up a vesicle is 10 to 1000 times larger than for a micelle, the lifetime of a vesicle must be extremely long and vesicles can probably be considered as “frozen” on the laboratory time scale (weeks to months or years).

Vesicles are known as liposomes when they are formed by phospholipids. This is discussed in the following chapter.

3. PHOSPHOLIPIDS AND LIPOSOMES

3.1. General Information about Lipids

Lipids, along with proteins and nucleic acids, are essential biomolecules for the structure and function of living matter. Lipids are chemically diverse group of complex organic compounds in both their respective structures and functions, including oils, fats, waxes, steroids, phospholipids and glycolipids (Figure 3.1) that are widely distributed throughout plant and animal kingdom. They form about 3.5% of the total chemical composition of a cell. Lipids are insoluble in water but soluble in non-polar solvents. These organic structures can be dispersed in water in the form of minute droplets. Such a complex is called an emulsion, a mixture of two or more immiscible liquids [83].

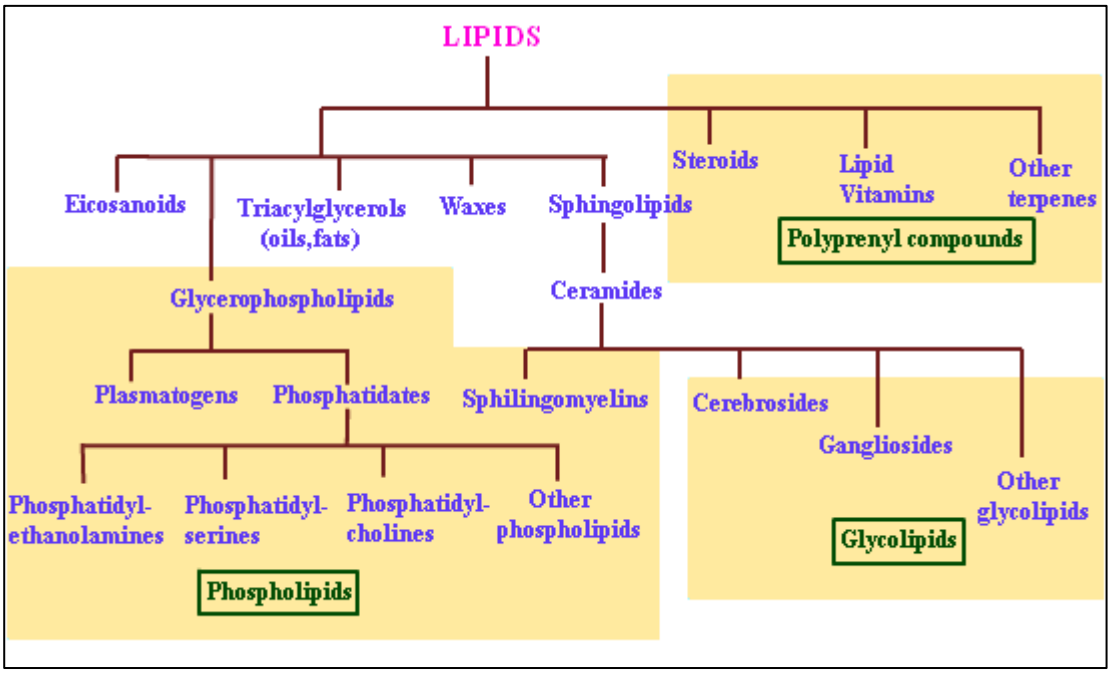


Figure 3.1. Lipid classification [84].

Lipids have diverse biological functions in living organisms. The main function is that they serve as structural components of biological membranes, as well as vitamins and

hormones or precursors. They act as energy reserves, mainly in the form of triacylglycerols. Also, lipids can act as thermal insulators.

The term lipid, used for the first time by Bloor in 1943, denotes a wide range of compounds that appear to have little obvious interrelation. However, although these compounds possess widely different structures, they are derived in part from similar biological precursors and exhibit similar physical and chemical characteristics. Furthermore, most lipids occur naturally in close association with protein, either in membranes as insoluble lipid-protein complex or as soluble lipoproteins of the plasma [85].

Out of all lipid compounds, three types of them are more common: triglycerides, steroids, and phospholipids.

3.2. Triglycerides

A triglyceride (triacylglycerol or triacylglyceride, TAG) is a naturally occurring ester derived from glycerol and three fatty acids that is the chief constituent of fats and oils.

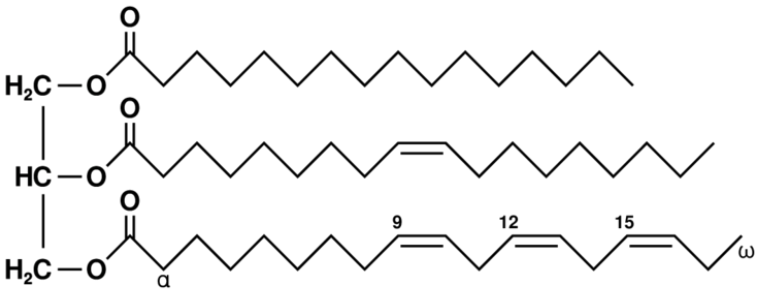


Figure 3.2. Example of an unsaturated fat triglyceride containing glycerol (left part) and palmitic acid, oleic acid, alpha-linolenic acid (right part from top to bottom) with a chemical formula $C_{55}H_{98}O_6$ [86].

TAGs are the simplest lipids, also referred to as fats or neutral fats. Because the polar hydroxyls of glycerol and the polar carboxylates of the fatty acids are bound in the ester linkages, TAGs are nonpolar and nonionic, hydrophobic molecules, essentially insoluble in water. The solid TAGs are named as fats which have high proportion of saturated fatty acids and the liquid ones are oils that contain high proportions of unsaturated fatty acids. Although triglycerides are not important membrane lipids, they are stored in most animals and plants as a metabolic energy reserve. TAGs are also important as intermediates in some metabolic reactions and may be component of other lipid class.

3.3. Steroids

This important class of lipids, called steroids, is actually metabolic derivatives of terpenes, but they are conventionally treated as a separate group. The core of steroids is composed of twenty carbon atoms bonded together that take the form of four fused rings: three fused six-membered (cyclohexane) and one five-membered ring (cyclopentane). The steroids vary by the functional groups attached to this four-ring core and by the oxidation state of the rings.

Hundreds of distinct steroids are found in animals, and plants. The compact hydrophobic steroid molecules are used as building blocks for certain vitamins like vitamin D and also some sex hormones such as estradiol and testosterone are steroids. The predominant steroid is cholesterol (Figure 3.3), with a significant quantity being found in subcellular membranes [87]. The common features for all the steroids are a 3β-hydroxyl group, a planar steroid nucleus, and an aliphatic side chain, all of which are essential for the characteristic behavior of steroids in membranes to be displayed. Cholesterol, inserted into the animal cell membrane, controls the cell fluidity and permeability and also, it is a precursor of several steroid hormones produced in animals [83].

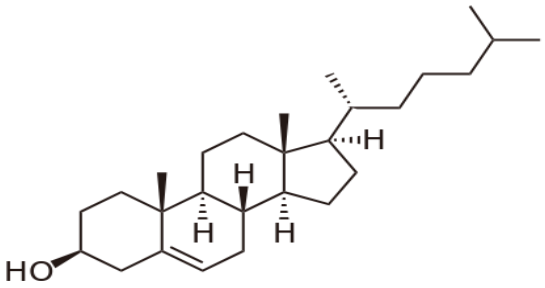


Figure 3.3. Chemical structure of the most abundant steroid, cholesterol [87].

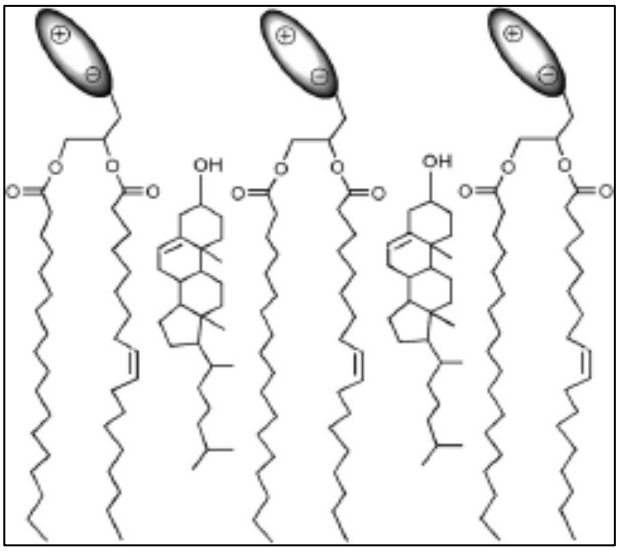


Figure 3.4. Position occupied by cholesterol in the membrane bilayer [88].

Cholesterol does not form bilayer structures by itself, but it can be incorporated into phospholipids membranes in very high concentrations-, up to 1:1 or even 2:1 molar ratios of cholesterol to phosphatidylcholine. In natural membranes, the ratio varies between 0.1 and 1, depending upon the anatomical and cellular location. Being an amphiphilic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface, and the aliphatic chain aligned parallel to the acyl chains in the center of the bilayer. The 3β-hydroxyl group is positioned level with the carboxyl residues of the ester linkages in the phospholipids, with very little vertical freedom of movement. The presence of the rigid steroid nucleus alongside the first ten or so carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining

carbons towards the terminal end of the chain (Figure 3.4) [89]. Above a certain concentration of cholesterol, the membrane area occupied by the acyl chains and steroids combined is greater than or equal to that taken up by the phosphocholine head group, so that phosphatidylcholine membranes with high levels of cholesterol do not show the chain tilt that is observed in the gel phase of liposomes composed of pure phosphatidylcholines (PC) (Figure 3.5).

The phase transition temperature (T_c) is defined as the temperature required to induce a change in the lipid's physical state from the ordered gel phase, where the hydrocarbon chains are fully extended and closely packed, to the disordered liquid crystalline phase where the hydrocarbon chains are randomly oriented and fluid. There are several factors that directly affect the phase transition temperature including hydrocarbon chain length, unsaturation, charge, and head group species. In the case of lipid membranes, addition of cholesterol to phosphatidylcholine membranes has a marginal effect on the position of the main T_c: for example; in dipalmitoyl phosphatidylcholine (DPPC) the T_c changes from 41°C to 44°C with 33 mol% cholesterol. With increasing concentration, however, cholesterol is able to eliminate evidence of a phase transition temperature. Below this temperature, the phospholipids are pushed apart, the packing of the head groups are weakened, and the fluidity of the ordered gel phase increased. Above the transition temperature, the reduction in freedom of acyl chains causes the membrane to condense, with a reduction in area, closer packing and a decrease in the fluidity. These changes in fluidity are parallel with the changes in the permeability of the membrane: decreased by high cholesterol at temperatures higher than T_c but increased at lower temperatures [88].

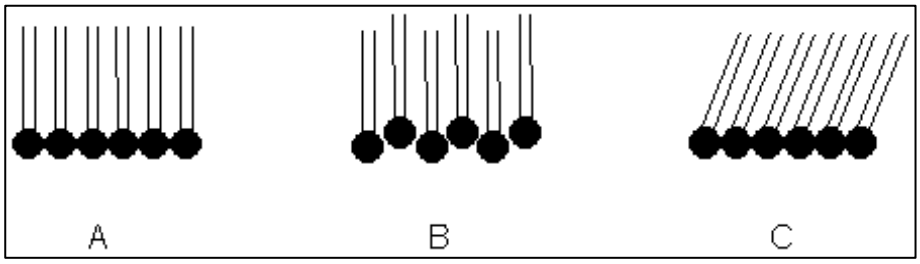


Figure 3.5. Different packing adaptations: (a) space requirement of head groups and tails is the same, (b) head group requires more space than tails, (c) tilted arrangement of tails allowing tighter packing [90].

3.4. Phospholipids

Phospholipids (phosphatids) are a family of lipids similar to TAGs except that one hydroxyl group of glycerol is replaced by the ester of phosphoric acid and an amino alcohol, bonded through a phosphodiester bond (Figure 3.6). The building block of phospholipids is the phosphotidic acid which results when hydrogen substituted to the other end phosphate group. Substitutions include ethanolamine (phosphatidylethanolamine), choline (phosphatidylcholine, also called as lecithin), and serine (phosphatidylserine) [91].

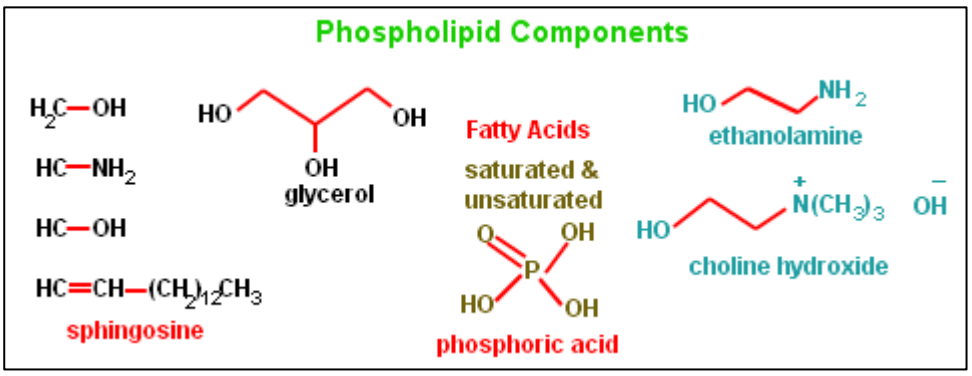


Figure 3.6. Components of phospholipids [84].

Phospholipids are amphiphilic molecules commonly called as polar lipids meaning that they are soluble in both oil and water [85]. The hydrophilic head contains the negatively charged phosphate group because of the oxygens with their entire unshared

electrons and may contain other polar groups. The hydrophobic tail usually consists of long fatty acid hydrocarbon chains (Figure 3.7).

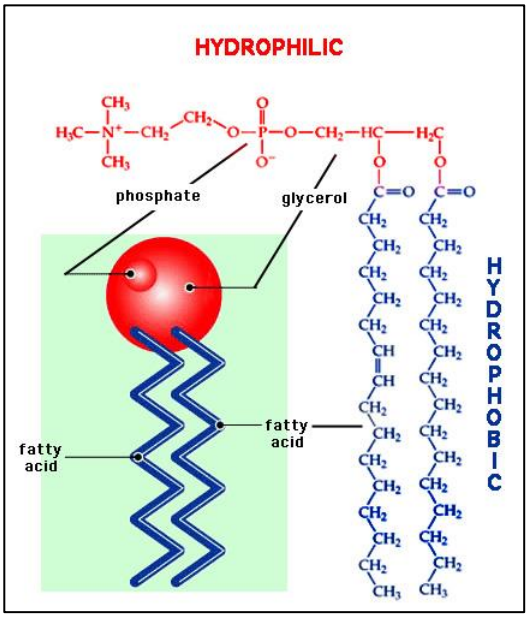


Figure 3.7. Schematic representation of a phospholipid molecule [92].

Phospholipids are the main constituents of the cell membrane lipids. They serve primarily as the structural components of all biological membranes. Besides being the components of bio-membranes, the phospholipids are also found in brain and cardiac tissues. They also act as pulmonary surfactant in lungs. Some phospholipids form the insulating sheath around the nerve tissues.

The basic structure of majority of phospholipids is very similar to that of the TAGs except that C3 carbon of the glycerol backbone is esterified to phosphoric acid. These phospholipids are commonly called glycerophospholipids or phosphoacyl-glycerols or phosphoglycerides. But in some phospholipids, the glycerol backbone is absent. Instead, they are the derivatives of sphingosine. These are commonly called as sphingomyelin or phosphosphingosides.

In this way, phospholipids can broadly be classified on the basis of their chemistry into following two classes: (i) glycerophospholipids or phosphoacyl-glycerols and (ii) sphingomyelin or phosphosphingosides [83]. In the following sections; only

glycerophospholipids are discussed in details since the phospholipids used in this study are the members of this category.

3.5. Glycerophospholipids or Phosphoacyl-glycerols

Glycerophospholipids which are the molecules composed of glycerol, a phosphate group, and two fatty acid chains are the most abundant structural components in cell membranes (Figure 3.8).

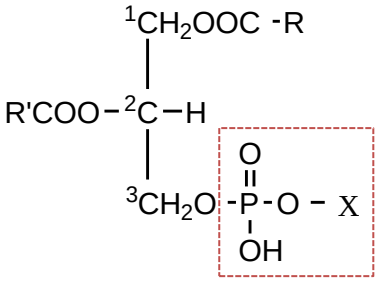


Figure 3.8. Chemical structure of a glycerophospholipids molecule [83].

The cell membrane seen under the electron microscope consists of two identifiable layers, each of which is made up of an ordered row of glycerophospholipids molecules. The composition of each layer can vary widely depending on the type of cell [93].

Like all lipids, they are insoluble in water, but their unique geometry, the hydrophobic tails and the hydrophilic head groups, causes them to aggregate into bilayers without any energy input. During the formation of bilayers, the hydrophobic tails point to each other and form a fatty, hydrophobic center and the ionic, hydrophilic head groups are placed at the inner and outer surfaces of the cell membrane [94]. The formed bilayer is a stable structure because the ionic hydrophilic head groups interact with the aqueous media inside and outside the cell, whereas the hydrophobic tails maximize the hydrophobic interactions with each other and are kept away from the aqueous environment. The overall aim of this structure is to construct a fatty barrier between the interior and exterior part of the cell.

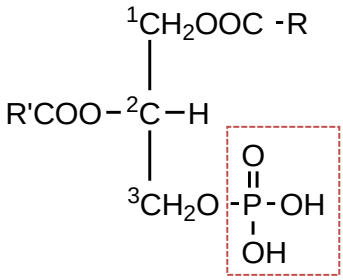


Figure 3.9. Chemical structure of a phosphotidic acid molecule [83].

The simplest phospholipid of this category is the *phosphotidic acid* which results when the *X* group in Figure 3.8 is substituted with a hydrogen atom (Figure 3.9). Other phospholipids of this group are the derivatives of phosphotidic acid, in which the *X* group is substituted by other groups and compounds such as, choline, ethanolamine, serine, glycerol, and myo-inositol. Some of the important groups of glycerophospholipids are PCs (known as lecithins), cephalins, phosphatidylinositol (PI), and plasmalogens. Only PCs and phosphatidylglycerols (PGs) are discussed in the following section, since they are the ones used in the liposome systems of this study.

3.5.1. Phosphatidylcholine (PC)

PCs are a class of phospholipids that have choline as a head group in their chemical structure. They are also a member of lecithin group of yellow-brownish fatty substances found in animal and plant tissue. Lecithin was first isolated in 1846 by the French chemist and pharmacist Theodore Gobley from egg yolk (*lekithos* in ancient Greek) [95]. In 1850, he named the phosphatidylcholine as *léchitine* [96] and in 1874; he established the complete chemical formula. Between the years 1850 and 1874, he demonstrated the presence of lecithin in a variety of biological matters like bile, fish eggs, and also in human, sheep and chicken brain [97]. PCs are such a major group of lecithin that in some contexts the terms are sometimes used as synonyms. However, lecithin is a mixture of phosphatidylcholine and other compounds.

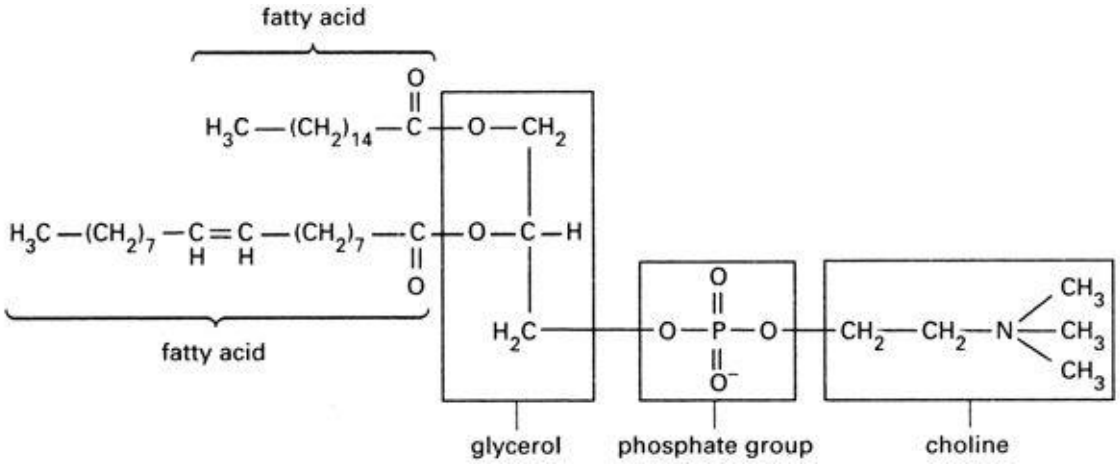


Figure 3.10. Chemical structure of PC [98].

PCs are obtained when a nitrogenous base choline gets attached to the hydroxyl (-OH) group of the phosphate of the phosphotidic acid with a variety of fatty acids, one being a saturated fatty acid and the other one being an unsaturated fatty acid (Figure 3.10). They are the most abundant phospholipid in animals and plants and the key building blocks of membrane bilayers. In particular, they make up a very high proportion of the outer leaflet of the plasma membrane. PCs are also the principal phospholipids circulating in plasma as an integral component of lipoproteins and the only phospholipids that are necessary for lipoprotein assembly and secretion. On the other hand, it is less often found in the bacterial cell membranes [98].

PCs are often used as the principal phospholipid in liposomes for a wide range of applications, both because of their low cost relative to the other phospholipids, and their neutral charge and chemical inertness.

3.5.2. Phosphatidylglycerol (PG)

PGs, a type of glycerophospholipids, are very common type of lipids that can be the main component of some bacterial membranes, and also found in the membranes of plants and animals where they appear to perform specific functions. Phosphatidylglycerol is present at a level of 1-2% in most animal tissues, but it can be the second most abundant phospholipid in lung surfactant at up to 11% of the total.

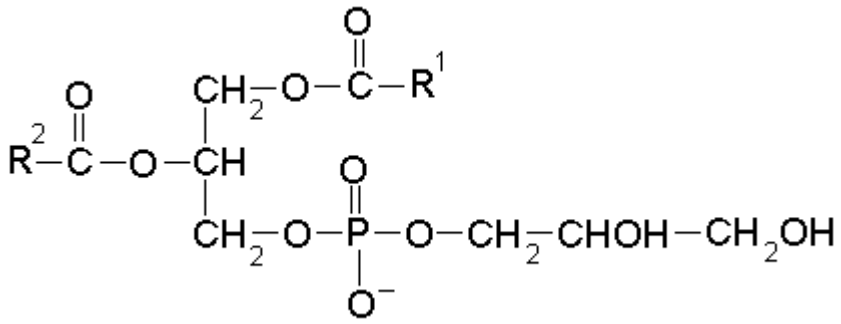


Figure 3.11. General structure of PG [99].

3.5.3. Phospholipids Technology and Applications

In pharmaceutical industry, phospholipids are used for a variety of emulsifying and dispersing applications as helpers. Besides of being fully natural, phospholipids are non-toxic additives with various physicochemical properties that offer excellent opportunities to the developers. Two basic groups of application have emerged in pharmaceutical sector: Phospholipids as active ingredients, having an effect on biochemical metabolic functions and phospholipids as helpers, in which they play an important part by virtue of their physicochemical properties.

Phospholipids are essential components of the human body and a natural ingredient for personal care products (like creams, lotions, soaps, hair shampoo and decorative cosmetics). Lecithin is an excellent skin restorative agent and moisturizer. It has the ability to penetrate the epidermis and carry substances to the right cell level. It can also form liposomes which are very similar in construction to an actual cell membrane. They are then able to pass through the epidermis and act as a carrier of substances that attach themselves [100].

Two other important application fields of phospholipids are the drug delivery and gene delivery where they are used in their self-assembled form, liposome. These two applications will be discussed under the topic of “Liposome”, in the following chapter.

3.6. Liposomes

3.6.1. General Information about Liposomes

Liposomes, or phospholipid vesicles, are self-assembling microscopic spherical lipid bilayers that array separating an aqueous internal compartment from the bulk aqueous phase. In contrast to lipid monolayer structures, liposomes are characterized by extended, two-dimensional, and clearly separated hydrophilic and hydrophobic regions. The hydrophilic portions of bilayer lipids are directed towards aqueous phases (external and internal), whereas the hydrophobic portions of both lipid layers are directed towards one another, forming the internal core of a membrane (Figure 3.13) [101]. The size of a liposome ranges from some 20 nm up to several micrometers and they may be composed of one or several concentric membranes, each with a thickness of about 4 nm.

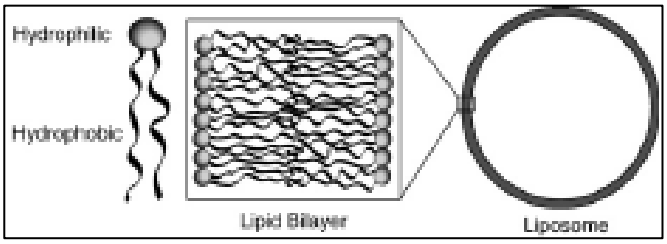


Figure 3.12. Schematic diagram of lipids incorporated into a bilayer membrane to form a liposome [101].

In 1963; Bangham demonstrated that phospholipids dispersed in aqueous media undergo hydration to form vesicular structures of concentric lamellae. Each lamella is a lipid bilayer. Bangham referred to these assemblies as liposomes, however, at first, they were also nicknamed as “Banghasomes” [102]. This discovery confirmed an earlier study that claimed that all plasma and intracellular membranes are based on phospholipid bilayers, and encouraged the use of liposomes as the main model system to study the physicochemical and other properties of biological membranes [103, 104]. Liposomes occur naturally as well as they can be prepared artificially, as shown by Bangham and his co-workers in the mid-1960s [105]. The early pioneers established the concept that this unique structure enables liposomes to be preferred carriers for a broad spectrum of agents,

including drugs [106-108], small interference ribonucleic acid (siRNA) [109, 110], plasmid deoxyribonucleic acid (DNA) [111-113], peptides [114, 115], proteins [116], and even subcellular organelles [117], viruses [118-120], and bacteria [121, 122]. The loaded molecules can be hydrophobic, hydrophilic, or amphiphilic in nature. Their location in the liposome will be dependent on the physicochemical properties of the loaded agent, being either in the liposome membrane, or in the intra-liposome aqueous phase. The lipid membrane of most liposomes used for drug, protein, or nucleic acid delivery is based mainly on specific phospholipids such as PC. Such lipids are referred to as liposome-forming lipids.

3.6.2. Forces between Lipid Molecules

Lipid molecules aggregate into various structures to minimize the free energy, ΔG, of the whole system by trying to maximize the hydrophilic interaction and minimize the hydrophobic one. Hydrocarbon chains bind with London-van der Waals forces, which lower their free energy gained while transferred from water to the nonpolar phase, whereas simultaneously, the polar head group hydration also greatly reduces ΔG. The total interfacial energy for molecules in an aggregate can be written in the first order as:

$$\mu = \gamma a + \frac{K_r}{a} \tag{3.1}$$

where γa is the hydrophobic contribution of interface with *a* being the surface area and γ free energy per unit area. The second term represents the repulsion of polar heads via empiric constant, K_r. Intramembrane and intermembrane forces which are responsible for the intra-bilayer and inter-bilayer interactions consist of hydrophobic forces (attractive, between neutral atoms or molecules, in the case of nonpolar molecules London dispersion forces), electrostatic forces (repulsive, systems with nonzero surface charge), hydration forces (repulsive, when the polar heads are hydrated) and steric forces (repulsive, thermal undulations of the bilayer, specifically or polar heads), which can all operate simultaneously [24, 123, 124].

3.6.3. Dynamics of Liposomes

Lipids that form liposomes are mainly those having a molecular packing parameter, *P* in the range of 0.74-1.0. When immersed in aqueous solution, at a concentration higher than the critical aggregation concentration (CAC) and at a temperature above the solid-ordered (SO) to liquid-disordered (LD) phase transition, the lipids aggregate spontaneously to form MLVs. These can then be downsized by various methods to form either LUV or SUV. When using lipid mixtures, it is possible to predict if liposomes will be formed by calculating the additive molecular packing parameter which is the sum of molecular packing parameter, *P* of each lipid component multiplied by its mole fraction. [125].

Liposomes and bilayers in general must be somewhat dynamic. Otherwise, they would be impenetrable barriers across which nothing could pass. The lipid bilayer of liposomes can be in one of three phases, based mainly on the packing of the lipid hydrocarbon chains, being in the SO (also referred to as crystalline, solid, or gel phase), LD (also called liquid crystalline, fluid, or liquid phase), or liquid-ordered (LO) phase (Figure 3.14) [126-130].

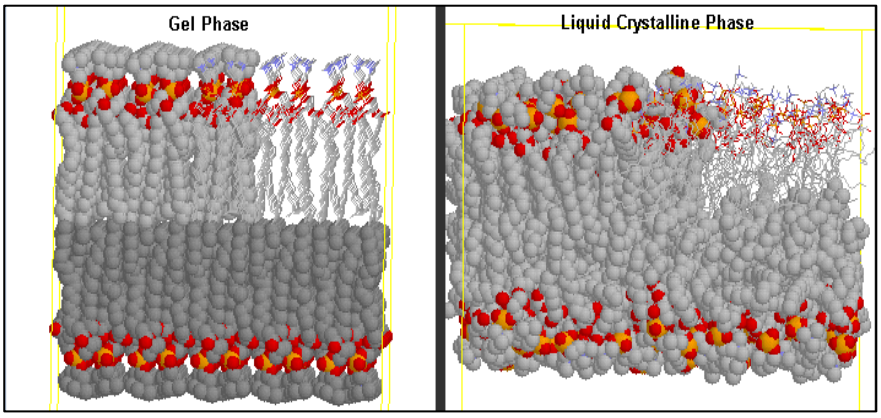


Figure 3.13. Differences in gel and liquid crystalline phases in phospholipid bilayers [131].

Most phospholipids have a phase transition of their lipid bilayer, from SO to the LD phase, and vice versa. The temperature range at which this transition occurs is dependent

on the exact molecular structure, polar heads and hydrocarbon chains, of the lipids assembling the bilayer. The main phase transition is characterized by several parameters, such as the temperature range at which it occurs, the temperature at which the maximum change in heat capacity occurs during the phase transition (referred to as T_c), the width at half height of the endothermic curve which represents the cooperativity of the process, while the area under the curve represents the total enthalpy involved in this phase transition. The most widely used method for determining the T_c is the differential scanning calorimetry (Figure 3.15) [126, 127].The influence of hydrocarbon chain length and unsaturation as well as the head group on the value of T_c for membranes composed of different phospholipids is considerable. In general, increasing the chain length, or increasing the saturation of the chains increases the transition temperature.

An understanding of phase transitions and fluidity of phospholipid membranes is important both in the manufacture and exploitation of liposomes, since the phase behavior of a liposome membrane determines such properties as permeability, fusion, aggregation, and protein binding, all of which can markedly affect the stability of liposomes, and their behavior in biological systems.

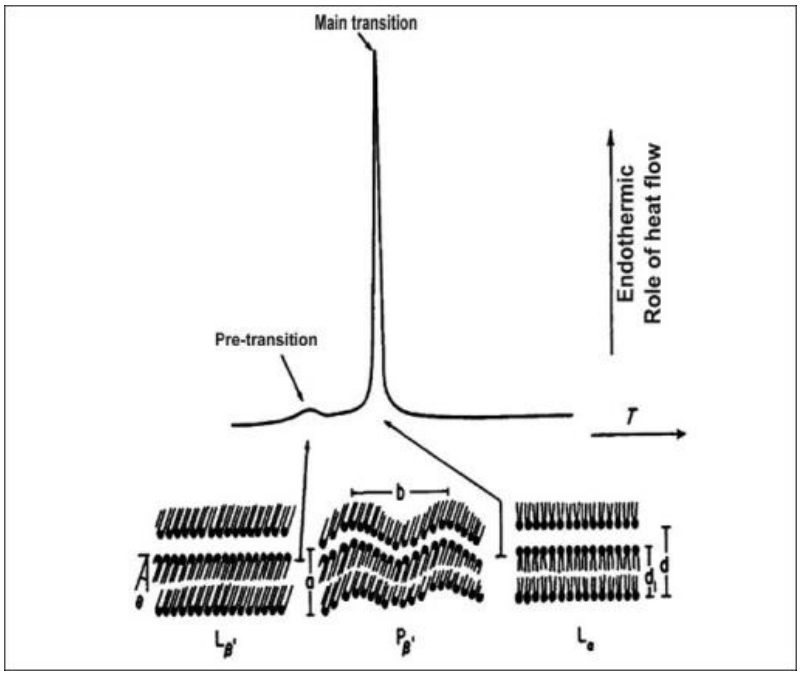


Figure 3.14. Typical differential scanning calorimetry (DSC) diagram of phospholipid bilayers undergoing gel-to-liquid crystal phase transition under controlled heating [132].

In general, liposomes in the LD phase are more permeable than the ones in the SO or LO phase. Liposomes undergoing phase transition are even more permeable than liposomes in the LD phase, due to large defects in the bilayer packing which are related to coexistence of SO and LD regions within the bilayer.

3.6.4 Preparation of Liposomes

The important concept in considering the manufacture of liposomes is that phospholipid membranes form spontaneously as result of unfavorable interactions between phospholipids and water. Therefore, the emphasis in making the liposomes is not towards assembling the membranes, but towards getting membranes to form liposomes of the right structure and size, and to entrap materials with high efficiency and such a way that these materials do not leak out of the liposome once formed [88].

There are lots of methods for making liposomes with common three or four stages: drying down of the lipids from organic solvents, dispersion of the lipids in an aqueous media after the drying step, purification of the resultant liposome, and analysis of the final product (Figure 3.16).

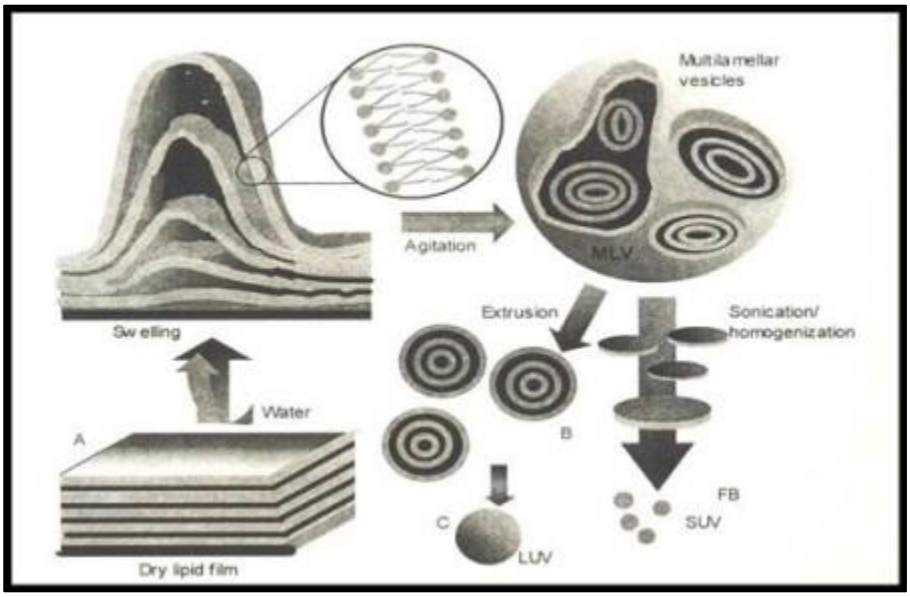


Figure 3.16. Mechanisms and processing steps to generate various types of liposomes [133].

The starting point of all the liposome preparation methods is an organic solution of membrane lipids. Even if the lipids need to be in a dry form at an early stage, it is good practice to dissolve them together in the same solution beforehand, in order to ensure complete and homogenous mixing of all the components as they are required in the final membrane preparation. In all the methods, without exception, compounds to be incorporated which are lipid soluble will be added to the organic solution, while compounds to be entrapped in the aqueous compartment of liposomes will be dissolved in the aqueous starting point.

The drying down of large volumes of organic solutions is most easily carried out in a rotary evaporator fitted with a cooling coil and a thermostatically-controlled water bath. The vacuum supplied by a good suction tap is sufficient for normal purposes; a low grade of vacuum pump can also be used if a suction tap is not available, but care must be taken not to allow solvents to build up in the pump oil. In cases where the vacuum attainable is not sufficient, or if the concentration of lipids is particularly high, it may be difficult to remove the last traces of the organic solvent from the lipid film. Therefore, it is recommended that after rotary evaporation, some further means (attachment of the flask to a lyophilizer and exposure to high vacuum overnight) is employed to bring the residue to complete dryness.

The main difference between the various methods of manufacture is the way in which membrane components are dispersed in the aqueous media, before being allowed to coalesce in the form of bilayer sheets. The liposome preparation methods can be classified according to the three basic models of dispersion; mechanical dispersion, solvent dispersion, and detergent solubilization.

3.6.4.1. Mechanical Dispersion Method. In mechanical dispersion method, which is the simplest in concept, after the lipids are dried down onto a solid support (usually the side of the glass container vessel) then they dispersed by addition of the aqueous medium, followed by methods of hand-shaking, non-shaking, freeze-drying, and pro-liposome. Even before the hydration of the thin film with the aqueous medium, the lipids in the dried form are thought to be oriented in such a way as to separate the hydrophilic and hydrophobic regions from each other, in a manner like their conformation in the finished

membrane preparation. Upon hydration, the lipids swell, and peel off the side of the glass container, generally to form MLV. In most of the mechanical dispersion methods, the aqueous volume enclosed within the lipid membrane is usually only a small proportion of the total volume used for swelling- about 5-10%. Therefore, the method is very insufficient for the water-soluble materials to be entrapped. However, the absolute yield of material, the captured volume in ml g^{-1} lipid, may be satisfactory for practical purposes. On the other hand, lipid-soluble materials can be encapsulated with 100% efficiency [88].

3.6.4.2. Solvent Dispersion Method. In this method, the lipids forming the liposome membrane are first dissolved in an organic solvent, which is then brought into contact with the aqueous phase containing the materials to be entrapped within the liposome. At the interface between the organic and aqueous media, the phospholipid molecules align themselves into a monolayer which forms the basis for half of the bilayer of the liposome. Methods employing solvent dispersion can be categorized into three groups: (i) those in which the organic solvent is miscible with the aqueous phase; (ii) those in which the organic solvent is immiscible with the aqueous phase, the latter being in a large excess; (*iii*) those in which the organic solvent is in large excess, and is again immiscible with the aqueous phase.

3.6.4.3. Detergent Solubilization Method. In this method, the phospholipids are brought into intimate contact with the aqueous phase via the intermediary of detergents, which associate with phospholipid molecules and serve to screen the hydrophobic portions of the molecule from water. The structures which form as a result of this association are micelles.

In all methods which employ detergents in the preparation of liposomes, the basic feature is to remove the detergent from pre-formed mixed micelles containing phospholipids, whereupon unilamellar liposomes form spontaneously. Because removal of detergents is carried out using techniques (e.g. dialysis, column chromatography) which inevitably remove other small water-soluble molecules, the detergent methods are not very efficient in terms of percentage entrapment values attainable; on the other hand, they are certainly the best general methods for preparing liposomes with lipophilic proteins inserted into the membranes, since these proteins can be introduced into the mixed

micelles in the presence of mild non-denaturing detergents, to achieve 100% incorporation without modification of the general method. Another special feature is the ability to vary the size of the liposomes by precise control of the conditions of detergent removal and to obtain liposomes of very high size homogeneity [89].

Table 3.1. Preparation methods for liposomes [134].

Classification of methods	**Sub-classification of methods**	**Liposomes obtained**
Mechanical dispersion	Hand-shaken method	LUV or MLV
	Pro-liposomes	MLV
	Freeze-drying method	MLV
Physical-hydration (modify or improve the characteristics of liposomes)	Micro-emulsification	MLV
	Sonication (bath or probe tip sonicator)	SUV
	French press extrusion	SUV
	Membrane extrusion	LUV
	Dried-reconstitute	LUV or MLV
	Freeze-thawing sonication	SUV or LUV
	Dehydration-rehydration vesicle	SUV
	pH-induced vesiculation	SUV or LUV
	Calcium-induces fusion	LUV
Solvent dispersion	Ether injection	SUV or LUV
	Double emulsification	LUV
	Multiple emulsification	SUV or LUV
	Ethanol injection	SUV or LUV
	Reverse-phase evaporation	LUV
Other methods	Detergent removal/dialysis	SUV or LUV
	Fusion of SUV	LUV
	Film-ultrasonic technique	Dependable on drug
	Amphiphiles-loading	Dependable on drug

3.6.4.4. Processing of Lipids Hydrated by Physical Means. After preparation of MLVs by hydration of dried lipid, it is possible to continue processing the liposomes in order to modify their size and other characteristics. For many purposes, MLVs are too large or too heterogeneous population to work with. There are so many methods devised to reduce their size, and in particular to convert liposomes in large size range to smaller ones. These include techniques such as micro-emulsification, extrusion, and ultrasonication. A second set of methods is designed to increase the entrapment volume of hydrated lipids, and/or reduce the lamellarity of the liposomes formed, and employ procedures such as freeze-drying, freeze-thawing or induction of vesiculation by ions or pH change.

In order to reduce hydrated lipids to liposomes of smaller size, it is necessary to use a method which imparts energy at a high level to the lipid suspension. This was first achieved by exposure of MLVs to ultrasonic radiation [135] and it is still the most widely used method for the production of small liposomes. The starting point is a suspension of multilamellar liposomes. Since these liposomes will be completely broken down in the ultrasonication process, the initial size of the MLVs, the percentage entrapment or the thickness of the lipid film are not too important to be concerned. Therefore; the lipid can be dried down in the same container as is used for sonication. There are two methods of sonication, using either a probe or a bath ultrasonication. The probe is applied for suspensions which require high energy in small volume while the bath is much more suitable for large volumes of dilute lipids, where it may not be necessary to reach the liposome size limit.

In the probe sonication, because of the high energy input, there is considerable risk of degradation of lipids resulting from the high temperature and increased gas exchange associated with operation of the probe. It is essential, therefore, that the sonicator vessel be cooled efficiently at all times. However, it is also desirable to sonicate the lipids above their T_c. Consequently, the method is not reproducible, and the only way to be sure of obtaining liposomes of standard size distribution is to sonicate for an over-long period of time, so that the bulk of the lipid is in the constitutes a relatively homogeneous population of liposomes in terms of size. The other drawback of the probe sonication is the limitation on the volume of sample. As the volume of the sample is increased, the energy distribution throughout the sample becomes much more uneven, and the energy imparted per milliliter

is greatly reduced so that it may not be possible to reach the minimum size limit for liposomes. For large volume of samples, therefore, there is probably little to be gained by probe sonication, and use of a bath sonicator is much more convenient method.

Bath sonication method is much milder than the probe sonication and has less risk of degradation of lipids. The volume of sample exposed to sonication is much larger, and reproducibility greater, provided the flask is positioned carefully in the same position each time. However, because the energy is dispersed over a much larger area, it may not be possible to reach the minimum size limit for bath sonicated liposomes. Special precautions for temperature control are not necessary since the small amount of heat generated is easily absorbed by the bath. Even after prolonged sonication, the population of liposomes obtained will not be homogenous, and in studies where there is a need for the size range of liposomes to be narrow or well characterized, it is necessary to remove the larger liposomes and MLVs from the predominating population of SUVs [135].

An even gentler method of reducing the size of the liposomes is to pass through a membrane filter of defined pore size [136]. This can be achieved at much more lower pressures and can give populations in which one can choose the upper size limit depending on the exact pore size of the filter used.

The membrane extrusion technique can be used to process LUVs as well as MLVs. With both these types of liposome, it should be kept in mind that during the extrusion process, liposome contents are exchanged with the suspending medium during breaking and resealing of the phospholipid bilayers as they pass through the polycarbonate membrane. In order to achieve as high an entrapment possible of water-soluble compounds, it is crucial to have these compounds present in the suspending medium during the extrusion. If MLVs are extruded through membranes of pore size 100 nm or smaller, then upon repeated extrusions, the liposome suspension becomes progressively more unilamellar in character, with the liposomes still maintaining a size distribution around the pore size of the membrane, but now possessing a considerable internal aqueous volume. An almost completely unilamellar population can be produced after 5-10 repeated extrusions through two stacked membranes [89].

3.6.5. Stability of Liposomes

The common attractive force between liposomes is Van der Waals attraction, while the long range repulsive force is electrostatic repulsion. The balance between these two factors determines the colloidal stability. The electrical forces increase exponentially as particles approach one and another and the attractive forces increase as an inverse power of separation. As a consequence, these additive forces may be expressed as a potential energy versus separation curve. A positive resultant corresponds to an energy barrier and repulsion, while a negative resultant corresponds to attraction and hence aggregation. Figure 3.18 states that the classic the Derjaguin-Landau-Verwey-Overbeek (DLVO) model is applicable for determining the stability of liposomes, considering that many of the physicochemical properties of liposomes resemble that of conventional colloidal particles [134].

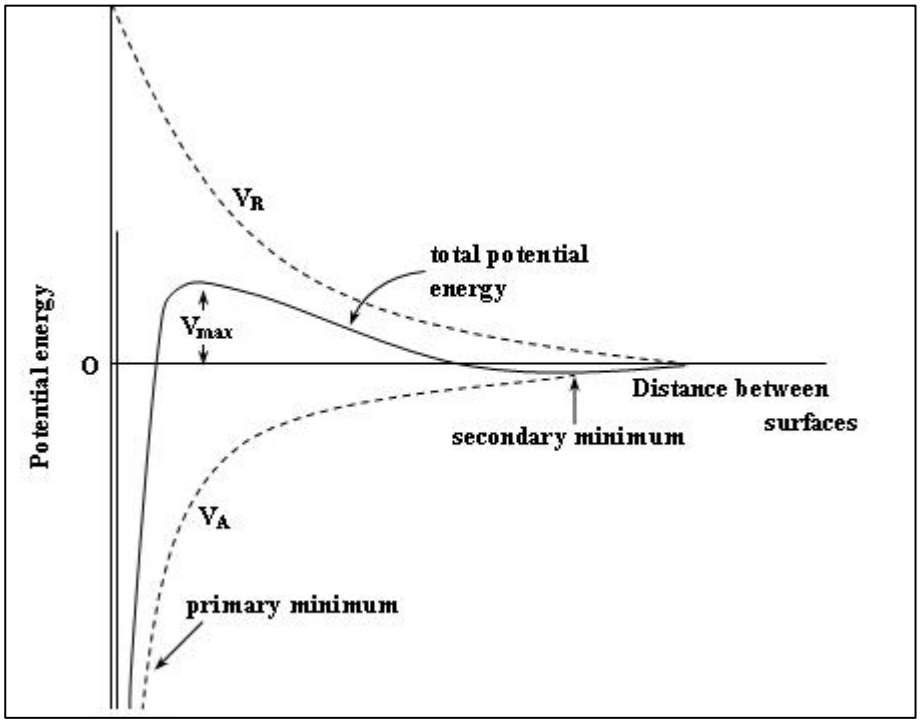


Figure 3.15. Classic DLVO model [134].

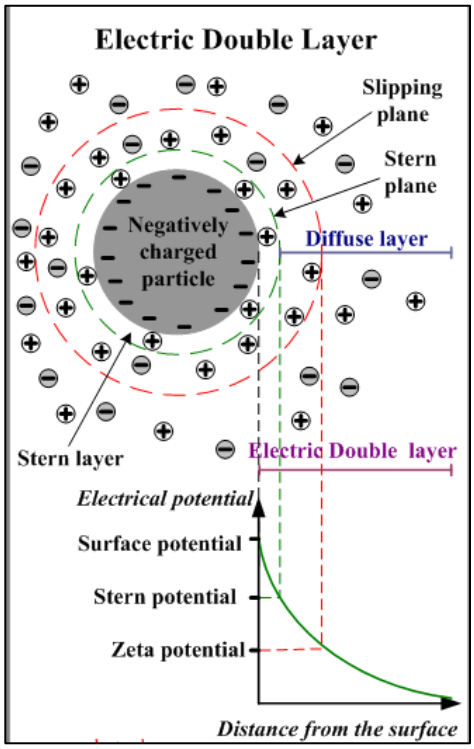


Figure 3.16. Electrical double layer [137].

Liposome stability can be subdivided into physical, chemical and biological stabilities, which are all inter-related. Generally, the shelf-life of liposomes is determined by the physical and chemical stability (uniformity of size distribution and encapsulation efficiency, and minimal degradation of all compounds, respectively). By optimizing the size distribution, pH and ionic strength, as well as the addition of antioxidants and chelating agents, liquid liposome formulations can be stable. As phospholipids usually form the backbone of the bilayer their chemical stability is important. Two types of degradation reactions can affect the performance of the phospholipids bilayers: hydrolysis of the ester bonds linking the fatty acids to the glycerol backbone and oxidation of the unsaturated acyl chains, if present. The oxidation and hydrolysis of lipids may lead to the appearance of short-chain phospholipids and then soluble derivatives will form in the membrane, resulting in the decrease of the quality of liposome products [138]. Moreover, physical processes such as aggregation/flocculation and fusion/coalescence that affect the shelf life of liposomes can result in loss of liposome-associated drug and changes in size. Aggregation is the formation of larger units of liposomal material; these units are still composed of individual liposomes. In principle, this process is reversible [139] e.g. by

applying mild shears forces, by changing the temperature or by binding metal ions that initially induced aggregation. However, the presence of aggregation can accelerate the process of coalescence of liposomes, which indicates the formation of new colloidal structures. As coalescence is an irreversible process; the original liposomes cannot be retrieved.

3.5.6. Methods for Enhancement of Liposome Stability

Methods devised to overcome the problems of liposome instability fall into two categories; those designed to minimize the degradation processes which may take place, and secondly, those which help liposomes to survive in the face of conditions which encourage these processes.

In the case of PC-lipids it is the hydrocarbon chains and especially the unsaturated ones that are subjected to oxidation. Saturated chains, however, be oxidized at high temperatures. The oxidation is a radical reaction, which finally results in the cleavage of the hydrocarbon chains or in the case of two adjacent double bonds, the formation of cyclic peroxides. The level of oxidation can be kept to a minimum by taking some precautions, such as starting with freshly purified lipids and freshly distilled solvents, avoiding processes which involve high temperature, carrying out the manufacture process in the absence of oxygen, deoxygenating aqueous solutions with nitrogen, storing all liposome suspensions in an inert atmosphere, and including an anti-oxidant as a component of the lipid membrane. An alternative approach to the oxidation problem might be to reduce the level of oxidizable lipids in the membrane by using saturated lipids instead of unsaturated ones. Liposomes may also be prepared from entirely synthetic saturated phospholipids such as DMPC and DPPC.

The ester bonds present in a phospholipid may all be subjected to hydrolysis in water. The hydrolysis rate of PC-lipids is both pH and temperature dependent. In general, the rate of hydrolysis has a “V-shaped” dependence, with a minimum at pH 6.5 and thus an increased rate at both higher and lower pH. Obviously, the rate of hydrolysis and temperature are directly proportional. By selecting the temperature and pH, the hydrolysis can be largely avoided.

The problems related to the lipid oxidation and hydrolysis during shelf life of the liposomal product can be reduced by the storage of liposomal dispersion in the dry state by freeze-drying (lyophilization), without compromising their physical state or encapsulation capacity [140]. However, freeze-drying of liposome systems without appropriate stabilizers will again lead to fusion of vesicles. To promote vesicle stability during the freeze-drying process, cycloprotectants, including saccharides (e.g. sucrose, trehalose, and lactose) and their derivatives are employed [141].

The physical degradation, leakage and fusion of liposomes, can occur as a result of the lattice defects in the membrane introduced during their manufacture. Although reported particularly to occur in SUVs when prepared below the membrane T_c, there is evidence for packing defects being maintained in other types of liposomes even above T_c. Aggregation of neutral liposomes occurs due to Van der Waals interactions, and tends to be more pronounced in large liposomes, where the increased planarity of the membranes allows greater areas of membrane to come in contact with each other. Although factors like residual solvent and trace elements can enhance the aggregation, for uncharged membranes it is a natural and unavoidable phenomenon. The simplest way to overcome is to include a small quantity of negative charge (e.g. 10% phosphatidyl glycerol, PG) in the lipid mixture [142].

In large liposomes which are properly made, there is no reason why fusion should occur. SUVs are prone to fusion as a means of relieving stress arising from the high curvature of the membrane. Since this can occur particularly at Tc, it is advisable to store the liposome suspensions at a temperature away from the Tc, and it could be completely advantageous to include sufficient amount of cholesterol in the membrane to reduce or completely remove the transition. Cholesterol prevents the phospholipids packing and induces orientation and more rigidity to those phospholipids and therefore prevent liposome aggregation.

Permeability of liposome membranes depends highly on the membrane lipid composition, as well as on the solute which has been entrapped. Large polar or ionic molecules will be retained much more effectively than low molecular weight lipophilic

compounds. In general, for both cases of compounds, a rigid, more saturated membrane with a higher ratio of cholesterol is the most stable with regard to the leakage of solutes.

Charge on the liposomes also determines the *in vivo* stability. Liposomes with neutral charge containing PC were the most stable and bound the lowest amount of protein. Liposomes with positive charge behaved similarly to those with neutral charge. However, the stability of negatively charged liposomes was very dependent on their composition. Those liposomes containing only one class of negatively charged phospholipids bound a great amount of protein and were very unstable. However, those liposomes containing PC bound less protein and are more stable [143].

3.5.7. Size Determination of Liposomes

There are some methods for the size determination of liposomes that vary in complexity and degree of sophistication. The two most common techniques are DLS and electron microscopy involving the direct observation of the liposomes. Undoubtedly the most precise method is that of electron microscopic examination, since it allows viewing each individual liposome so that one can obtain exact information about the profile of a liposome population over the whole range of sizes. Unfortunately, it can be very time-consuming. In contrast, DLS is very simple and rapid method, but suffers from the disadvantage of measuring an average property of the bulk of the liposomes, and even with the most advanced refinements it may not pick up or describe in any detail small deviations from a mean value or the nature of the residual peaks at extremes of the size range. The two methods mentioned above require items of very costly equipment. If only an approximate idea of size range is required, then gel exclusion chromatographic techniques can be used, since the only expense is that of buffers and gel materials [89].

3.5.8. Applications of Liposomes

Due to their structure, chemical composition and colloidal size, all of which can be controlled by preparation methods, liposomes exhibit several properties which may be useful in various applications (Table 3.2). The most important properties are colloidal size (in the range of 20 nm to 10 μm), special membrane and surface characteristics. They

include bilayer phase behavior, its mechanical properties and permeability, charge density, presence of surface bound or grafted polymers, or attachment of special ligands, respectively. Additionally, due to their amphiphilic character, liposomes are a powerful solubilizing system for a wide range of compounds. In addition to these physico-chemical properties, liposomes exhibit many special biological characteristics, including interactions with biological membranes and various cells [144].

The industrial applications include liposomes as drug delivery vehicles in medicine, gene delivery vehicles in gene therapy, adjuvants in vaccination, signal enhancers/carriers in medical diagnostics and analytical biochemistry, solubilizers for various ingredients as well as support matrix or various ingredients and penetration enhancer in cosmetics.

3.5.9 Liposomal Drug Delivery Vehicles

Liposomes have been widely investigated since 1970 as drug carriers for improving the delivery of therapeutic agents to specific sites in the body. As a result, numerous improvements have been made, thus making this technology potentially useful for the treatment of certain diseases in the clinics. The success of liposomes as drug carriers has been reflected in a number of liposome-based formulations, which are commercially available or are currently undergoing clinical trials.

Table 3.2. Applications of liposomes in the sciences [63].

Discipline	**Application**
Mathematics	Topology of two-dimensional surfaces in three-dimensional space governed only by bilayer elasticity
Physics	Aggregation behavior, fractals, soft and high-strength materials
Biophysics	Permeability, phase transitions in two-dimensions, photophysics

Table 3.3. Applications of liposomes in the sciences [63] (cont.).

Physical Chemistry	Colloid behavior in a system of well-defined physical characteristics, inter- and intra-aggregate forces, DLVO
Chemistry	Photochemistry, artificial photosynthesis, catalysis, microcompartmentalization
Biochemistry	Reconstitution of membrane proteins into artificial membranes
Pharmaceutics	Studies of drug action
Medicine	Drug-delivery and medical diagnostics, gene therapy

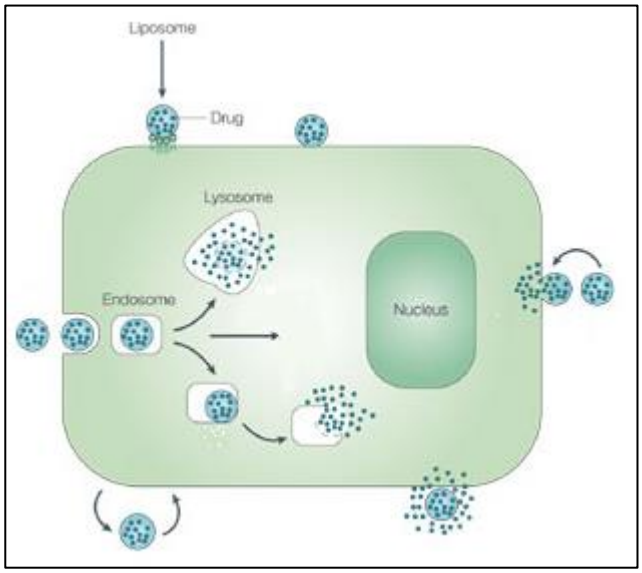


Figure 3.17. Liposome-cell interactions: liposomal adsorption (upper left), uptake of liposomes by endocytosis (middle left), fusion (middle right), and the exchange of lipids (lower left) [145].

Liposome applications in drug delivery depend, and are based on, physicochemical and colloidal characteristics such as composition, size, loading efficiency and the stability of the carrier, as well as their biological interactions between liposomes and cells [121, 146]. There are four major interactions between liposomes and cells: (i) exchange of lipids or proteins with cell membranes, (ii) adsorption or binding of liposomes to cells, (iii)

internalization of liposomes by endocytosis or phagocytosis, and (iv) fusion of bound liposome bilayer with the cell membrane. All four types of interactions are dependent on lipid composition, type of cell, presence of specific receptors and targeting vectors, etc.

For drug delivery, liposomes can be formulated as a suspension, as an aerosol or in a (semi) solid form such as a gel, cream or dry powder; *in vivo*, they can be administered topically or parentally. After systematic administration (usually intravenous), which seems to be the most promising route for this carrier systems, liposomes are typically recognized as foreign particles and consequently endocytosed by the reticular endothelial system (RES) [146, 147]. This fate is very useful for delivering drugs to these cells but, in general, it excludes other applications, including site-specific drug delivery by using ligands expressed on the liposome surface in order to bind the receptors (over)expressed on the diseased cells [121]. For this reason, a search for liposomes that could evade rapid uptake by the mononuclear phagocytic system, (MPS) started a few lipid compositions that prolonged liposome blood-circulation times were discovered [148, 149], culminating in the development of polyethylene glycol (PEG) coated, sterically stabilized liposomes [147].

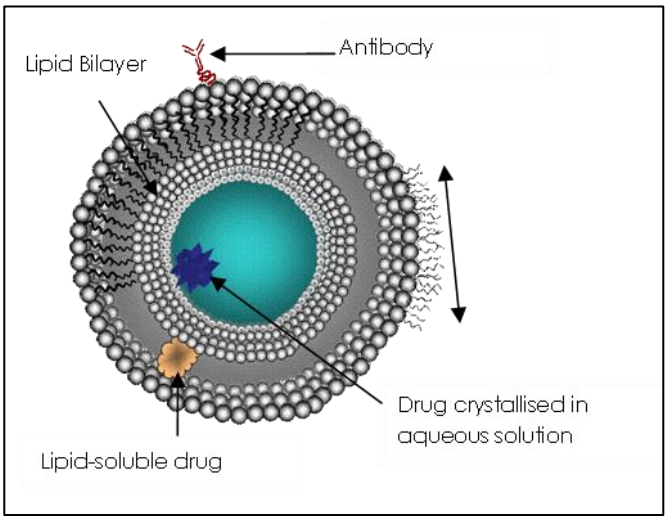


Figure 3.18. Schematic representation of a drug loaded liposome [150].

3.5.10. Liposomes in Gene Therapy

Gene therapy is a promising method to cure genetic deficiencies as well as numerous acquired diseases [151-154]. The principal of gene therapy is simple. If a patient is suffering from a disease caused by a known genetic defect, then the delivery of a correct copy of the defective gene to the diseased cells or body organ, by means of a specially designed vector, would be expected to correct directly for the genetic defect and hence cure the disease. Alternatively, if a patient is suffering from a disease for which there is no clear genetic cause but whose pathophysiology is well understood, then the vector might be used to deliver corrective gene(s) or some other nucleic acid agent to the diseased cells and so disrupt the known disease pathophysiology in some other way. Hence, generally speaking, gene therapy may be defined either as "the use of genes as medicines to treat disease" or "the delivery of nucleic acid (with a vector) to patients for some therapeutic response.

Gene delivery systems are designed to control the location of administered therapeutic genes within the patient's body. Successful *in vivo* gene transfer may require: *(i)* condensation of the plasmid (a small DNA molecule that is physically separate from, and can replicate independently of, chromosomal DNA within a cell) and its protection from the nuclease degradation, *(ii)* cellular interaction and internalization of condensed plasmid, *(iii)* escape of the plasmid from endosomes (if endocytosis is involved), and *(iv)* plasmid entry into cell nuclei [155].

Direct administration of genes to patients may be virally or non-virally mediated. As viruses represents a highly suitable vector for gene transfer, several viruses including retrovirus, adenovirus, adeno-associated-virus and herpes virus have been investigated for their potentials in gene delivery [156].

The drawbacks associated with the use of viral vectors, namely those related to safety problems, have prompted investigators to develop alternative methods of gene delivery, cationic lipid-based systems being the most representative ones. Plasmid liposome complexes have many advantages as gene transfer vehicles over viral based vectors [156]: *(i)* these complexes are relatively nonimmunogenic because they lack

proteins, *(ii)* liposomes or lipid complexes can be used for transfection of large-sized genetic material, and *(iii)* viruses, unlike plasmid liposome complexes, may replicate and cause infection. Despite extensive research in the last decade on the use of cationic liposomes as gene transfer vectors and the development of elegant strategies to enhance their biological activity, these systems are still far from being viable alternatives to the use of viral vector in gene therapy [157].

3.5.11. Limitations of Liposome Technology

Liposomes have a great potential in the areas of drug delivery and gene therapy. However, liposome-based formulations have some problems such as, stability issues, batch to batch reproducibility, sterilization method, low entrapment capacity, particle size control, production of large batch sizes and short circulation half-life of liposomes [158].

One of the major problems limiting the widespread use of liposomes is stability-both physical and chemical. As it is discussed in Section 3.5.6, depending on their composition, the final liposome formulations may have short shelf-lives partly due to chemical (hydrolysis of ester linkages or oxidation of unsaturated acyl chains) and physical instability (drug leakage from the liposome and/or aggregation or fusion of liposomes to form larger particles). Both of the instability sources influence the *in vivo* performance of the drug formulation, and therefore may affect the therapeutic index (TI) of the drug. Some of the stability problems may be overcome by lyophilization in which the final liposome product is freeze-dried with a cryoprotectant (mostly a sugar like trehalose) and is reconstituted with a vehicle prior to administration. Lyophilization increases the shelf-life of the finished product by preserving it in a relatively more stable dry state.

A number of technical problems have to be overcome when liposomal formulations are used as vectors in gene therapy. For instance, liposomes are significantly less efficient than viral vectors in their transfection ability. Furthermore, the DNA-lipid complexes are not stable in terms of particle size [159, 160] for long periods of time. In addition, there is lack of *in vivo* targeting after systemic administration, and the toxicity of the cationic lipids limits the administered dose of the DNA-lipid complex.

4. GENE DELIVERY

4.1. Methods for Delivering DNA to Target Tissues and Cells

Gene therapy provides a unique approach to medicine as it can be adapted towards the treatment of both inherited and acquired diseases. Gene delivery relies upon the encapsulation of a gene of interest, which is then ideally delivered to the target cells. After uptake up by endocytosis, the DNA must be released into the cell so that transcription and translation may occur to produce the protein of interest. To achieve successful gene delivery, significant barriers must be overcome at each step of this process in order to optimize gene activity while minimizing the potential for inhibitory inflammatory responses.

There are many different methods of gene delivery developed for a various types of cells and tissues, from bacterial to mammalian. Generally, the methods can be divided into two categories, viral and non-viral. Particular interest has been paid in recent years to the development of efficient non-viral vectors. Viral vectors (i.e., retroviruses and adenoviruses) may provide superior gene delivery to target cells compared to their non-viral counterparts, but viral vectors also have significantly increased risk of triggering a specific immune response, which under extreme circumstances could result in death [161, 162]. Non-viral vectors may trigger an inflammatory response but are not likely to elicit specific recognition, making these types of vectors less hazardous in terms of antigen-specific immune responses. Although non-viral vectors are more appealing in this respect, there are several factors that must be considered in vector design, including specific cell targeting, optimized uptake, and efficient intracellular release of the vector, in addition to minimizing the immune response.

4.1.1. Viral Vectors for DNA Delivery

Through millions of years of evolution as infective agents, viruses can transfer DNA molecules into cells with consummate ease. For therapeutic purposes, the transgene of interest is assembled in the viral genome and the virus uses its innate mechanism of

infection to enter the cell and release the expression package. The gene then enters the nucleus, is integrated into the host gene pool, and is eventually expressed. Viruses are obligate parasites and cannot replicate without the help of the host cell. Thus they are, by nature, adapted to efficiently transmit genetic information to cells.

Gene expression using viral vectors has been achieved with high transfection efficiencies in tissues. However, before viruses can be used to deliver therapeutic genetic material, they first must be modified to prevent the uncontrolled replication of the engineered viral vector and the potential for reversion of the therapeutic virus into an actively pathogenic form.

Viruses are currently used in more than 70% of human clinical gene therapy trials world-wide. Gene therapy using viral systems has made considerable progress for the treatment of a wide range of diseases, such as muscular dystrophy, AIDS, and cancer. Despite such impressive statistics for gene transfer, there are several concerns over the use of viruses to deliver DNA therapeutics in humans. The main concern is the toxicity of the viruses and the potential for generating a strong immune response owing to their proteinaceous capsid. Such toxicities have been observed in numerous animal models [163-165]. Many other factors may limit the use of the viral vectors for therapeutic applications. Since the viral envelope has a finite capacity, there is a limit on the size of the expression plasmid that it can incorporate.

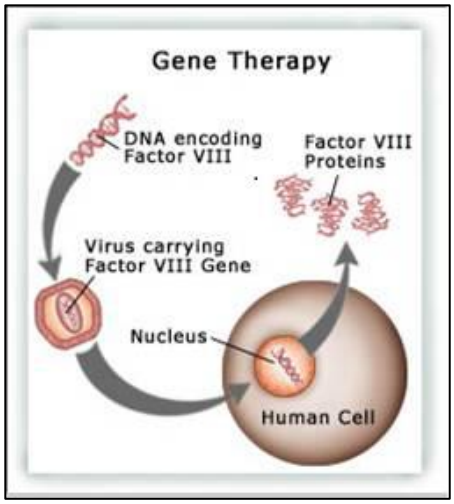


Figure 4.1. Schematic representation of viral gene therapy [166].

4.1.2. Non-viral Methods for DNA Delivery

Although viral-based vectors are highly efficient at delivering DNA into cells, concerns about their safety have led to the development of a number of non-viral vectors as a more suitable alternative. Non-viral methods of DNA delivery into cells have been used for the delivery of DNA *in vitro* in the laboratory for many decades and can circumvent some of the problems associated with viral vectors. Among the greatest advantages of non-viral gene vectors are lack of immune response and ease of formulation and assembly. Commonly used non-viral vectors for the delivery of DNA-based therapeutics can be classified into 2 major types based on the nature of the synthetic material: (i) Polymeric delivery systems (DNA-polymer complexes) and (ii) Liposomal delivery systems (DNA entrapped in and/or complexed to liposomes).

One of the first chemical reagents used to transfer nucleic acids into cultured mammalian cells was 2-(diethylamino) ether (DEAE)-dextran in 1965 [167]. DEAE-dextran is a cationic polymer that tightly associates with negatively charged nucleic acids. An excess of positive charge, contributed by the polymer in the DNA: polymer complex, allows the complex to come into closer association with the negatively charged cell membrane. However, this technique is not generally useful for stable or long-term transfection studies.

Calcium phosphate co-precipitation protocol [168] involves mixing DNA with calcium chloride, adding this in a controlled manner to a buffered saline/phosphate solution and allowing the mixture to incubate at room temperature. The controlled addition generates a precipitate that is dispersed onto the cultured cells. However, calcium phosphate co-precipitation is prone to variability and is not suited for *in vivo* gene transfer to whole animals. In addition, small pH changes (± 0.1) can compromise the efficacy of calcium phosphate transfection.

The non-viral methods like DEAE-dextran and calcium phosphate co-precipitation methods cannot readily be employed for *in vivo* use mainly because of problems associated with low delivery efficiency and high cytotoxicity. Despite these limitations, non-viral delivery systems do offer many advantages over viral-based technologies and,

therefore, the improvement and development of non-viral DNA delivery technology has been the focus of intense research. Non-viral methods of DNA delivery are simple to use and the synthetic components of these systems, such as cationic lipids or polymers, can be produced in large quantities with relative ease. Perhaps the most desired feature of non-viral vectors is their safety for use *in vivo* as a specific host immune response to the vector is not encountered. Additionally, non-viral mechanisms of DNA delivery do not disrupt the genetic material of the targeted host cell and provide short-term expression of the therapeutic gene.

4.1.2.1. Naked DNA. The direct transfer of naked DNA that encodes a potentially therapeutic gene or DNA sequence is one of the simplest and safest methods of DNA delivery. Naked plasmid DNA provides a promising mechanism for gene delivery as it is less immunogenic than most non-viral vectors currently used. Complications arise, however, in that naked plasmid DNA has no target-specific recognition and is more susceptible to nuclease degradation than encapsulated DNA. These issues limit the quantity of naked DNA that is able to reach the target cells, thereby limiting the efficiency of gene expression. Naked DNA can be administered possible routes, either by *ex vivo* delivery or by *in vivo* delivery (gene gun, electroporation and laser beam gene transduction, LBST) [169, 170].

4.1.2.2. Cationic Lipids. The use of DNA into target cells is not feasible in many cases. In such instances, a delivery vehicle, such as cationic lipids or cationic polymers, must be used to facilitate efficient entry of DNA into cells. Numerous studies have demonstrated the use of cationic liposomal formulations for the delivery of different plasmid constructs in a wide range of cells, both *in vivo* and *in vitro*. The nonimmunogenic nature and ease of industrial production of these systems makes them appealing for gene transfer.

Table 4.1. Comparison of viral- and non-viral gene delivery systems.

Property	**Viral Delivery**	**Non-viral delivery**
Efficiency	Highly efficient gene delivery Potential for long-term gene expression (retroviral delivery)	Low efficiency of gene transfer
Size of therapeutic DNA	Limited by size that can be accommodated by viral genome and packaging of viral particles	Largely unlimited
Safety issues	Some viral vectors stimulate severe immune responses in the patient Concern regarding use of viral vectors based on HIV	Low toxicity to host
Longevity of expression	Transient (adenoviral-based-systems) or long-term (retroviral- or Adeno-associated virus, AAV-based systems) expression	Transient expression

Liposomes and DNA combine to form complexes referred to as *lipoplexes*. Since the initial investigations of lipoplexes nearly 30 years ago by Felgner *et al*., various cationic lipids and combinations of cationic and neutral lipids have been thoroughly examined for their potential as non-viral vectors [32] and subsequently this technology has been widely adopted and shown to be suitable for the transfection of many different cell types. Also, the method is comparable in efficiency to most other non-viral approaches to gene transfer *in vivo*. The first human gene therapy trail using cationic liposomes was conducted in 1992. The trail used 3β-[N-(Dimethylaminoethane) carbamoyl] cholesterol (DC-chol)/dioleoylphosphatidylethanolamine (DOPE) cationic liposomes to deliver the transgene of interest [171]. In recent years, optimization of lipoplexes has focused primarily on improving the targeting of these vectors to specific cells as well as increasing the release of DNA into the cytoplasm.

The cationic lipids in the liposomal formulation serve as a DNA complexation and DNA condensation agent during the formation of the lipoplex [172, 173]. The positive charge also helps in cellular association. The zwitterionic lipids help in membrane perturbation and fusion. Proprietary formulations of cationic lipids such as Lipofectamine (Invitrogen, Carlsbad, CA), Effectene (Qiagen, Valencia, CA), and Tranfectam (Promega, Madison, WI) are commercially available, but most of the transfection kids are useful only for *in vitro* experimentation.

Despite the appreciable success of cationic lipids in gene transfer, toxicity is of great concern. Cytotoxicity of cationic lipids has been established in numerous *in vitro* [174, 175] and *in vivo* [176, 177] studies. Additionally, the transfection efficiencies of non-viral cationic liposomal vectors are significantly lower than those of viral-vectors. Low transfection efficiencies have been attributed to the heterogeneity and instability of cationic lipoplexes [178]. Another drawback in the use of cationic lipids is their rapid inactivation in the presence of serum [173-178].

There are three types of lipids, anionic (negatively charged), neutral, or cationic (positively charged). Initially, anionic and neutral lipids were used for the construction of lipoplexes for non-viral vectors. However, in spite of the facts that there is little toxicity associated with them, that they are compatible with body fluids and that there was a possibility of adapting them to be tissue specific; they are complicated and time consuming to produce so attention was turned to the cationic versions.

All cationic lipids possess a hydrophobic group which may either be one or two fatty acid or alkyl moieties of 12-18 carbons in length or a cholesteryl moiety, in addition to an amine group. The hydrophobic moieties ensure that the cationic lipids assemble into bilayer vesicles on dispersion in aqueous media, effectively shielding the hydrophobic portion of the molecule and exposing the amine head groups to the aqueous medium. The amine group is an absolute necessity for transfection competence as this is the DNA binding moiety interacting electrostatically with DNA and condensing the large anionic molecule into small transportable units- lipoplexes. Structure-activity relationship studies have shown that increasing the number of amine groups per molecule [179] and the distance between the amine groups and the hydrophobic units [180] is advantageous to

gene delivery. This arrangement of atoms allows an intimate level of DNA binding in the lipoplex (by increasing contact size) as well as a separation of the bound DNA from the cohesive interaction of the hydrophobic units.

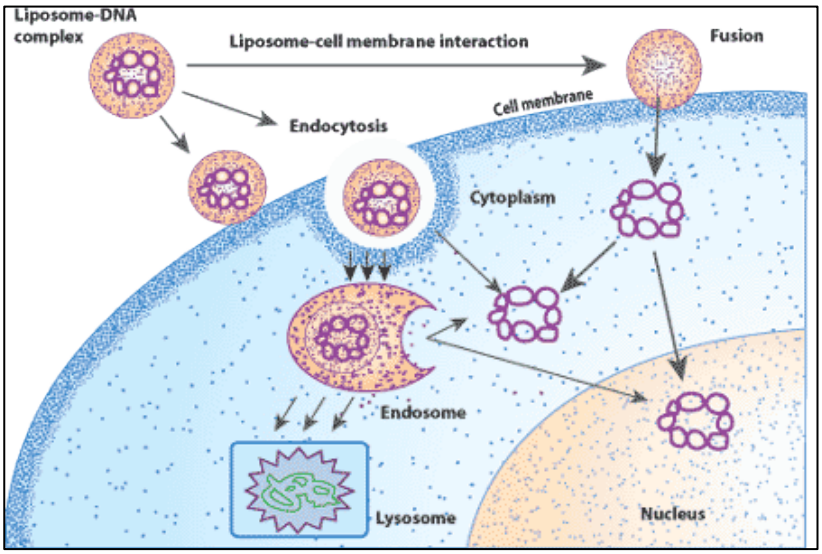


Figure 4.2. Liposome-mediated DNA delivery [181].

Cationic lipids, due to their positive charge, naturally form complex with the negatively charged DNA. Also as a result of their charge they interact with the cell membrane, endocytosis of the lipoplex occurs and the DNA is released into the cytoplasm. The cationic lipids also protect against degradation of the DNA by the cell. Cationic liposomes also have been used in clinical trials to deliver therapeutic DNA; for example, the liposomal-mediated delivery of the cystic fibrosis transmembrane conductance regulator (CFTR) gene to the nasal epithelium in patients with cystic fibrosis [182-184]. The cationic lipids in use currently are a mixture of neutrally and positively charged molecules. When the DNA and cationic lipids are mixed, DNA molecules condense and the positively charged lipid molecules bind to negatively charged phosphate groups on the DNA, which in turn form a lipid/DNA complex (Figure 4.2). Cells that come into contact with the DNA complexes take them up via a process of non-specific endocytosis or via fusion of the liposome/DNA complex with the cell membrane. Once inside the cell, the complexes are released and enter the target cell nucleus, where the therapeutic DNA is transcribed and translated to express the therapeutic gene product.

There are major barriers that must be overcome to improve the efficiency of cationic lipids as DNA-delivery vehicles. First, formation of the DNA/lipid complex can be a variable process and variation in such complexes might influence the efficiency of DNA delivery. Secondly, the efficiency by which the lipid/DNA complex is taken up by targeted cells must be improved.

4.1.2.3. Cationic Polymers. Cationic polymers are commonly used in gene delivery because they can easily complex with the anionic DNA molecules. Encapsulation of naked DNA by cationic polymers to form *polyplexes* is one of the primary non-viral vector systems that have been examined for the optimization of gene delivery. Versatility of physicochemical properties and easy manipulation are some of the most important advantages of polymeric gene carriers [185]. Polymeric matrices with varying properties can be designed by choosing an appropriate distribution of different molecular weights and degree of cross-linking of the polymer, and/or by the incorporation of the targeting ligands [186]. Industrial-scale manufacturing is feasible at low cost.

The general mechanism of action of polyplexes is based on the generation of a positively charged complex owing to electrostatic interaction of these cationic polymers with anionic DNA [186]. Generally, the cationic polymer first functions as a condensing agent, to collapse DNA into compact bundles of a size suitable for delivery. After the complex arrives at the target cells, the polymer must promote the release of DNA from the endosomal compartments into the cell. To accomplish these tasks, the cationic polymer must form a strong association with the DNA but release it under the appropriate conditions as well as have the ability to recognize specific cells. Synthetic polymers, such as protective interactive non-condensing polymers (PINC), poly-L-lysine (PLL), polyethyleneimine (PEI), cationic polymers or dendrimers, and arginine-rich proteins or peptides offer an alternative to cationic lipids as a vehicle for DNA delivery into target cells [187-191]. Agents such as folates, transferrin, antibodies, or sugars such as Galactose and mannose can be incorporated for tissue targeting [185]. In recent years, efforts have been directed towards optimizing the polymer to become more efficient at these functions, often by increasing the biocompatibility, primarily through modifications to existing polymers.

Polymeric transfection systems have advantages, but they are not ideal. In addition to low transfection capabilities, polymeric delivery systems suffer from problems in the control of molecular weight distributions, dispersities of the polyplexes, and other quality control issues [185].

4.1.2.4. Anionic Liposomes. As an alternative to cationic lipids, the potential of anionic lipids for DNA delivery has been investigated [35, 36, 192-195]. The safety of anionic lipids has been demonstrated when administered to epithelium lung tissue [176]. In recent years, a few studies [35, 36] using anionic liposomal DNA delivery vectors have been reported. They include the delivery of oligonucleotides to hippocampal neurons using anionic liposomes [36]. Similarly, an anionic lipid formulation called fluid liposomes, composed of DPPC and 1,2-dimyristoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)] (DMPG), was capable of delivering fluorescently labeled oligonucleotides into bacterial cells [195]. In this case, the entrapment of oligonucleotides within the liposomes was facilitated by Na^+ and K^+ ions. There have been attempts to incorporate anionic liposomes into polymeric delivery systems [196, 197]. For example, LPDII vectors are non-viral vehicles for gene delivery that consist of a complex between anionic pH-sensitive liposomes and polycation-condensed plasmid DNA (polyplexes) [196, 197]. However, these vectors have limited applications, mainly because of (i) inefficient entrapment of DNA molecules within anionic liposomes [193] and (ii) lack of toxicity data [198]. Lack of further progress of these systems may be attributed, in part, to the poor association between DNA molecules and anionic lipids, caused by electrostatic repulsion between these negatively charged species [193].

The interaction of divalent cations, particularly calcium, with phospholipid membranes has been a subject of interest for some time [199]. Such interest arises from the well-known regulatory role played by calcium in many biological phenomena, as well as from the ability of calcium to induce aggregation and fusion in phospholipid vesicular dispersions [200]. The phospholipid membrane-binding properties of Ca^{2+} versus those of other divalent cations have also been examined [199]. It has become clear in such investigations that, particularly for the case of membranes that are electrically charged, electrostatic forces exert an important influence on the ion-membrane interaction [201]. In its simplest form, such interaction may be considered in two parts, involving long-range

Coulombic and short-range interfacial "binding" (or adsorption) forces. The former have been treated successfully in terms of the Gouy-Chapman theory of the diffuse double layer [202, 203], whereas the latter generally are treated in terms of a Langmuir adsorption isotherm [204], in which the strength of the binding (or adsorption) interaction is expressed by a phenomenological association constant. The connection between the electrostatics and the binding lies in use of the interfacial value of the aqueous ion concentration in the adsorption isotherm. The interfacial value is determined from the ion concentration in the bulk aqueous phase by a Boltzmann relation involving the membrane electrostatic surface potential. The surface potential, in turn, is affected both by ions in the aqueous phase (screening) and ions that are bound or adsorbed by the membrane (alteration of surface-charge density). The "total" adsorption isotherm, giving the adsorbed ion density versus bulk aqueous ion concentration, including electrostatic effects, is known as the Stern isotherm [205].

Recently, the development of a novel anionic lipoplex delivery system with high transfection efficiency and low toxicity has been reported [175, 192, 193, 195]. This delivery system is composed of a mixture of anionic lipid 1,2-dioleoyl-*sn*-glycero-3-[phosphor-*rac*-(1-glycerol)] (sodium salt) (DOPG) and zwitterionic DOPE. These lipids are known to occur endogenously *in vivo* and are complexed to DNA molecules using divalent Ca^{2+} [36, 192]. The resulting ternary complex of lipids, calcium ions, and DNA was shown to be capable of transfecting mammalian cells with a model green fluorescent reporter protein (GFP) [196].

4.1.2.5. Toxicity of Non-Viral Vectors. Non-viral vectors, although less lethal than viral vectors, still may elicit a strong, non-specific immune response. Toxicity frequently results from characteristics of the encapsulating polymer or lipid such as the length, saturation, or branching of the polymer. Efforts to reduce the toxicity of non-viral vectors have largely resulted in efforts to make the vectors more biodegradable and biocompatible. Many of the systems previously mentioned (i.e. triggered release with disulfides, PEG copolymers) incorporated more biologically active components into the system, thereby reducing the immune response induced by the delivery of the vector. Further efforts to reduce toxicity have involved a method explored by Tan *et al*. which includes the sequential injection of liposome, then DNA as opposed to delivery of lipoplex. By injecting the liposome prior to

the plasmid, the toxicity levels were reduced by 80% compared to lipoplex [206]. Thus, significant advances have been made towards decreasing the toxicity of these non-viral vectors.

5. AIM OF THE STUDY

This work is based on the ability and efficiency of encapsulation materials by liposomes. The first part of the study involves the use of synthetic phospholipid mixtures, DMPC (zwitterionic phospholipids) and DMPG (anionic phospholipid); their preparation by different methods; variations in their compositions; insertion of cholesterol in their bilayer; their ability and efficiency in encapsulating hydrophilic (cytochrome-C) and hydrophobic material (Vitamin-E); and their characterization by Particle Size/Zeta Analyzer.

The second part of the study introduces the usage of these DMPC/DMPG liposomes as non-viral vectors in gene delivery. The slightly anionic liposomes, with cholesterol as a helper lipid, which are generally known as anionic lipoplexes, have been tested as non-viral vectors, DNA alone or in the form of a DNA-divalent cation (Ca^{2+}) complex, and analyzed on the targeted cell populations in terms of transfection efficiency and toxicity. SIK2, a serine/threonine kinase protein, has been chosen as the DNA segment and the MIO-M1, Müller cells have been the targeted cell populations for the gene delivery experiments.

6. MATERIALS AND METHODS

6.1. Materials

6.1.1. Phospholipids

DMPC (Sodium salt) was supplied from Sigma Aldrich. As a zwitterionic phospholipid (Figure 6.1 and Figure 6.2), it was found in white powder form (>99 % purity) and used without purification.

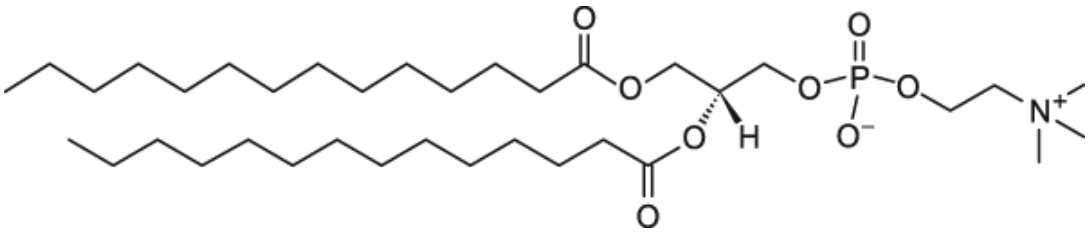


Figure 6.1. Structure of DMPC [207].

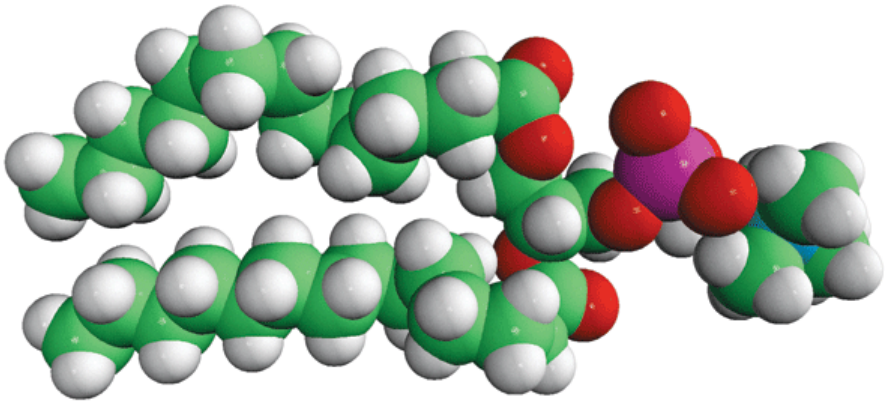


Figure 6.2. Three-dimensional structure of DMPC [207].

DMPG was supplied from Sigma Aldrich. As an anionic phospholipid (Figure 6.3 and Figure 6.4), it was found in white powder form (99% purity) and used without purification.

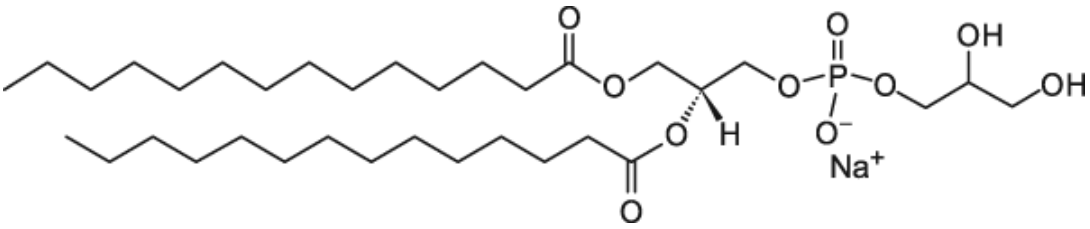


Figure 6.3. Structure of DMPG [207].

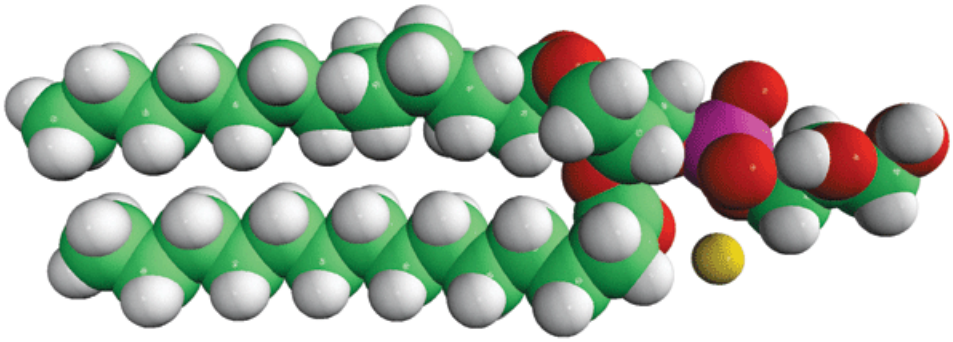


Figure 6.4. Three-dimensional structure of DMPG [207].

6.1.2. Cholesterol

Cholesterol (Figure 6.5. and Figure 6.6) was purchased from Sigma Aldrich (standard for chromatography). As a helper lipid during the preparation of liposomes, it was found in white powder form (99% purity) and used without purification. It is an essential structural of animal cell membranes that is required to establish proper membrane permeability and fluidity. In addition to its importance within cells, cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D.

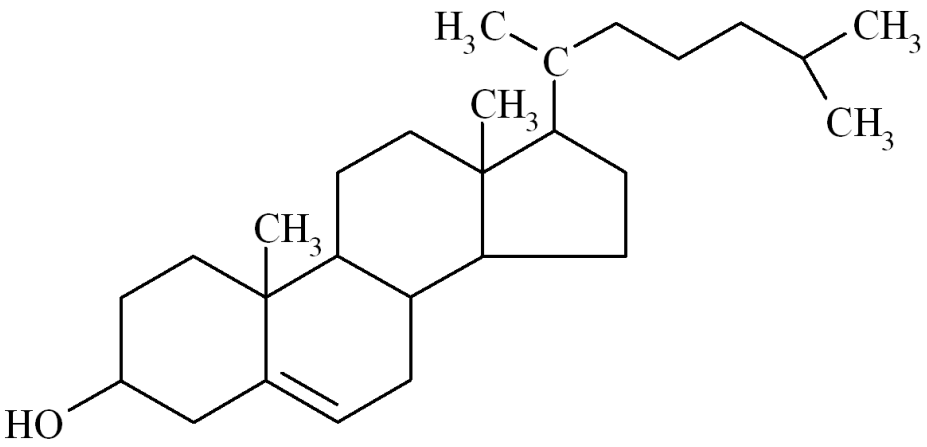


Figure 6.5. Structure of cholesterol [208].

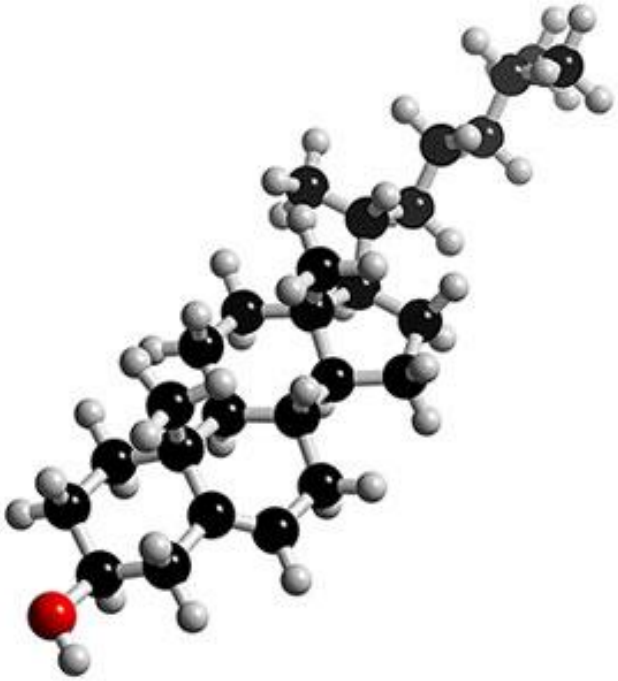


Figure 6.6. Three-dimensional structure of cholesterol [209].

6.1.3. Encapsulated Materials

6.1.3.1. Cytochrome-C (cyt-C). Cyt-C is a small heme protein found loosely associated with the inner membrane of the mitochondrion (Figure 6.7 and Figure 6.8). It belongs to the cytochrome c family of proteins. Cyt-C is a highly soluble protein, unlike other cytochromes, with a solubility of about 100 g/L and is an essential component of the electron transport chain (the site of oxidative phosphorylation in eukaryotes), where it carries on electron. It is capable of undergoing oxidation and reduction, but does not bind oxygen; it just transfers electrons between complexes. It was obtained from Sigma Aldrich. As an iron containing protein, it was found in red powder form (99%) and used without purification.

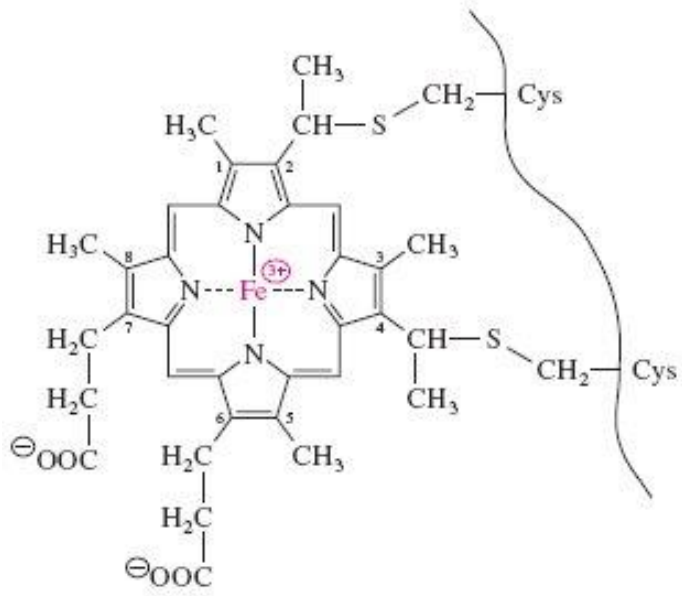


Figure 6.7. Structure of cyt-C [210].

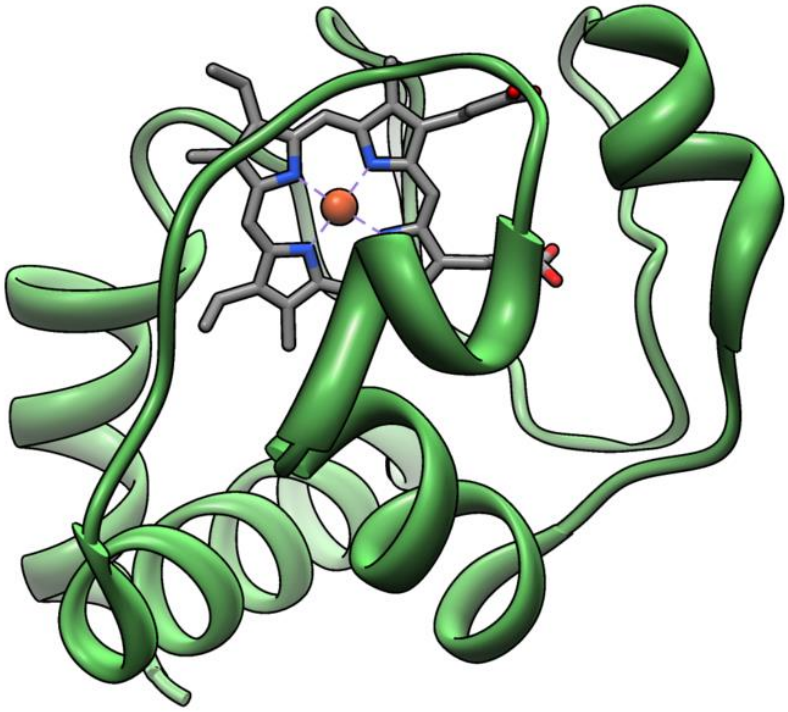


Figure 6.8. Three-dimensional structure of cytochrome c (green) with a heme molecule coordinating a central iron atom (orange) [211].

6.1.3.2. Vitamin-E (Vit-E, α-tocopherol). Vit-E (Figure 6.9) was purchased from Fluka. It was found in white powder form (≥95.5%) and used without purification. Tocopherols (TCP) (Vit-E) are a series (α, β, γ and δ) of chiral organic molecules that vary in their degree of methylation of the phenol moiety of the chromanol rings. TCPs are lipid soluble anti-oxidants that protect cell membranes from the oxidative damage but also seem to have many other functions in the body. A-Tocopherol is the form of tocopherol preferentially absorbed and accumulated by humans.

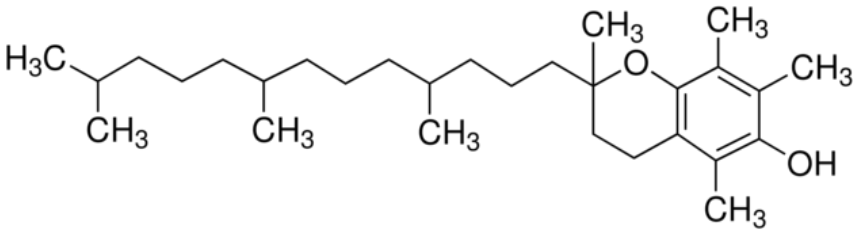


Figure 6.9. Structure of Vit-E [212].

6.1.4. Chloroform

Chloroform (99-99.4%), used to dissolve the phospholipids and cholesterol prior to the the thin film preparation by rotary evaporator, was purchased from Merck.

6.1.5. Phosphate Buffer (PBS) Materials

For the preparation of PBS, 1.36 g of potassium dihydrogen phosphate (KH_2PO_4, Merck), was dissolved in 100 mL deionized water and followed by the addition of 58.2 mL 0.1 M sodium hydroxide (NaOH, Merck). The solution was completed to 200 mL by the addition of of 41.8 mL of deionized water in order to have 0.05 M KH_2PO_4 buffer solution with a pH of 7.01.

PBS was used as an aqueous medium during the hydration of lipid thin film for the preparation of liposomes as well as in the washing steps of the cell lines in the transfection experiments.

6.1.6. Cholic Acid, Sodium Salt (Sodium Cholate)

DMPC/DMPG liposomes were broken down by the saturation of the bilayers with sodium cholate solutions. Sodium cholate, with 99% purity was supplied from Acros Organics. Sodium cholate (Figure 6.10) is a bile salt, a white crystalline substance insoluble in water. It is one of the two major bile salts produced by the liver where it is synthesized from cholesterol.

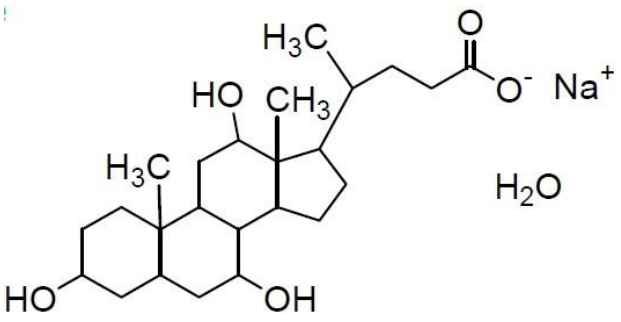
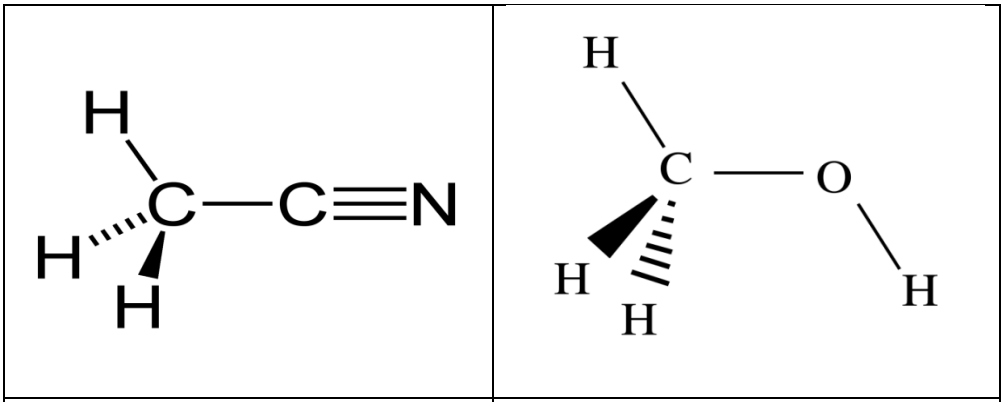


Figure 6.10. Structure of sodium cholate [213].

6.1.7. High-performance Liquid Chromatography (HPLC) Analysis Materials

Deionized water, methanol, and acetonitrile were used as mobile phases during the HPLC analysis. Methanol and acetonitrile (Figure 6.11) were obtained from Merck and were of liquid chromatographic grade.



Acetonitrile	Methanol

Figure 6.11. Structures of mobile phases of HPLC analysis, acetonitrile and methanol [214, 215].

6.1.8. Transfecting Reagent

The transfection experimental results were compared with the commercial transfecting reagent X-tremeGENE HP (Roche) which is a cationic non-liposomal solution with a high performance that transfects a broad range of eukaryotic cells, including insect cells and hard-to-transfect cell lines. The reagent is stable at room temperature, filtered through 0.2 μm pore size membrane, and active in serum containing medium.

6.1.9. Dulbecco's Modified Eagle's Medium (DMEM)

In the immortalization of MIO-M1 Müller glia cells, they were maintained in the DMEM, high glucose, GlutaMAX, pyruvate which was supplied from Gibco, Invitrogen. DMEM is a widely used basal medium for supporting the growth of the many mammalian cells. DMEM is unique from other media as it contains 4 times the concentration of amino

acids and vitamins than the original Eagle's Minimal Essential Medium (EMEM). DMEM with GlutaMAX supplement minimizes toxic ammonia build-up and improves cell viability and growth in an easy-to-use format.

6.1.10. Penicillin-Streptomycin 100X Solution

The antibiotics penicillin and streptomycin were used to prevent bacterial contamination on cell cultures due to their effective combined action against gram-positive and gram-negative bacteria. This solution contains 10000 units of penicillin, 10000 μg of streptomycin, and 29.2 mg / ml of L-glutamine in a 10 mM citrate buffer for pH stability and it was obtained from Gibco, Invitrogen.

6.1.11. Fetal Bovine Serum (FBS)

FBS, supplied from Sigma, was used in the cell culture during the immortalization of MIO-M1 Müller glial cells. It is the most widely used growth supplement for cell culture media because of its high content of embryonic growth promoting factors.

6.1.12. Paraformaldehyde (PFA) Fixative

In cell biology, fixation is a chemical process by which biological tissues are preserved from decay, thereby preventing autolysis and putrefaction. Fixation preserves a sample of biological material (tissue or cells) as close to its natural state as possible in the process of preparing tissue for examination. In this study, 4% PFA solution, obtained from Sigma, was used to fix the MIO-M1 Müller cells (Figure 6.12).

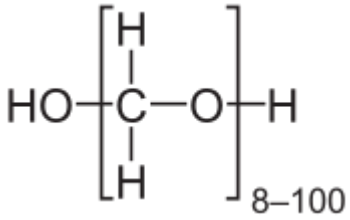


Figure 6.12. Structure of PFA [216].

6.1.13. GFP

GFP is a protein composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. Generate N- or C-terminal GFP fusion proteins to express and visualize a protein of interest fused to a green fluorescent protein in mammalian cells. In this study, GFP was fused to plasmid SIK2 so that the transfected cells could be visualized and counted via fluorescence microscopy. The GFP was supplied from Clontech Laboratories with excitation and emission maxima 475 and 505 nm, respectively (Figure 6.13).

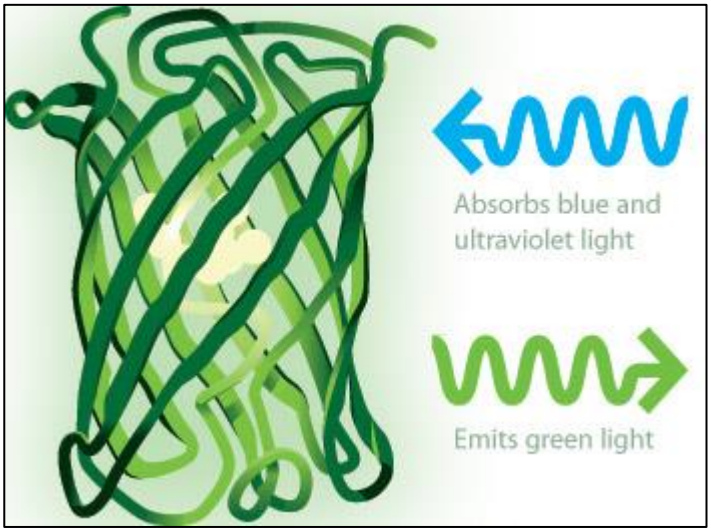


Figure 6.13. Schematic representation of GFP [217].

6.1.14. 4ˈ6-Diamidine-2ˈ-Phenylindole Dihydrochloride (DAPI)

The fluorescent dye, DAPI (Roche) (Figure 6.14 and Figure 6.15), was used to stain the cell nuclei in order to obtain the number of total cells present in the medium by fluorescence microscopy during the transfection experiments. DAPI selectively binds to adenine (A)-thymine (T) rich regions in DNA to form strongly fluorescent DNA-DAPI complexes with high specificity. On adding DAPI to tissue culture cells it is rapidly taken up into cellular DNA yielding highly fluorescent nuclei and detectable cytoplasmic fluorescence. When bound to double-stranded DNA, DAPI has a maximum absorption wavelength at 358 nm (ultraviolet) and a maximum emission wavelength at 461 nm (blue).

Therefore for fluorescence microscopy, DAPI is excited with ultraviolet light and is detected through a blue/cyan filter. The emission peak is fairly broad. DAPI's blue emission is convenient for microscopists who wish to use multiple fluorescent stains in a single sample. There is some fluorescence overlap between DAPI and green-fluorescent molecules like fluorescein and green fluorescent protein (GFP) but the effect of this, is small.

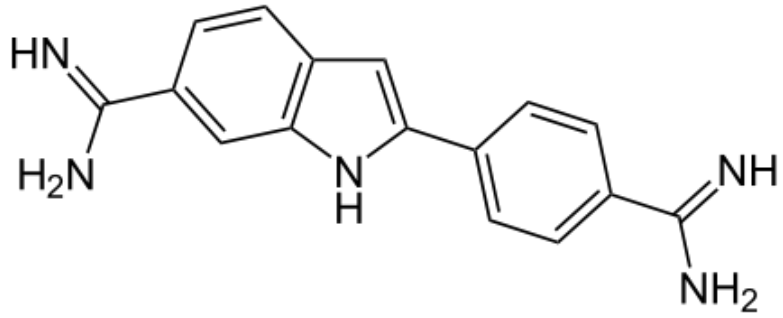


Figure 6.14. Structure of DAPI [218].

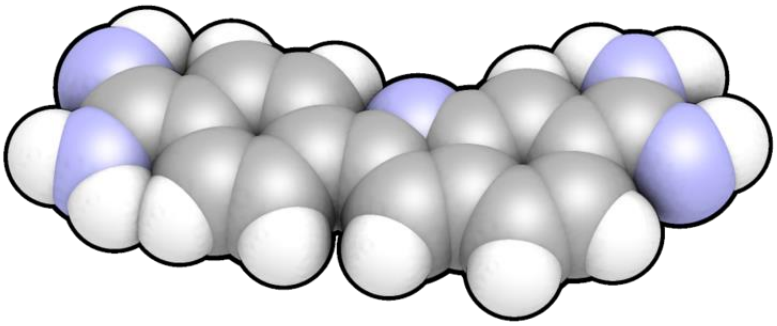


Figure 6.15. Three-dimensional structure of DAPI [218].

6.1.15. Rotary Evaporator

The thin film was obtained by evaporating the chloroform from the lipid mixture by using rotary evaporator (BIBBY, Rotary Evaporator, RE100).

6.1.16. Water Bath

The lipid mixture in the form of thin film was subjected to hydration with PBS in a water bath (Julabo SW 22), at a temperature higher than the T_c of the phospholipids, 55 ^{0}C.

6.1.17. Vortex Mixer Fusion Whirlimixer

Vortex Mixer Fusion Whirlimixer was used to homogenize the suspended lipid solution.

6.1.18. Liposome Down-Sizing Methods

In order to down-size the prepared liposomes, two different methods were used; extrusion and sonication. The former method was carried out by using Avestin Lipofast mini-extruder (mechanical energy), with two Hamilton syringes (Figure 6.16). Liposomes were extruded through 100 nm polycarbonate filters to ensure uniform sizes.

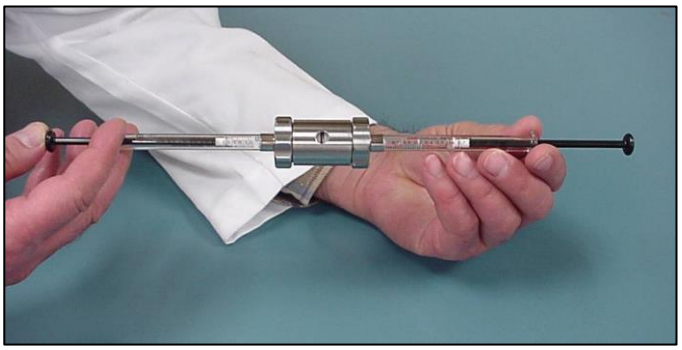


Figure 6.16. Avestin Lipofast mini-extruder [219].

The latter method, sonication (sonic energy), was also used to down-size the liposome solutions by the usage of sonicator, Bandelin Sonorex RK 52.

6.1.19. Centrifuge and Filtration

Centrifugation was carried out by Rotafix 32 centrifuge (Hettich, Zentrifugen), using Vivascience Vivaspin 2 mL concentrator or Millipore Centricon 30 kDa MWCO centrifugal filters (Figure 6.17) in order to remove the protein, Cyt-C in the bulk (exterior of the liposome structure).

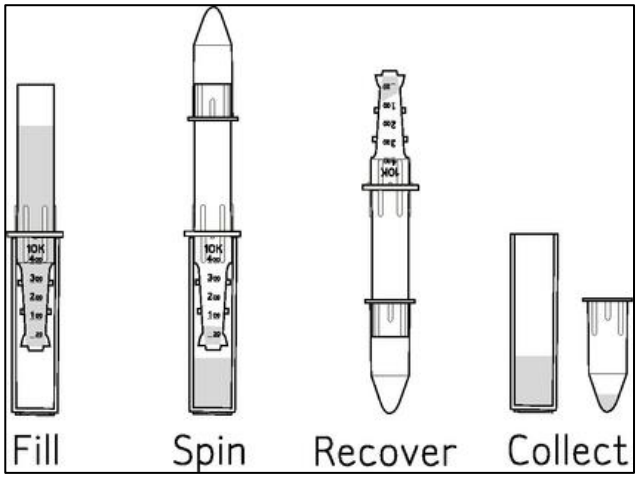


Figure 6.17. Schematic representation of centrifugal filters [220].

6.1.20. Ultraviolet Visible (UV/VIS) Spectrophotometer

Turbidity and protein encapsulation analysis were measured on a TU-1880 Double Beam UV/VIS Spectrophotometer.

6.1.21. HPLC

Encapsulated Vit-E amount was measured with a Water 1525 Binary HPLC which consists of a Water 2489 UV/VIS Detector, controlled by Water 1525 Pump, equipped with a 717 Autosampler, operating at room temperature. The analytical column was filled with the stationary C8 reversed-phase was SunFire (150×4.6 mm; Waters) made of stainless steel with a particle size of 5 μm.

6.1.22. Particle Size and Zeta Analyzer, DLS

Brookhaven Instruments 90 Plus Particle Size/Zeta Analyzer was used to measure the size and surface charge (zeta potential) of the liposomal systems.

6.1.23. The Neubauer Improved Haemocytometer

The Neubauer improved haemocytometer, Lumicyte Bright-Lined Ruling T.M. REG. 1/400 SQ MM, 1/10 MM DEEP, was used to count the total number of MIO-M1 Müller cells seeded.

6.1.24. Centrifuge

During the maintenance of MIO-M1 Müller cells, one of the steps included the usage of centrifuge in order to sediment the cell pellets. The instrument model was B. Braun Biotech International Centrifuge B5.

6.1.25. Fluorescence Microscopy

Fluorescence microscopy measurements were carried out to observe the transfected cells in the presence of GFP and count the total number of cells stained with DAPI using the Alexa Fluor 488 and DAPI filters, respectively. The instrument was Z1 AxioVision Inverted Fluorescent Microscope, Zeiss Observer.

6.2. Methods

6.2.1. Preparation of Liposomes

Liposomes were prepared using thin film evaporation method [221]. In this method, the lipids were first dissolved in an organic solvent, chloroform, in order to assure a clear and homogenous mixture of lipids. Once the lipids were thoroughly mixed in the chloroform, chloroform was removed by rotary evaporator to yield a thin film on the sides of a round bottom flask. The usage of a round-sided glass container of large volume makes

the lipids dried down onto a large surface area as possible to form a very thin film. The lipid film was thoroughly dried to remove the residual chloroform by placing the flask on a vacuum pump overnight. Phospholipids DMPC and DMPG were mixed with a molar ratio of 9:1. In all preparations, the initial DMPC and DMPG concentrations were 5 mM and 0.55 mM, respectively. Cholesterol-containing liposomes were prepared with a molar ratio of 9:1:3 DMPC: DMPG: cholesterol, although alternative ratios were also tried.

Hydration of the thin film was accomplished by the addition of an aqueous medium, phosphate buffer (pH 7.01), to the container of dry lipid and keeping the mixture at 55 ^{0}C in water bath for 12 hours. To obtain a homogenous colloidal system, the solution was vortexed for 10-20 minutes, which is one of the mechanical dispersion methods for the liposome preparation. When a stable, hydrated MLV suspension was produced, the particles were downsized through a polycarbonate filter with a defined pore size by mini-extruder. Prior to extrusion through the final pore size, MLV suspensions were subjected to 10 cycles of freezing (liquid nitrogen) and thawing (37 ^{0}C water bath). This method helps to prevent the membranes from clogging and improves the homogeneity of the size distribution of the final suspension. The extrusion was done about 10 ^{0}C above the T_c of the lipids. Large unilamellar liposomes by extrusion techniques (LUVETs) were generated by pressing the lipid dispersion through the 100 nm polycarbonate filters mounted in the mini-extruder, fitted with 0.5 mL Hamilton syringes. The samples were subjected to 11 passes through the filter. An odd number of passages were performed to avoid contamination of the sample by large liposomes which might not have passed through the filter.

Sonicated liposomes were prepared by sonicating the MLVs, which were obtained after thin film preparation, hydration and freeze and thaw cycles, for 15 minutes in sonic bath.

For both preparation methods, all liposome solutions formed had the typical bluish turbid appearance and remained so for at least two weeks. Once phospholipid liposomes were produced, they were kept at 27 ^{0}C, above the T_c of the phospholipids.

Liposomes containing Cyt-C were prepared by dissolving the required amount of Cyt-C in phosphate buffer prior to hydration of the dry lipid film. Liposomes containing Vit-E were prepared by dissolving the vitamin along with the lipids in chloroform, prior to evaporation in preparation of the thin film.

The presence and the concentration determination of encapsulates were carried out using both a UV/VIS double beam spectrophotometer (Cyt-C) and an HPLC (Vit-E).

6.2.2. HPLC Analysis

The mobile phase, having a volume ratio (64.5):(33):(2.5) of acetonitrile, methanol and water, respectively, was filtered and degassed under reduced pressure prior to use. Chromatographic separation was carried out isocratically at ambient temperature (25 ± 1 ^{0}C), and a flow rate of 1.0 mL/min, with UV detection at 260 nm. Run time was set to 12 minutes.

6.2.3. Removal of Excess Cyt-C

When lipid thin film was hydrated with Cyt-C containing PBS buffer (pH=7.01), some protein was encapsulated inside the liposome, whereas a large amount remained free on the outside. The protein in the bulk (exterior of the liposome) was removed by ultrafiltration. After each filtration, concentrated liposome sample containing Cyt-C was diluted with PBS. The process was repeated until no protein trace was detected in the filtrate spectrophotometrically since the maximum absorption wavelength (λ_{max}) of Cyt-C is 408 nm.

6.2.4. Breakdown of Liposomes

Malloy and Binford [222] have shown that the breakdown of DMPC/DMPG liposomes is possible when the bilayer is saturated with bile salt. This is done by mixing liposome and sodium cholate solutions at a 1:1 (v/v) ratio. After the addition of sodium cholate, the breakdown of the liposomes can be followed by the disappearance of the turbidity using a UV/VIS spectrophotometry at around 350 nm.

6.2.5. Parameterized Model Number 3 (PM3)

PM3 is a semi-empirical method for the quantum calculation of molecular electronic structure in computational chemistry. It is based on the Neglect of Differential Diatomic Overlap (NDDO) integral approximation. The PM3 methodology was used for the size calculations of the lipid molecules DMPC and DMPG, making use of the program package Spartan (30).

The total surface area of a spherical liposome can be calculated using;

$$A_{TOT} = 4\pi r^2 \tag{6.1}$$

where *r* is the hydrodynamic radius of the liposome, measured by the DLS.

Assuming that all the lipids are associated with the liposomes and each liposome is covered by lipid head groups, the number of lipids on the exterior of the liposome, N_{lipid}, is given by;

$$N_{lipid} = \frac{A_{TOT}}{A_{lipid}} \tag{6.2}$$

In equation 6.2, A_{lipid} is calculated head group area of the lipid.

Total number of lipids in the bilayer is $2\times N_{lipid}$.

Number of moles of lipids per liposome, M_{lipid}, is given by;

$$M_{lipid} = \frac{2\times N_{lipid}}{N_{AV}} \tag{6.3}$$

where N_{AV} is Avogadro's number.

The total volume of a liposome is given by;

$$V_{TOT} = \frac{4}{3}\pi r^3 \tag{6.4}$$

and the internal volume of the liposome is;

$$V_{inner} = \frac{4}{3}\pi r_{inner}^3 \tag{6.5}$$

where;

$$r_{inner} = r - 2l \tag{6.6}$$

and *l* is the length of the phospholipid including the head group.

The amount of liposomes in 1 L solution can be calculated;

$$\frac{\text{Liposome}}{\text{Litre}} = \frac{[\text{lipid}]_{\text{initial}}}{M_{\text{lipid}}} \tag{6.7}$$

Total "inner" volume in 1 L solution is calculated using;

$$V_{\text{total inner}} = V_{inner} \times \frac{[\text{lipid}]_{\text{initial}}}{M_{\text{lipid}}} \tag{6.8}$$

where $[\text{lipid}]_{\text{initial}}$ is the initial lipid concentration used in liposome preparation.

6.2.6. MIO-M1 Cell Culture Maintenance

Spontaneously immortalized MIO-M1 Müller glial cells were maintained in DMEM cell culture with glutamine supplemented with 10% FBS and 0.1% penicillin/streptomycin in CO_2 incubator. When the cell plates reached confluence, the cells were washed with 1×PBS, treated with 0.05% trypsin solution for 3 minutes and scraped to re-suspend cells adherent to the cell culture dish wall during the process of harvesting cells. The cells were pelleted by centrifugation at 2000×g for 5 minutes and after re-suspension in complete

medium, they were divided into three plates once a week in order to have exactly the same number in each well of the plate.

6.2.7. Transfection Experiments

One day before the transfection experiment, MIO-M1 Müller cell density was measured with haemocytometer. 50000 cells were seeded on 24-well plates, and grown in DMEM containing 10% FBS and 0.1% streptomycin/penicillin. At the day of transfection, prepared and filtersterilized phospholipid liposomes were added into 25 µL of DMEM and incubated for a determined time at room temperature. The SIK2-GFP vector was mixed with this liposome/DMEM complex and after incubation at room temperature, the mixture was added to the wells in a drop-wise manner. The plate contents were swirled on a rotating platform shaker for 30 seconds. After 48 hours transfection time, culture medium was decanted and cells were washed with 1xPBS and fixed with 4% PFA. Then cells were washed with PBS twice and finally incubated with DAPI in 1xPBS for 5 minutes to stain the cell nuclei. Cells were visualized with inverted fluorescence microscope by using Alexa 488 filter for GFP and DAPI filter for nuclei visualization to count the total number of cells in the medium.

7. RESULTS AND DISCUSSION

7.1. Liposomes for Drug Encapsulation Studies

7.1.1. Comparison of Preparation Methods In Terms of Liposome Size

As mentioned in Section 6.2.1, DMPC/DMPG liposomes, with or without cholesterol, were prepared by two different traditional methods: extrusion and sonication. The size measurement results, carried out by Brookhaven Instruments 90 Plus Particle Size/Zeta Analyzer, were compared. No significant size difference was determined between the two methods. As an example; the effective diameters were found to be 111.8 and 117.1 nm for extruded and sonicated liposomes, respectively (see Appendix A, Figure.A1 and Figure.A2).

7.1.2. Liposome Stability and Zeta Potential Analysis

Liposomes might undergo physical degradation, such as aggregation or coalescence, which affect the shelf-life of liposomes and causing in loss of liposome-drug association and change in size. Aggregation process is reversible and the small particles still retain their liposome identity while the aggregation moves as a single unit. Coalescence, in which the new larger particle moves as a single unit, is an irreversible, spontaneous process and it occurs in the direction of decreasing the Gibb's free energy.

The size results of the prepared liposomes, extruded or sonicated, with or without the cholesterol, were analyzed in order to understand their stability over a period of time. There is no significant difference between the immediate size of the liposome and the one measured after 3 weeks. Table 7.1 gives an example for the stability of cholesterol containing sonicated liposome.

Table 7.1. DLS analysis results to determine the stability of cholesterol containing sonicated liposome (see Appendix A, Figure.A3-Figure.A6).

Time	Effective Diameter (nm)
Immediately	148.3
After 1 week	145.4
After 2 weeks	143.3
After 3 weeks	145.8

Average results of hydrodynamic diameters, d_H, after 3 days are tabulated in Table 7.2. The hydrodynamic diameter of a particle is the effective diameter of an irregularly shaped particle that is used when describing the manner in which particles in suspension diffuse through the suspension medium. As can be seen from Table 7.2, independent of the method of preparation, when cholesterol is inserted into the bilayer, the liposomes become larger. The extruded liposomes are generally larger than the sonicated ones, however the difference is less pronounced when cholesterol is present in the lipid bilayer.

The zeta potential measurements were also carried out by the same instrument in the size measurements for DMPC: DMPG: cholesterol (9:1:3 molar ratio, respectively) liposome solutions. As it can be followed from Figure 7.1, the zeta potential of the colloid was determined approximately -20 mV.

Table 7.2. Liposome size measurements (× = not present, √ = present).

Cholesterol	Vit-E	Cyt-C	Extrusion	Sonication	d_H (nm)
×	×	×	√	×	89 ± 8
×	×	×	×	√	32 ± 5
√	×	×	√	×	120 ± 13
√	×	×	×	√	114 ± 10
×	√	×	√	×	77 ± 8

Table 7.3. Liposome size measurements (× = not present, √ = present). (cont.)

Cholesterol	Vit-E	Cyt-C	Extrusion	Sonication	d_H (nm)
×	√	×	×	√	55 ± 5
√	√	×	√	×	66 ± 6
√	√	×	×	√	54 ± 9
×	×	√	√	×	68 ± 10
×	×	√	×	√	50 ± 10
√	×	√	√	×	157 ± 40
√	×	√	×	√	152 ±25

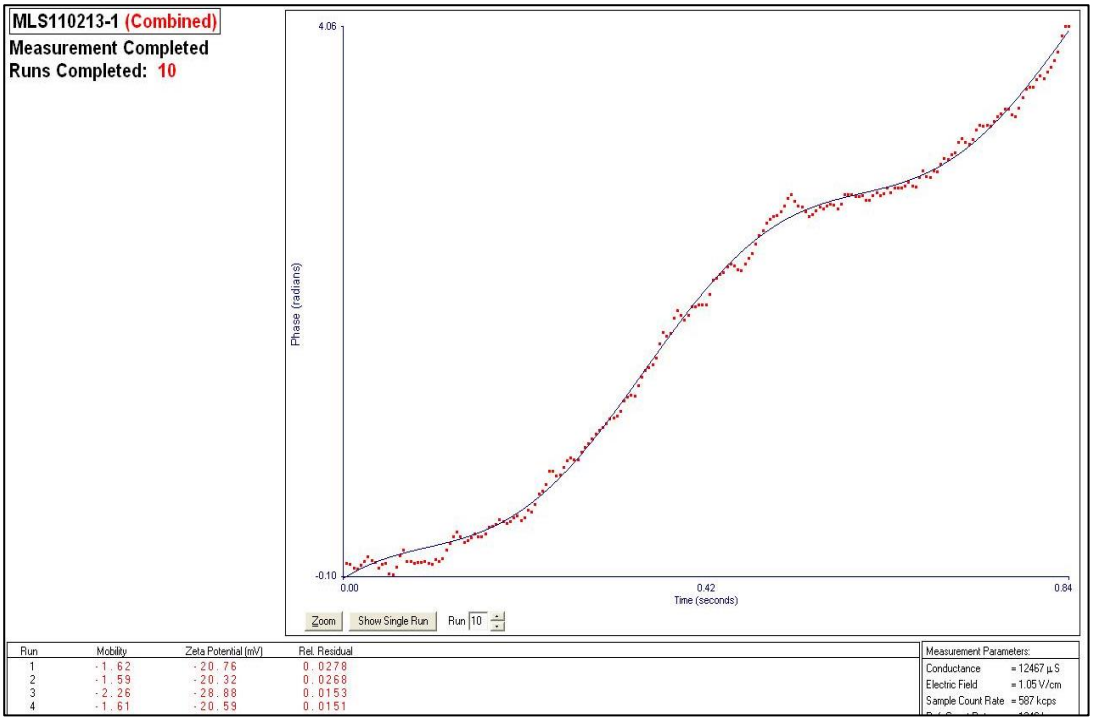


Figure 7.1. An example to zeta potential measurement of DMPC: DMPC: cholesterol, 9:1:3, liposome.

The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in the suspension have a large negative or positive zeta potential, then they will tend to repel each other and there will be no tendency for the particles to come together. However, if the particles have low zeta potential values then there will be no force to prevent the particles coming together and flocculating. Dispersions with a low zeta potential value will eventually aggregate and due to Van der Waals inter-particle attractions. Therefore, at low zeta potential values the colloidal systems are considered to be unstable and their size values might show an increment with time.

When the results of size and zeta potential analysis are considered together, although the zeta potential value of -20 mV indicated an incipient instability, the size measurements showed that the liposome systems preserved at least 3 weeks stability for each of the preparation methods, with or without cholesterol.

7.1.3. Turbidity Measurements of Liposomes

After the preparation steps of the liposomes, the solution is always bluish in color and turbid. This turbidity, indication of the presence of the liposome associations, of the liposomal solution can be followed by UV/VIS spectrophotometer by analyzing at 350 nm.

The turbidity of the liposomal solutions determined by absorbance values, given in Table 7.3, showed a variance even though each liposome was prepared with approximately the same amount of lipids and the same method of 15 minutes sonication.

The graph of the measured turbidity values, in terms of absorption, is given in Figure 7.2.

Table 7.4. Turbidity comparison for liposome solutions.

Liposome	DMPC (mg)	DMPG (mg)	Cholesterol (mg)	Absorbance at 350 nm.
1	34.4	4.1	6.7	1.3930
2	34.2	4.0	6.8	1.7250
3	34.3	3.8	6.3	1.0020
4	33.8	3.6	6.7	1.0036
5	34.1	3.9	6.7	1.5080
6	34.2	4.0	6.7	1.3990

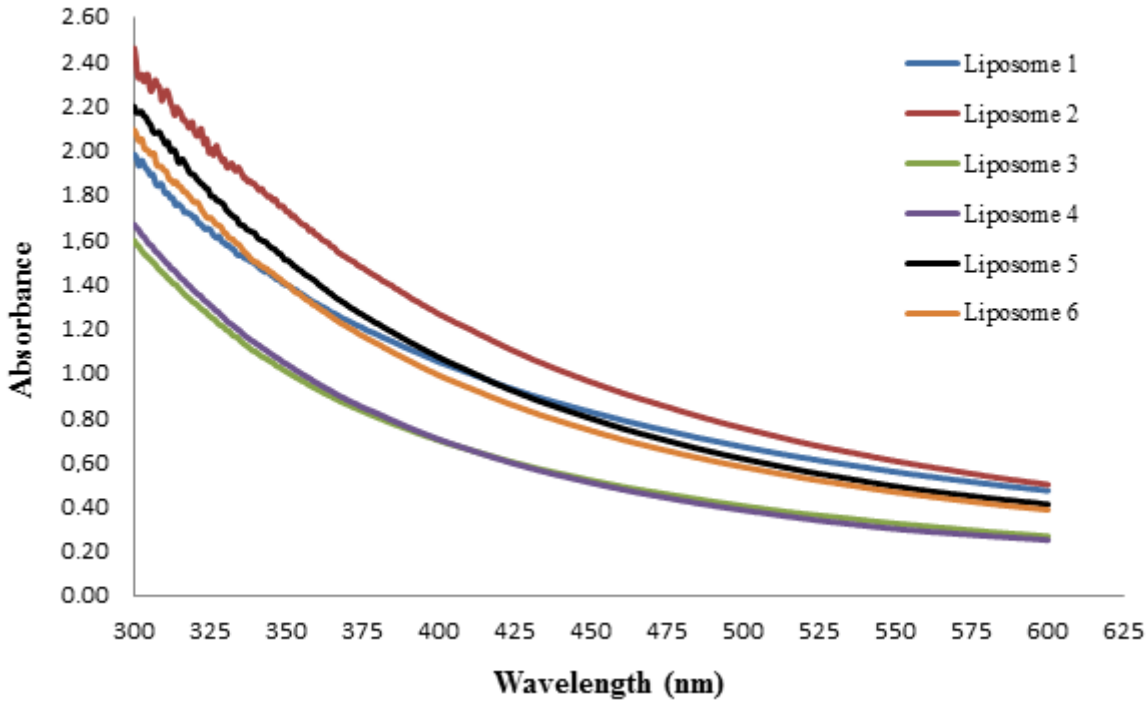


Figure 7.2. UV/VIS graph for the turbidity measurements of six different liposomes prepared with approximately same lipid weights and the same method.

7.1.4. Breakdown of Liposomes

DMPC/DMPG phospholipids liposomes were titrated with sodium cholate at room temperature. The suspensions became transparent, and a sharp drop in the absorbance was observed as incremental addition of the bile salt, sodium cholate.

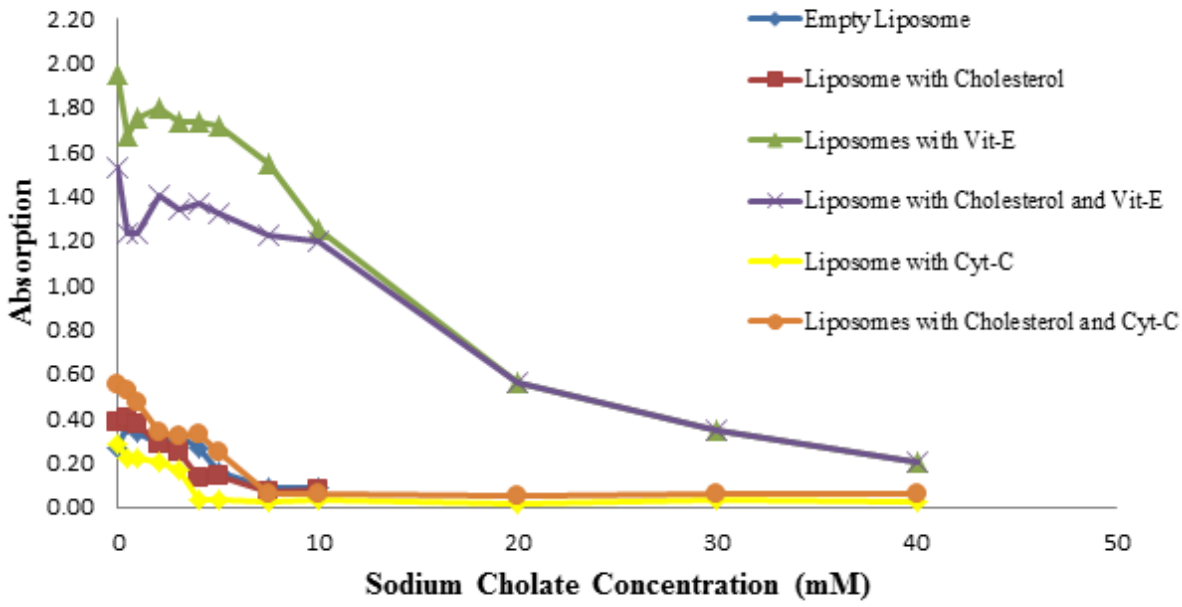


Figure 7.3. Breakdown of DMPC/DMPG liposomes upon addition of sodium cholate the bile salt at 350 nm.

Initially, minimum amounts of sodium cholate required to break down the liposomes were determined for all the systems. The behavior and the concentrations required were found to be independent of preparation. The results presented in the Figure 7.3 are for the "extruded liposomes" only.

It can be clearly seen from the Figure 7.3, 10 mM sodium cholate was sufficient for all liposomes except for those containing Vit-E. It was more difficult to break the Vit-E containing systems, irrespective of the presence of cholesterol, and 20 mM sodium cholate was required.

7.1.5. Encapsulation of Hydrophilic Cyt-C and Hydrophobic Vit-E in Liposomes

A sample solution, prepared at a weight ratio of 9:1:3 DMPC: DMPG: cholesterol, containing 0.4 mg/mL cyt-C, and extruded as described before, was filtered 9 times to remove the free cyt-C from the exterior of the liposomes. In the absorbance versus wavelength graph in Figure 7.4, the dashed lines show the decreasing absorbance (i.e. the amount) of cyt-C in the liposome solution after each filtration. The protein has a maximum absorbance wavelength of 408 nm. F0 corresponds to the cyt-C in the original solution, and F9 to the cyt-C in the solution after 9 filtrations.

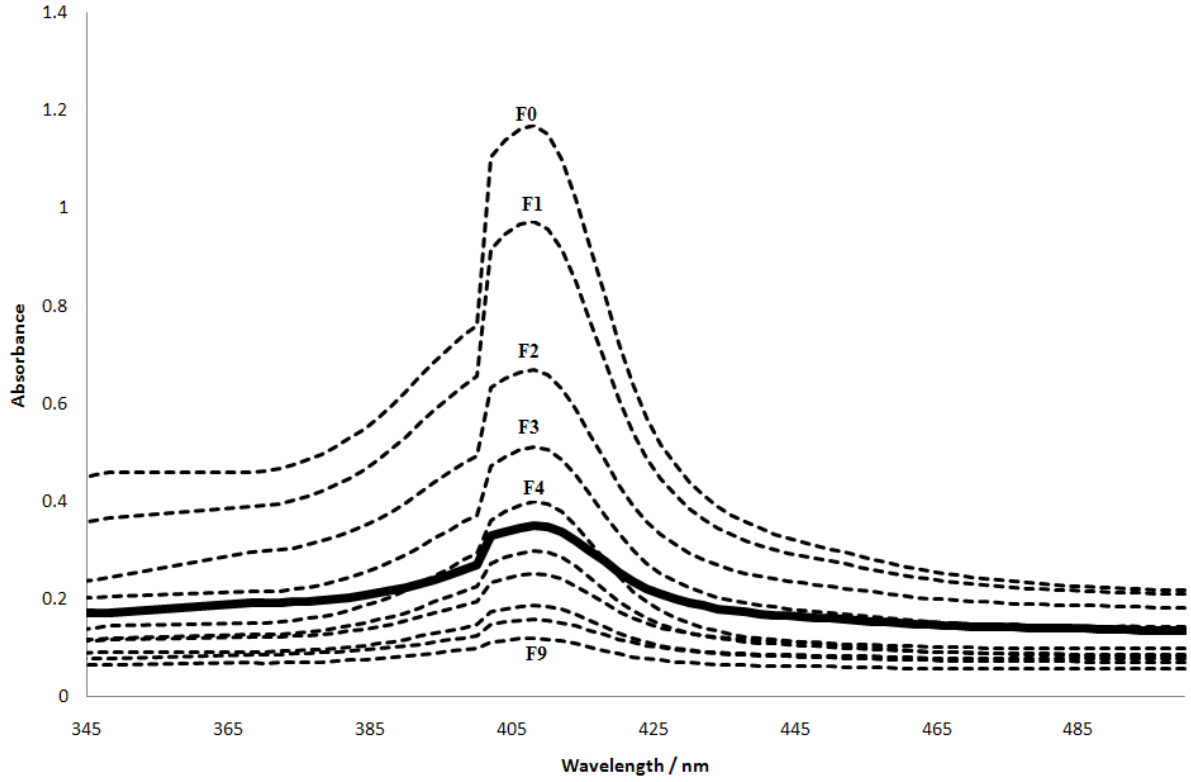


Figure 7.4. Absorbance plot of cyt-C in liposome filtrates and of release cyt-C [223]

The final solution of liposomes containing encapsulated cyt-C in the inner core was then broken down, by the addition of sodium cholate, to release the protein. The absorption of the released cyt-C was determined spectrophotometrically. The result is shown by the solid bold line which coincides with the absorption pattern of the protein and the amount of the released protein could be determined quantitatively (Figure 7.4).

Water insoluble Vit-E was encapsulated in the bilayer of the liposome system and the amount of encapsulation was measured with HPLC.

Table 7.5. Encapsulated amounts of cyt-C and Vit-E in 10 mL solutions.

Cholesterol	**Vit-E**	**Cyt-C**	**Extrusion**	**Sonication**	**Encapsulated Amount**
×	10 mg	×	√	×	All
×	10 mg	×	×	√	All
6.4 mg	10 mg	×	√	×	All
6.4 mg	10 mg	×	×	√	All
×	×	0.4 mg/mL	√	×	None
×	×	0.4 mg/mL	×	√	None
6.4 mg	×	0.4 mg/mL	√	×	0.077 mg/mL
6.4 mg	×	0.4 mg/mL	×	√	0.0071 mg/mL

The amounts of encapsulated cyt-C and Vit-E in extruded and sonicated liposomes, prepared with or without cholesterol, are tabulated in Table 7.4. The volume of each liposome solution was 10 mL, and the amounts of phospholipids, DMPC and DMPG, in them were kept constant at 34.4. and 3.7 mg, respectively.

There is a limited amount of Vit-E that can be solubilized in the bilayer with a certain lipid composition, and 38.1 mg lipid was found to solubilize 10 mg Vit-E. The solubilized Vit-E amount was independent of the method of liposome preparation and the presence of cholesterol in the bilayer. The lipid-soluble materials can be encapsulated with 100% efficiency.

The liposomes, in the absence of cholesterol, do not encapsulate any hydrophilic protein cyt-C whether it was prepared by extrusion or sonication. However, encapsulation of cyt-C was possible in the presence of cholesterol in the bilayer of the liposome. The amount of cyt-C encapsulated was found to be determined by the method of preparation, where extruded liposomes result in the encapsulation of 10 times more cyt-C than the sonicated ones.

7.1.6. Calculations of Available Volume for Encapsulation

In order to calculate the inner volume available for encapsulation values of head group area and length of the lipids were needed. The size calculations of the lipid molecules, DMPC and DMPG, were carried out semi-empirically with the PM3 methodology, making use of the program package Spartan (30). The head group area of DMPC and DMPG were 187 $Å^2$ and 156 $Å^2$, respectively. The total length, *l*, including the head group, for DMPC was calculated to be 26 Å. Since the lipid bilayer was predominantly composed of DMPC, all calculations were done using the DMPC size values. Using the measured hydrodynamic diameters, d_H of the liposomes (Table 7.2), and the equations given in Section 5.2.5, percent inner volume available for encapsulation was calculated for cholesterol containing liposomes.

At the lipid concentration of interest and in the presence of cholesterol, the ratio of the inner volume available for encapsulation for extruded and sonicated liposomes was found to be approximately 1:1.

From the encapsulation experiments, it has been shown that extruded liposomes encapsulate approximately 10 times more cyt-C than the sonicated ones. The higher encapsulation efficacy by the extruded liposomes cannot be attributed to the inner volume. Sonication appears to disrupt the liposomes and cause the leakage of cyt-C from the inner core.

In most of the mechanical dispersion methods, the inner aqueous volume enclosed within the bilayer is usually a small proportion of the total volume used for swelling-about 5-10%. Inner aqueous volume of sonicated DMPC/DMPG liposomes was calculated

to be 4.5% of the total volume whereas only 1.8% of the available cyt-C encapsulation was obtained. On the other hand, extruded liposomes with 4.8% inner available volume, encapsulated 19% of the available cyt-C. This high encapsulation is likely to be a result of higher tendency for the protein to be incorporated with the liposome, which might be due to electrostatic interactions between the negatively charged lipids and the positively charged cyt-C under the given pH conditions (pH= 7).

All these results, given in Section 7.1, have been published by Cagdas *et al.* [224].

7.2. Liposomes for Gene Delivery Studies

7.2.1. Determining the Type of Liposome as a Gene Delivery Vector

Four types of liposomes, extruded and sonicated empty liposome, extruded and sonicated cholesterol containing liposome, were tested for gene delivery experiments. These liposome types were all used in order to deliver the genetic material, SIK2-GFP vector. While keeping the SIK2-GFP vector amount (0.5 μg) and the incubation time (15 minutes) of the liposome with the vector constant, the volume of the liposome added into the cells was changed (0.5, 1, 2, and 5 μL).

Table 7.6. Summary of transfection efficiency results for the different types of liposomes with a volume of 5 μL (see Appendix B, Figure.B1-B7).

Liposome Type	**Transfection Efficiency (%)**
Sonicated liposome	1
Extruded liposome	1
Extruded cholesterol-containing liposome	1
Sonicated cholesterol-containing liposome	5

The volumes other than 5 μL and the liposomes prepared with extrusion, with or without cholesterol, failed to transfect any cells, meaning that the transfection efficiencies were nearly zero. In the presence of sonicated cholesterol-containing-liposomes with 5 μL

volume however, gave 5% transfection efficiency (Figure 7.5) (Table 7.5) whereas the same type of liposome in the absence of cholesterol showed no transfection. In this experiment, 2 μL sonicated cholesterol containing liposome solution was also promising in transfection but the efficiency was lower than the transfection with 5 μL.

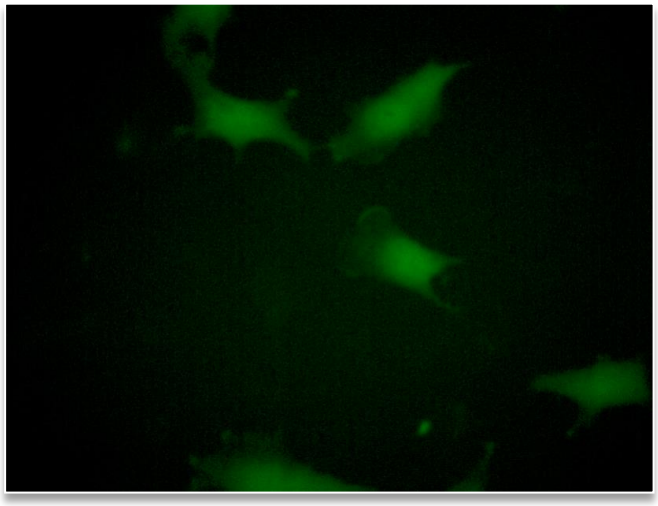


Figure 7.5. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated cholesterol containing liposome and SIK2-GFP vector (transfection efficiency approximately 5%).

In this set of experiments, the type of the liposome was tested as well as the volume effect. While obtaining the best result with sonicated cholesterol containing liposome, it was observed that as the liposome volume increased the transfection efficiency also increased. In the control experiments carried out by just adding the liposome solution in the cell media, no bacterial contamination was observed. The same experiments with the same parameters were also performed by using the cationic non-liposomal transfecting agent, X-tremeGENE HD (Roche), and the transfection efficiency was approximately 35% but it caused the cell death within 24 hour-time period.

7.2.2. Effect of Liposome Volume on Transfection Efficiency

In order to find the optimum volume of liposome solution that should be used in the transfection experiments, different volumes (between 2 and 20 μL) were used while

keeping the SIK2-GFP vector amount (0.5 μg) and the incubation time constant (15 minutes).

Transfection was observed only for solutions for 2 μL and 5 μL liposomes keeping all other variables constant. Since the best result (approximately 5%, as in the previous experiment) was obtained for 5 μL cholesterol containing sonicated liposome, this type with this volume of liposome system was used for the rest of the experiments (Appendix: B). It was determined that the extrusion method is not suitable for the transfection experiments and also the absence of cholesterol prevents liposomes from being a good transfecting agent.

As a control experiment, 5 μL transfecting reagent X-tremeGENE HD (Roche) in the presence of 0.5 μg SIK2-GFP vector with 15 minutes incubation time was used and again approximately the same transfection efficiencies, 35%, mentioned for the preceding experiments, were obtained.

7.2.3. Effect of Incubation Time on Transfection Efficiency

The incubation period is an essential determinant for a lot of experimental work in molecular biology for growing and maintaining cell cultures, as well as for complexing the transfecting agent and the genetic material.

Up to this point, the incubation time was kept constant at 15 minutes. Since it is the time taken for the liposome and SIK2-GFP vector association, it was thought that a longer incubation time might have a positive effect on the transfection efficiencies. The times were changed between 20 to 60 minutes, and keeping all other conditions unchanged (0.5 μg SIK2-GFP vector and 5 μL cholesterol containing sonicated liposome).

Table 7.7. Summary of transfection efficiency results for the effect of incubation time (0.5 μg SIK2-GFP and 5 μL liposome) (see Appendix B, Figure.B8 and Figure.B9).

Incubation time (min.)	**Transfection efficiency (%)**
20	5
30	12
60	17

It was determined that as the incubation time increased, the transfection efficiency obtained with 5 μL liposome in the presence of 0.5 μg SIK2-GFP vector increased (Table 7.6). For the case of 20 minute incubation time, the result did not differ than the one obtained with 15 minute incubation time. The transfection efficiency was again 5%, approximately. In the longer incubation time experiments, 30 and 60 minutes, the transfection efficiencies were determined as 12 and 17%, respectively (Figure 7.6).

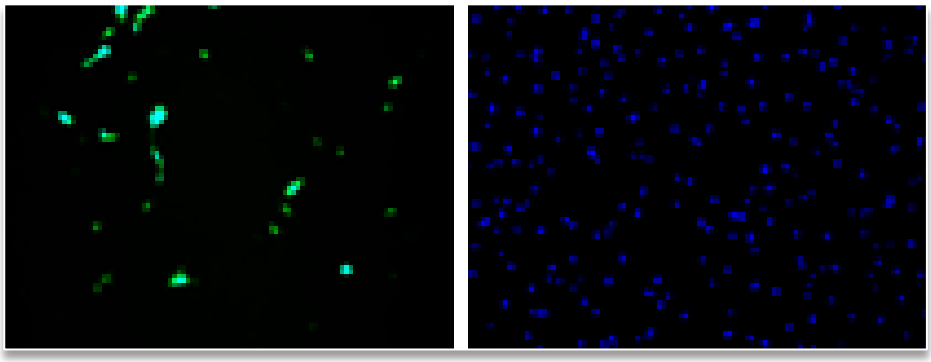


Figure 7.6. Fluorescence microscopy images for incubation time 60 minutes (17% transfection efficiency).

As a control experiment; 5 μL transfecting reagent X-tremeGENE HD (Roche) in the presence of 0.5 μg SIK2-GFP vector with 15 minutes incubation time was used and again approximately the same transfection efficiencies, mentioned for the preceding experiments, were obtained.

The incubation times of 90, 120, and 150 minutes were also tested with the liposome systems but no transfection efficiency greater than 17% was obtained.

7.2.4. Effect of Liposome Size on Transfection Efficiency

Up to this step, the size of the liposomes were kept between 120 and 165 nm as a result of the 15 minutes sonication, in the experiments carried out by the cholesterol containing sonicated liposome systems.

In order to see the effect of the liposome size on transfection efficiencies, the prepared DMPC: DMPG: cholesterol liposomes were further down-sized to 40 nm by increasing the sonication time. While keeping the rest of the experimental conditions constant, these smaller sized liposomes were experimented but none of them showed any transfection efficiency higher than 1%.

By lowering the size of the liposomes, it was thought that if the liposomes were small enough, they could enter the cell much more efficiently via endocytosis. Since the larger liposomes show much more transfection efficiency, there is a possibility of passing through the cell membrane via fusion (higher surface area makes it possible to fuse with the cell membrane much easily).

7.2.5. Effect of Cholesterol on the Transfection Efficiency

It is known that the presence of a neutral and helper lipid, cholesterol or its derivatives, in the liposomal structure enhances the gene transfer ability, increases the liposome biodegradation and subsequent release of “cargo” inside the cell. This fact was also confirmed with the initial experiments of this study where no transfection was observed by the liposomes without cholesterol.

The original liposome system was in the molar ratio of 10:3, phospholipid: cholesterol. The cholesterol amount was both decreased down to 10:1 and increased up to 10:5, phospholipid: cholesterol molar ratio, while keeping the size values between 120 and 165 nm at the end of 15 minutes sonication time, in order to see the effect of cholesterol

presence. The experiments carried out with these liposomes showed nearly no transfection (transfection efficiency was less than 1%). This result showed that the optimum molar ratio between phospholipids and cholesterol was 10:3. When the cholesterol amount was decreased, the liposome might lose its gene transfer ability and when it was increased there might be a strong interaction causing not to release the complexed DNA inside the cell.

7.2.6. Effect of Liposome Composition on the Transfection Efficiency

The liposome system DMPC: DMPG: cholesterol with a molar ratio of 9:1:3, as it was previously mentioned, has a slight negative charge. By changing the lipid weights, while keeping the total weight of the lipid mixture constant, the molar ratio of the liposome system DMPC: DMPG: cholesterol was changed to 10:1:3 and 8:1:3, with the same sizes (between 120 and 165 nm). The transfection experiments were carried out with 5 μL liposome and 0.5 μg SIK2-GFP vector at 60 minute incubation time.

The highest transfection efficiency was obtained with the original liposome composition DMPC: DPMG: cholesterol, 9:1:3 molar ratios at 60 minute incubation time. The ones with the different negative moieties showed no transfection efficiencies.

As a control experiment; 5 μL transfecting reagent X-tremeGENE HD (Roche) in the presence of 0.5 μg SIK2-GFP vector with 15 minutes incubation time was used and again 35% efficiency was obtained for the transfection of MIO-M1 cells.

As a neutral liposome at pH 7, DMPC: cholesterol with a molar ratio 10:3 was also prepared. The size of this lipid system was decreased approximately to 120 nm with 15 minutes of sonication. Before the transfection experiments, the lipid mixture was observed to coagulate which indicated the failure of the liposome formation. As expected, the transfection of the cells could not be observed.

7.2.7. Effect of DNA Amount on the Transfection Efficiency

The position of the hydrophilic DNA molecule is not clear. It could either be encapsulated within the core or at the interface of the liposome. So far, the amount of the DNA vector, SIK2-GFP, was kept constant (0.5 μg) while changing the other parameters in order to increase the transfection efficiency. This amount was increased to 1 and 2 μg, and the delivery systems were experimented after incubation of 60 minutes. After 48 hours of transfection time, none of the cells were transfected. The possible reason of the negative effect could be the increasing like-charge repulsions as the DNA and liposome were both anionic in nature.

7.3. Complexing DNA-Liposome Systems with Divalent Cation Ca^{2+}

Anionic lipids occur naturally in eukaryotic cell membranes and they can be complexed with anionic DNA via interaction with multivalent cations such as Ca^{2+}, Ba^{2+}, Mg^{2+}, and Mn^{2+} to form a DNA delivery system. As it is previously mentioned, Ca^{2+} is the most abundantly used divalent cation because of its regulatory role in many biological phenomena and ability to induce aggregation and fusion in phospholipid liposomal dispersions.

7.3.1. Determining the Complexation Sequence of Anionic Liposome, DNA and Divalent Cation, Ca^{2+}

The anionic liposomes, prepared using DMPC: DMPG: cholesterol (9:1:3 molar ratio), were initially incubated with Ca^{2+} cations for 15 minutes, and then re-incubated with DNA before applying them on the cells as non-viral delivery systems. When this incubation sequence was used, none of the MIO-M1 cells were transfected. It is possible that Ca^{2+} previously combined with the liposome system might have been disturbing the charge balance of the liposome, thus breaking down the system.

In order to prevent the disturbance of the charge balance on the liposome surface, DNA and Ca^{2+} were incubated initially for 15 minutes and then combined with the liposome solution prior to the addition to the cell medium. With this complexing

sequence, the cells were transfected and the rest of the experiments, to increase the transfection efficiency, were carried out using this sequence.

7.3.2. Determination of the Optimum DNA (SIK2-GFP) Amount in the Presence of Ca^{2+}

When Ca^{2+} is used as a complexing agent, the higher amount of positive moiety presence might increase the possibility of forming complex between negatively charged DNA and the liposome. In order to find the maximum SIK2-GFP amount that could be delivered into the cells, 0.5, 1, 2, and 4 μg were experimented with constant liposome volume (5 μL) and different Ca^{2+} concentrations in the range between 10 and 50 mM at 90 minutes of incubation times.

Promising results were obtained with 2 and 4 μg of SIK2-GFP amounts which showed that, in the presence of Ca^{2+}, the encapsulated amount of SIK2-GFP increases when compared to the 0.5 μg of SIK2-GFP complexing with liposome alone.

According to the results given in Table 7.7, it was clear that there was not a significant difference between the two SIK2-GFP amounts, 2 and 4 μg and with the same Ca^{2+} concentrations, approximately the same transfection efficiency results, were obtained.

Another deduction from these results could be that when the 20, 30, and 40 mM of Ca^{2+} solutions were used, appreciable transfection efficiencies were obtained but with 10 mM, at each SIK2-GFP amount transfection could not be obtained. In the case of 50 mM, the results were not consistent like the others.

Table 7.8. Transfection efficiency results with 2 and 4 µg of DNA at 90 minutes incubation time for different Ca^{2+} concentrations (see Appendix B, Figure.B10-B15).

SIK2-GFP Amount	**Transfection Efficiencies (%) at 90 minutes incubation time**				
	10 mM Ca^{2+}	**20 mM Ca^{2+}**	**30 mM Ca^{2+}**	**40 mM Ca^{2+}**	**50 mM Ca^{2+}**
2 µg	0	15	30	26	0
4 µg	0	15	33	25	10

As a positive control for this set of experiment, 2 µL X-tremeGENE HP in the presence of 0.5 µg SIK2-GFP was used for the transfection of MIO-M1 cells and a transfection efficiency of 18% (Figure 7.7). The liposome systems used in this experiment with 30 and 40 mM Ca^{2+} clearly showed significantly higher transfection efficiency.

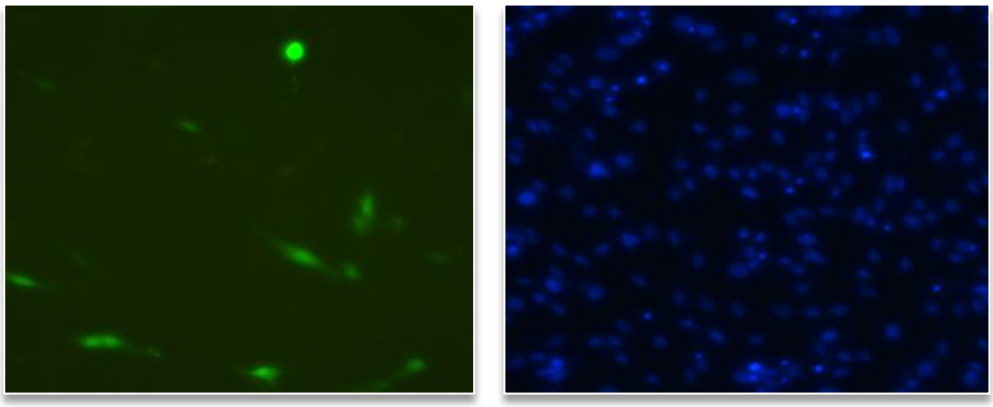


Figure 7.7. Fluorescence microscopy images for 0.5 µg SIK2-GFP with 2 µL X-tremeGENE HP (positive experiment) (18% transfection efficiency).

The same experiment was also carried out by increasing the incubation time from 90 to 180 minutes. The ones in the presence of 20 and 30 mM Ca^{2+} showed appreciable amounts of transfection efficiency whereas with the concentrations 10, 40, and 50 mM of the divalent cation, no transfected cells were observed (Table 7.8). When the SIK2-GFP amounts were compared, again there was no significant difference between them in terms of transfection efficiencies. The efficiencies obtained with 20 mM Ca^{2+} at 180 minutes

incubation time were higher than the ones at 90 minutes, on the other hand with 30 mM Ca^{2+} at 180 minutes were lower than the ones at 90 minutes.

Table 7.9. Transfection efficiency results with 2 and 4 μg of SIK2-GFP at 180 minutes incubation time for different Ca^{2+} concentrations (see Appendix B, Figure. B16-B21).

SIK2-GFP Amount	**Transfection Efficiencies (%) at 180 minutes incubation time**				
	10 mM Ca^{2+}	**20 mM Ca^{2+}**	**30 mM Ca^{2+}**	**40 mM Ca^{2+}**	**50 mM Ca^{2+}**
2 μg	2	26	14	0	0
4 μg	2	33	15	0	0

In the control experiment; 2 μL X-tremeGENE HP in the presence of 0.5 μg SIK2-GFP, the transfection efficiency was determined as 20% which is lower than the transfection efficiencies obtained with 20 mM Ca^{2+} system at each SIK2-GFP amounts.

Other control experiments were also carried out in order to see the effect of each parameter individually. From these control experiments (Table 7.9), it was determined that the SIK2-GFP (naked DNA) could not manage to transfect any cells due to the repulsion between the negatively charged cell membrane and the DNA itself (Experiment 3, in Table 7.9). SIK2-GFP with calcium ion with or without liposome showed 15 and 35% transfection efficiencies (Experiment 1 and 4, in Table 7.9), respectively. According to these results; liposome presence has a positive effect on the number of transfected cells. Again, a higher transfection efficiency was determined with liposome system containing calcium than the non-liposomal X-tremeGENE HP transfecting reagent (Experiment 1 and 5, in Table 7.9).

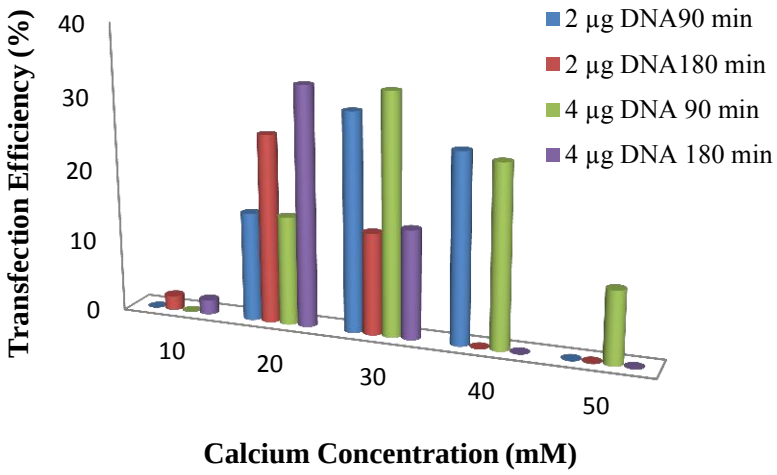


Figure 7.8. The transfection efficiency percentage results while changing the SIK2-GFP amount and the incubation time at each Ca^{2+} concentration.

Table 7.10. Control Experiments (see Appendix B, Figure.B22-B27).

Control Experiment	Experimental Parameters	Transfection Efficiency (%)
1	30mM Ca^{2+}- 4 µg SIK2-GFP -5 µl liposomes-90 minute incubation time	35
2	4 µg SIK2-GFP -5 µl liposomes-90 minute incubation time	1
3	4 µg SIK2-GFP-90 minute incubation time	0
4	30 mM Ca^{+2}-4 µg SIK2-GFP-90 minute incubation time	15
5	0.5 µg DNA-2 µL X-tremeGENE HP (positive control)	20

7.3.3. Determination of the Optimum Liposome Volume in the Presence of Ca^{2+}

When no cationic mediator was present, the volume of liposomes were changed between 2-20 µL and only the solutions with 2 and 5 µL liposomes were observed to show transfection (see Section 7.2.2). The same experiment was repeated in the presence

of the divalent cation, Ca^{2+}. The SIK2-GFP vector was kept constant at 4 μg, the Ca^{2+} concentrations were between 10 and 50 mM, and the incubation time was 90 minutes. 10, 40, and 50 mM Ca^{2+} concentrations were unable to transfect the cells. The transfected cells were only observed with 20 and 30 mM Ca^{2+} when the liposome volumes were 2, 5, or 8 μL (Table 7.10). With the other volumes (10, 12, and 20 μL), no transfected cells were observed.

Table 7.11. Transfection efficiency percentages when changing the liposome volume in the presence Ca^{2+} with 4 μg SIK2-GFP and 90 minutes incubation time (see Appendix B, Figure. B28-B34).

	Transfection Efficiencies (%)		
[Ca^{2+}]	**2 μL liposome**	**5 μL liposome**	**8 μL liposome**
20 mM	1.8	7.5	11
30 mM	22.7	17.4	30

Even though 30% transfection efficiency was observed with 8 μL liposome, approximately the same value was also achieved with 5 μL liposome volume (Table 7.9). Although the experiments carried out with liposome volumes < 2 μL and > 8 μL showed very low or no transfection, 2-8 μL of liposome volumes obtained up to 30% transfections. Taking this into account, 5 μL was taken as the optimum liposome volume and used thereafter.

7.3.4. Effect of Liposome Composition on the Transfection Efficiency in the Presence of Ca^{2+}

DMPG in the liposome formulation has a negative character. The liposome composition has been 9:1:3 molar ratio with respect to DMPC: DMPG: cholesterol as it has been previously mentioned. If the DMPG amount is increased in this formulation, the positively charged calcium ion might be expected to form a complex between the liposome and negatively charged SIK2-GFP vector. For this purpose, a set of liposome solutions with increasing amount of DPG, hence increasing negative charge density, was prepared. The molar ratios DMPC: DMPG: cholesterol used were 7:3:3, 5:5:3, 3:7:3, as

well as 1:9:3 molar ratios (Figure 7.9-7.13). These new solutions were tried on the MIO-M1 cells for transfection, in the presence of both 2 and 4 µg SIK2-GFP vector and each Ca^{2+} concentration at 90 minutes incubation time as well as 180 minutes.

The highest transfection efficiencies (between 15 and 25%) were obtained with the original liposome formulation where the molar ratio was 9:1:3 molar ratio of DMPC: DMPG: cholesterol, respectively. In consistence with the previous experiments, 20 and 30 mM of Ca^{2+} concentrations were the most suitable ones for each SIK2-GFP amount and at each incubation time.

On the other hand, the other liposome formulations with higher anionic character showed maximum transfection efficiency around 10% except the liposome formulation of DMPC: DMPG: cholesterol with a molar ratio of 1:9:3, respectively. In this 1:9:3 liposome formulation, the anionic character is the highest among the others. When the Ca^{2+} concentration was 40 mM, it showed very appreciable transfection efficiencies around 20% at each SIK2-GFP amount but at higher incubation time, 180 minutes.

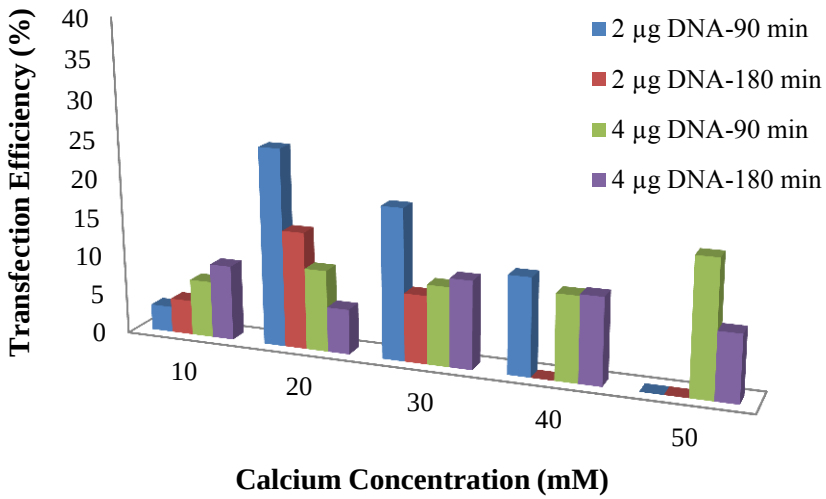


Figure 7.9. Transfection efficiency percentage results for 9:1:3 molar ratio DMPC:DMPG:cholesterol.

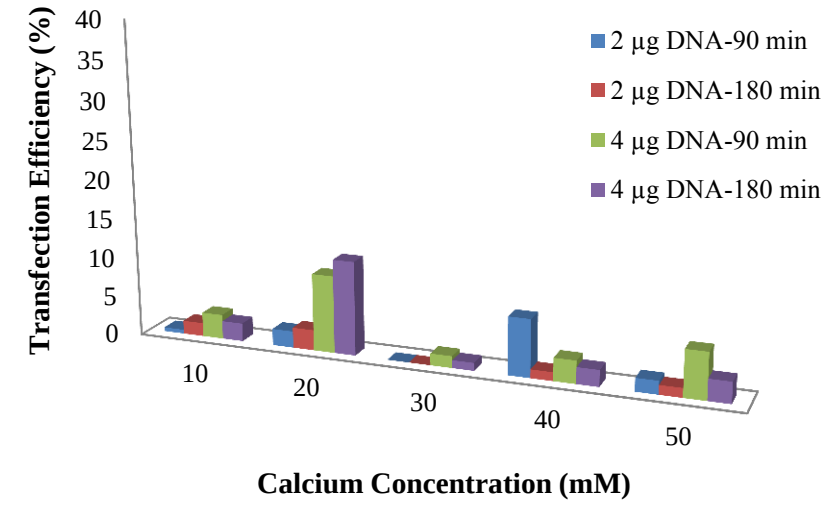


Figure 7.10. Transfection efficiency percentage results for 7:3:3 molar ratio DMPC:DMPG:cholesterol.

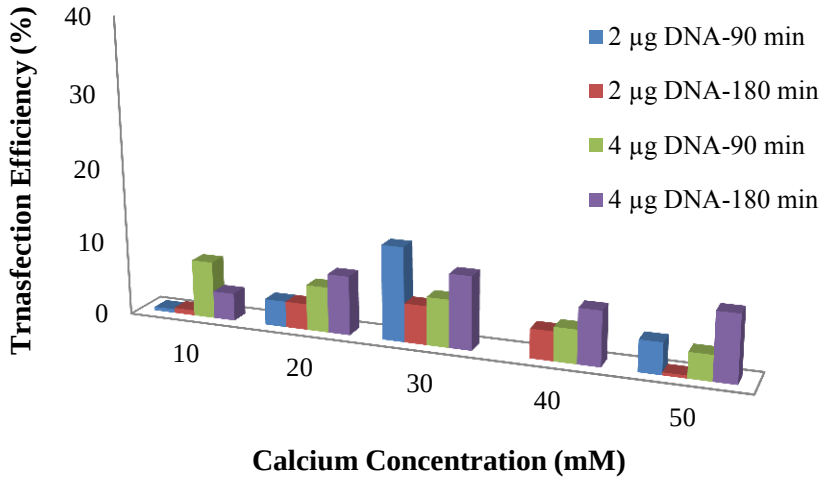


Figure 7.11. Transfection efficiency percentage results for 5:5:3 molar ratio DMPC:DMPG:cholesterol.

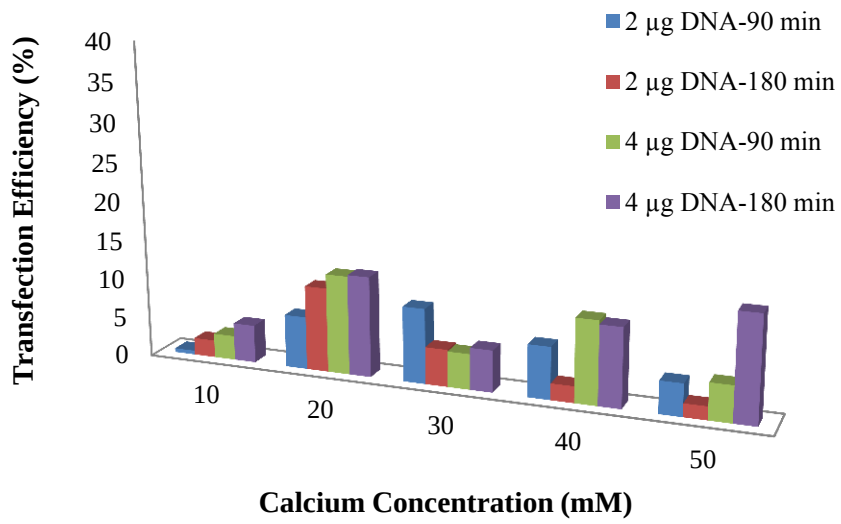


Figure 7.12. Transfection efficiency percentage results for 3:7:3 molar ratio DMPC:DMPG:cholesterol.

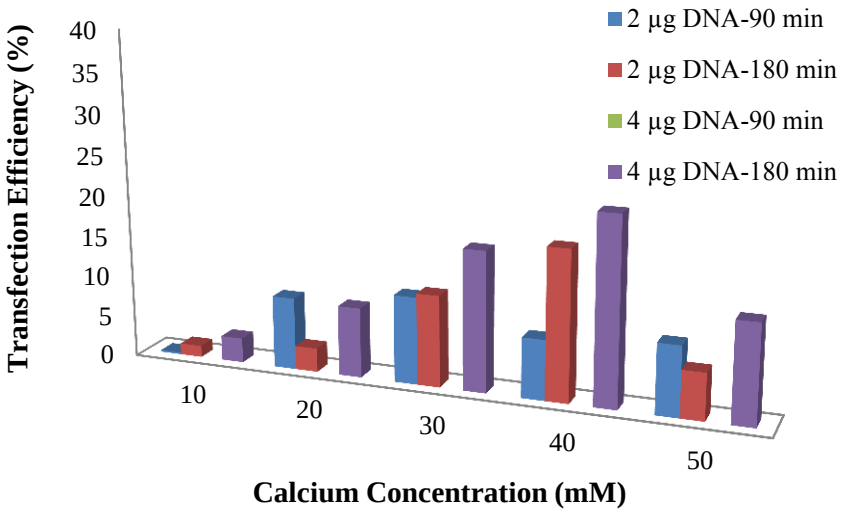


Figure 7.13. Transfection efficiency percentage results for 1:9:3 molar ratio DMPC:DMPG:cholesterol.

7.3.5. The Liposome Effect on the Transfection Efficiency in the Presence of Ca^{2+}

Two liposome solutions having the original lipid composition (DMPC: DMPG: cholesterol with 9:1:3 molar ratio) were prepared with approximately the same size and the same turbidity behavior. In Table 7.11, absorbance values are given as a measure of turbidity.

Table 7.12. Turbidity and size value of two liposomes; Liposome 1 and Liposome 2.

	Turbidity at 350 nm	**Effective Diameter (nm)**
Liposome 1	1.005	115.2
Liposome 2	1.041	118.2

The transfection experiments carried out for 20 and 30 mM Ca^{2+} concentrations with 2 μg SIK2-GFP and at 90 minutes of incubation time.

According to the given transfection efficiency values given in Table 7.12, there is a difference between the numbers of transfected cells when the liposome systems are compared. However, they had approximately the same size and same turbidity absorbance at 350 nm. This could be due to some disruptions occurred during the preparation steps of the liposomes or some coagulation. Also, when the transfection efficiencies of the same liposome on two different cell batches are compared, it can be determined that different transfection efficiencies could be obtained depending on the MIO-M1 cells. The passage number of the cells, which is the number of times the cells in the culture have been subcultured, is very influential on the transfection. If the passage number is high, then there could be this kind of inconsistencies with the transfection efficiencies.

Table 7.13. Transfection efficiency percentages of the two liposomes on different batches of MIO-M1 cells.

	Transfection Efficiency %			
	20 mM Ca^{2+}		**30 mM Ca^{2+}**	
	Batch 1	**Batch 2**	**Batch 1**	**Batch 2**
Liposome 1	10.4	5	3.5	4.0
Liposome 2	7.7	6.6	8.6	7.4

7.3.6. The Effect of Changing the Cell Type on the Transfection Efficiency in the Presence of Ca^{2+}

Human Embryonic Kidney 293 (HEK 293) which are known to be transfected more readily than the MIO-M1 cells were also examined for transfection efficiencies with the anionic liposome and the calcium ion system.

Although the fluorescence microscopy images obtained in the presence of 20 and 30 mM Ca^{2+} were not clear enough to count the tranfected cells in these preliminary result, the ability of this system to transfect different types of cells have been shown. These cells are promising for further investigation in these transfection studies.

8. CONCLUSION

i) Unilamellar liposomes, prepared either by extrusion or sonication from synthetic phospholipid mixture of DMPC and DMPG, with or without cholesterol, have been used to entrap water-soluble and -insoluble molecules. The ones prepared by extrusion method were generally larger than the sonicated liposomes, and hence had a larger inner volume. This situation changed when cholesterol was introduced into the bilayer and the liposomes prepared by either method became more comparable in size, and hence in their inner volume.

ii) When the liposomes were down-sized from MLVs to SUVs immediately after preparation, their sizes were determined as 148.3 nm. The same system was measured to have 145 nm liposomes after 3 weeks. Although the measured, -20 mV, zeta potential value is in the "incipient stability" range, the size measurements confirm the stability for this period of time.

iii) Encapsulation of hydrophilic protein cyt-C was only possible when the bilayer contained cholesterol. Cholesterol stabilizes the bilayer, hindering the transfer of hydrophilic molecules in between the inner and outer aqueous regions. However, despite similar available inner volume for cyt-C encapsulation, the encapsulated amount was at least 10 times higher with extruded liposomes. As a preparation method, sonication appears to disrupt the bilayer and cause the leakage of cyt-C from the inner core, since it has the risk of degradation of lipids. In extrusion; repeated cycles make the liposome solution progressively more unilamellar in character with the size distribution, but now possessing a considerable amount of internal aqueous volume.

iv) A maximum amount of hydrophobic Vit-E, on the other hand, could be encapsulated in the bilayer, regardless of the presence of cholesterol. A similar amount could be taken up by the liposomes prepared by either method. The lipid: Vit-E ratio seems to be more of a determinant in the maximum encapsulation than the method of preparation.

v) The liposomes, DMPC: DMPG: cholesterol, were slightly negative due to presence of the anionic phospholipid, DMPG at physiological pH 7. DNA is also negatively charged due to the presence of phosphate groups in each nucleotide and it was observed that naked DNA (with no vectors) cannot transfect the MIO-M1 cells. A transfection observed with these liposome vectors in the preliminary experiments was very promising in spite of the possible repulsion between the negative moieties in each structure. Therefore, the follow up experiments were built on to increase the transfected efficiencies initially obtained.

vi) Among the sonicated, extruded, sonicated cholesterol containing, and extruded cholesterol containing liposomes; the highest transfection efficiency was numbered when the sonicated liposomes with cholesterol were used whereas the extruded ones did not show any transfection in the presence or absence of cholesterol. The suitable method of liposome preparation was sonication. Since the sonicated liposomes could not manage to transfect any of the cells in the media, the presence of the cholesterol inside the bilayer of the liposome is a must for the gene delivery experiments.

vii) When the liposome volumes were experimentally changed, the best transfection efficiency results were obtained when 5 μL was used. This optimum volume was used for transfection experiments.

viii)With the increased incubation time, for bringing the liposome in contact with the cells, from 15 to 60 minutes, the transfection efficiency was increased up to 17% whereas it was determined around 35% with the cationic transfecting reagent. The lower transfection efficiency than the cationic transfecting reagent could be attributed to the electrostatic repulsion between the negatively charged DNA and anionic liposome during the complex formation and further repulsion with the negatively charged cell membrane. One explanation for this might be that after the incubation of liposome and DNA, the size of the complex becomes higher than the initial one. This might cause the rapid “digestion” of the large sized complex by the reticuloendothelial system (RES) of the cell.

ix) The cell viability for the ones transfected with the liposome system were obtained for more than 2 weeks whereas the cell death was observed less than in 48 hours when the transfecting reagents were used.

x) When the composition of the liposomes was changed with respect to DMPG, so that the anionic charge could also be changed, no significant change was observed.

xi) The small size liposomes (around 40 nm) which could be taken up via endocytosis provided no transfection. This strengthens the argument that the liposomes pass through the cell membrane via fusion.

xii) When the liposomes with different compositions in terms of the negative charge (molar ratio of anionic phospholipid, DMPG was changed) did not show any better transfection efficiency than the original liposome composition, DMPC: DMPG: cholesterol, 9:1:3 molar ratio.

xiii) In order to increase the transfection, cationic mediator calcium was added to the system. The presence of cation, Ca^{2+} increases the amount of DNA that can be encapsulated by the liposome.

xiv) When naked DNA was used, the cells were not transfected. When the liposome alone was used as a vector to transfer DNA, 17% of transfection efficiency was determined. Further increase in the transfection efficiency, up to 35%, was obtained when the cationic mediator, Ca^{2+} was added to the liposome-DNA complex.

xv) Changing the amount of encapsulated DNA from 2 to 4 μg, and the incubation time from 90 to 180 minutes, did not change the experimental outcome. The best transfection results were obtained when the calcium concentrations were 20 or 30 mM.

xvi) In the case of DNA-liposome systems with no Ca^{2+}, the best results were obtained for liposome volume, 5 μL only. When Ca^{2+} was added to these systems, 2-8 μL volume range was used and successful results were obtained.

xvii) As in the case of the systems with no calcium, the composition was altered as to change the charge of the liposomes. Increasing the anionic character of the liposomes, in the presence of Ca^{2+}, did not change the experimental outcome.

9. FUTURE WORK SUGGESTIONS

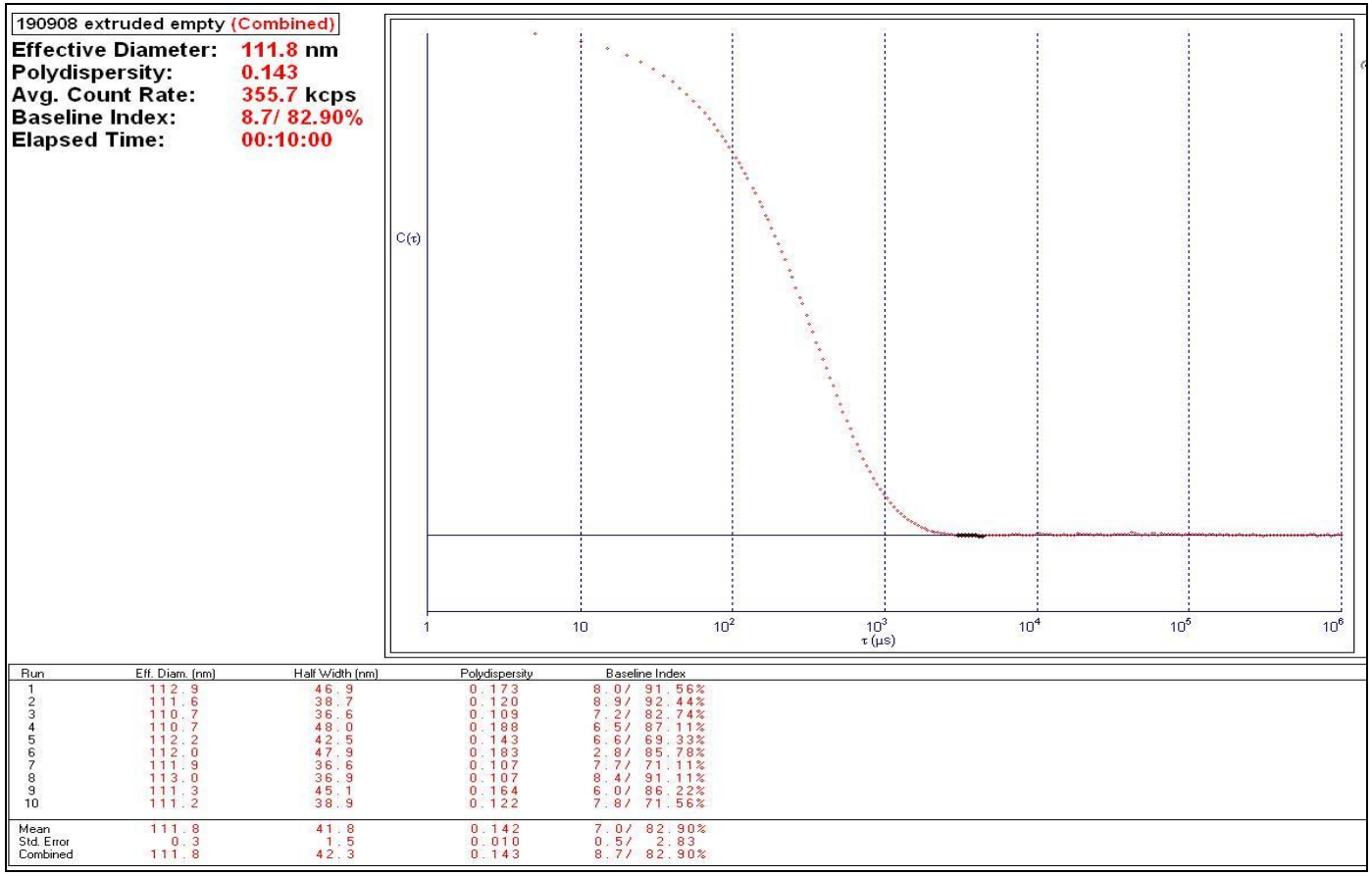
i) Promising results were obtained in the transfection experiments of MIO-M1 cells with the anionic liposomes with cationic mediator, i.e. liposome-DNA-Ca^{2+} system. In order to see the applicability of this system, different types of cells, such as HEK 293 which were used in preliminary experiments should also be further examined

ii) The slightly anionic liposome was complexed with divalent cation, Ca^{2+} to overcome the repulsion between the liposome, DNA, and the cell membrane during the gene delivery. Another choice could be to use a cationic, biodegradable polymer, such as PLL to coat the negatively charged surface of the liposome.

iii) Liposomes have a great potential in the areas of drug and gene delivery but they have a limitation like batch-to-batch reproducibility. Further work should be done to produce more reproducible solutions.

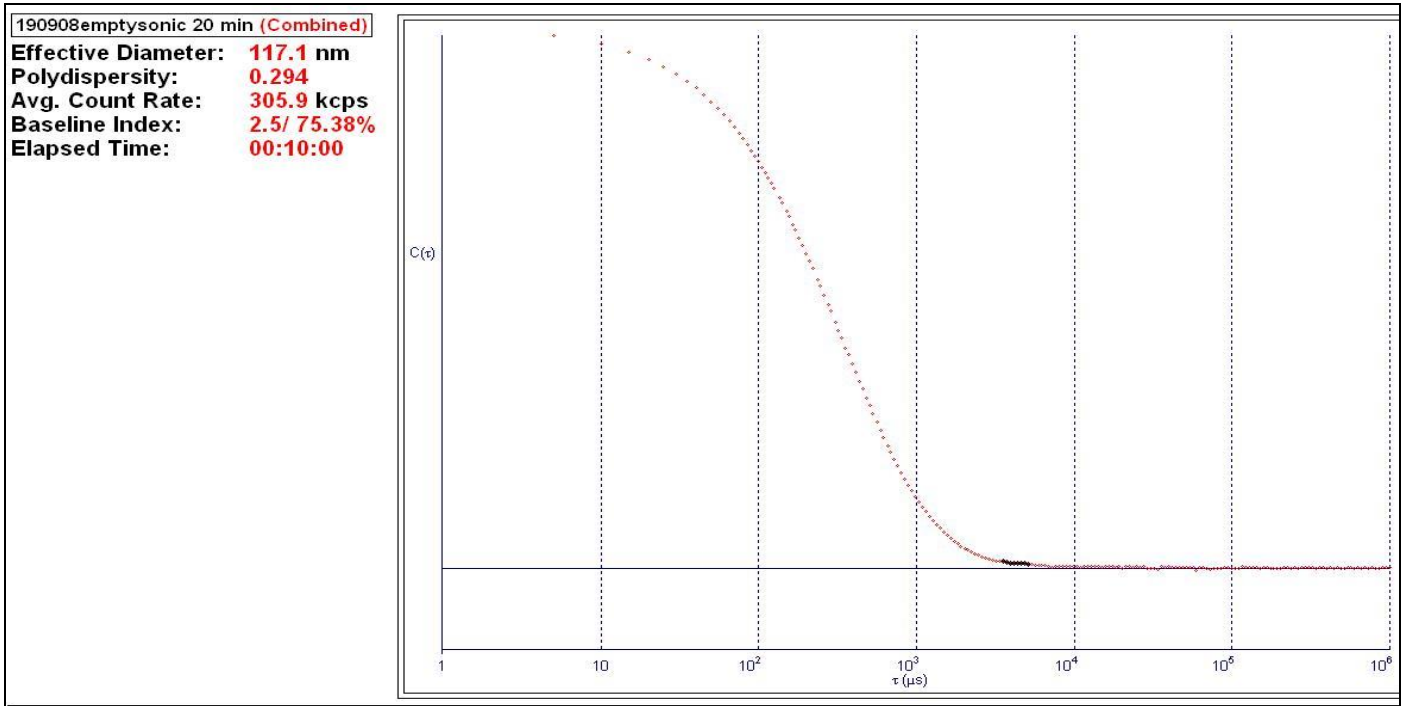
APPENDIX A: DLS ANALYSIS RESULTS

The following pages (115-120) show the DLS analysis results of the samples mentioned in Section 7.1.1 and 7.1.2.



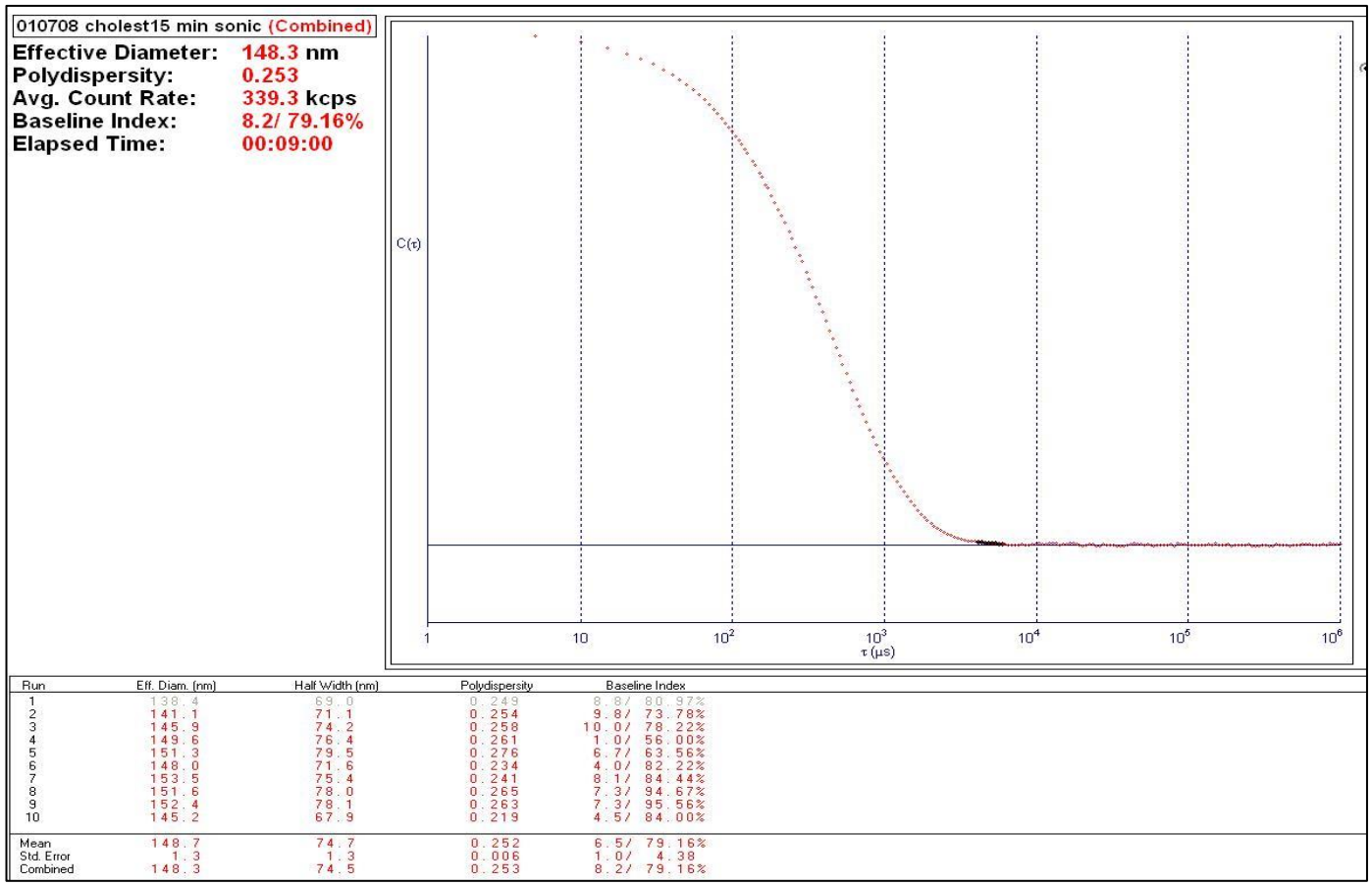
Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	112.9	46.9	0.173	8.0/ 91.56%
2	111.6	38.7	0.120	8.9/ 92.44%
3	110.7	36.6	0.109	7.2/ 82.74%
4	110.7	48.0	0.188	6.5/ 87.11%
5	112.2	42.5	0.143	6.6/ 69.33%
6	112.0	47.9	0.183	2.8/ 85.78%
7	111.9	36.6	0.107	7.7/ 71.11%
8	113.0	36.9	0.107	8.4/ 91.11%
9	111.3	45.1	0.164	6.0/ 86.22%
10	111.2	38.9	0.122	7.8/ 71.56%
Mean	111.8	41.8	0.142	7.0/ 82.90%
Std. Error	0.3	1.5	0.010	0.5/ 2.83
Combined	111.8	42.3	0.143	8.7/ 82.90%

Figure A. 1. DLS analysis of an empty, extruded liposome.



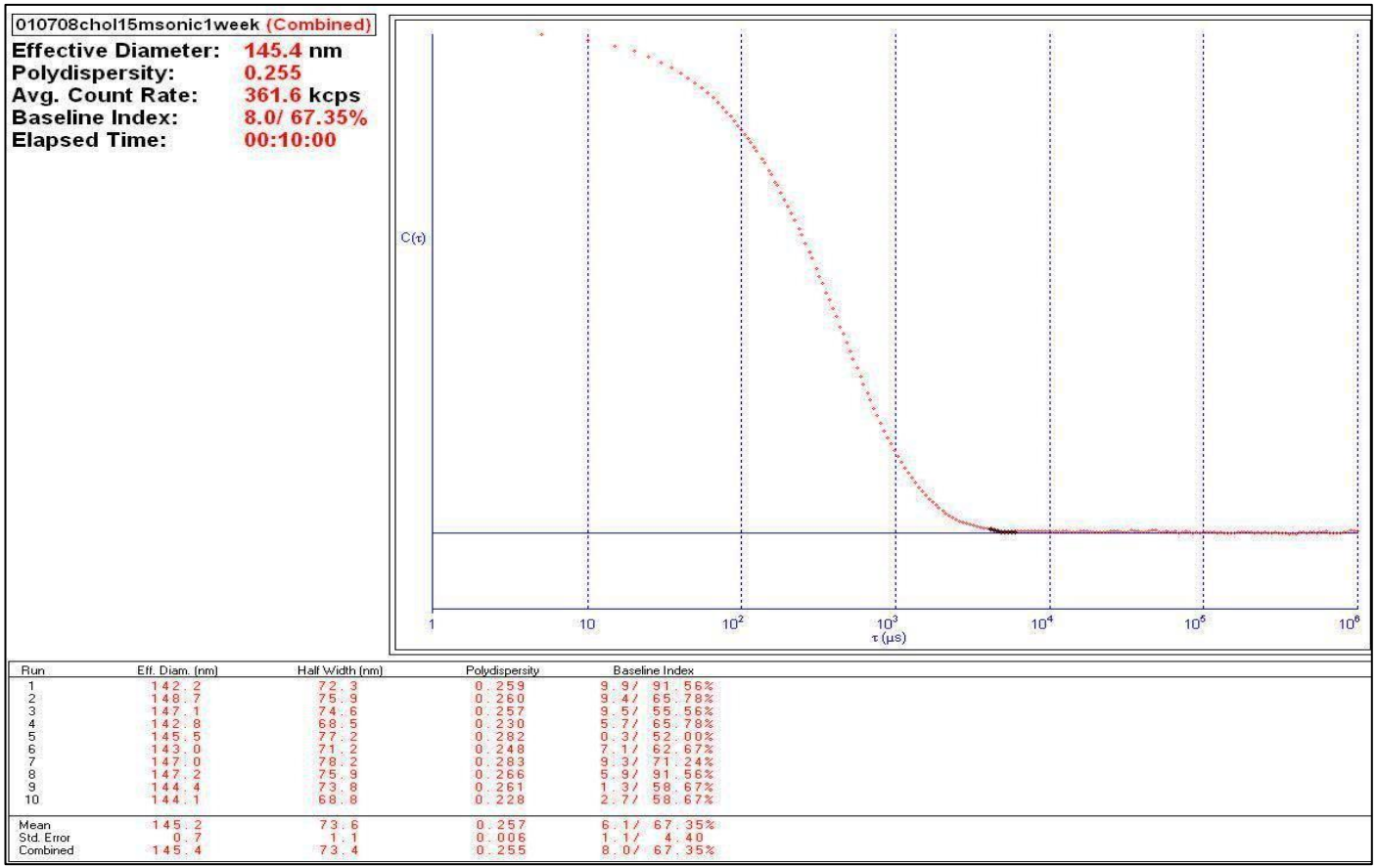
Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	112.7	61.9	0.302	7.6/ 50.22%
2	119.6	65.2	0.297	6.4/ 64.00%
3	115.9	63.5	0.300	2.6/ 82.22%
4	118.0	61.0	0.268	3.7/ 62.67%
5	116.4	61.9	0.283	0.7/ 83.11%
6	116.7	64.3	0.304	1.0/ 82.67%
7	116.6	65.1	0.311	3.5/ 76.44%
8	120.3	66.9	0.309	3.9/ 92.00%
9	114.8	61.2	0.284	0.0/ 82.22%
10	118.5	63.6	0.288	0.3/ 78.22%
Mean	116.9	63.4	0.295	3.0/ 75.38%
Std. Error	0.7	0.6	0.004	0.8/ 3.97
Combined	117.1	63.6	0.294	2.5/ 75.38%

Figure A.2. DLS analysis of a sonicated liposome.



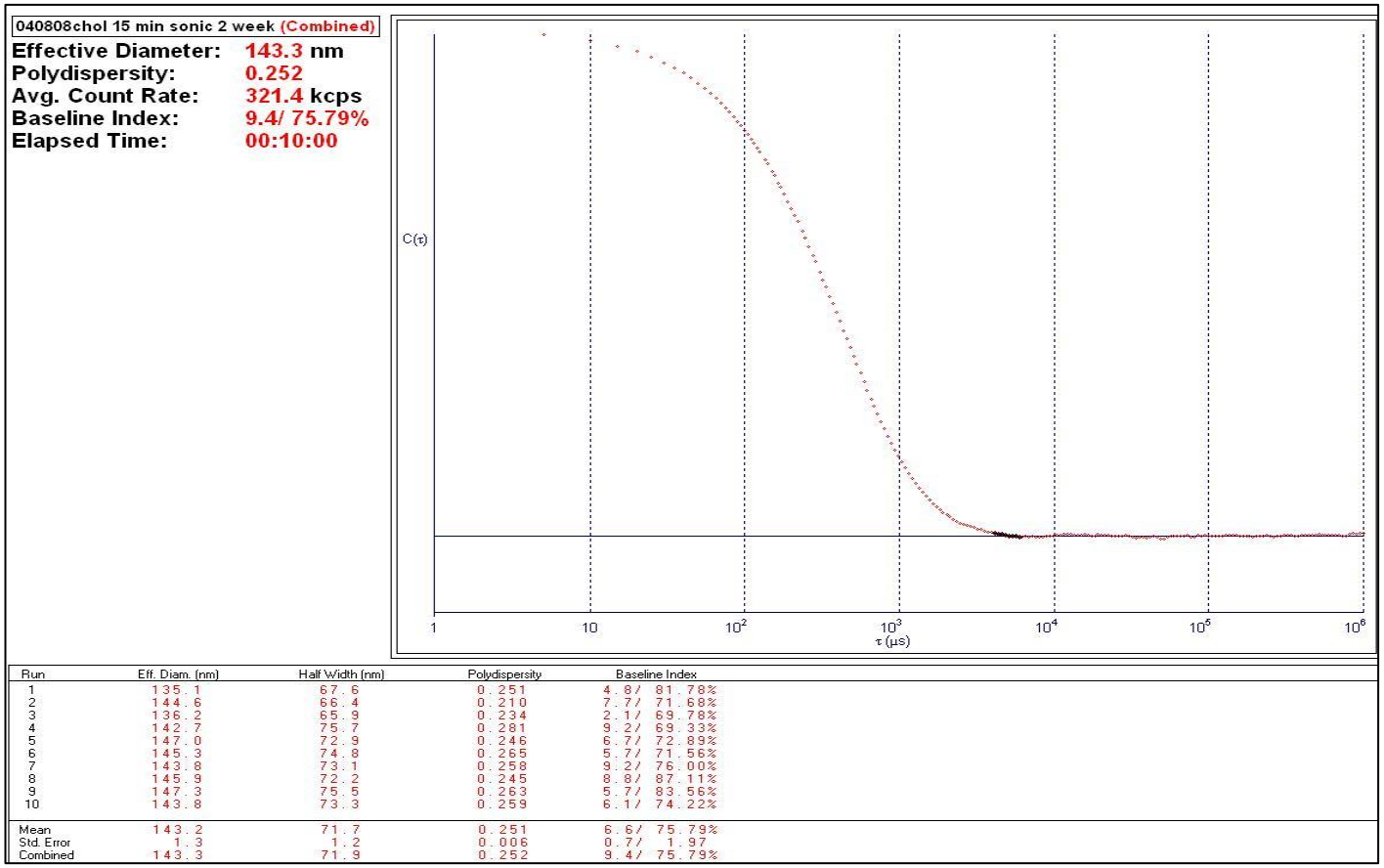
Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	138.4	69.0	0.249	8.8/ 80.97%
2	141.1	71.1	0.254	9.8/ 73.78%
3	145.9	74.2	0.258	10.0/ 78.22%
4	149.6	76.4	0.261	1.0/ 56.00%
5	151.3	79.5	0.276	6.7/ 63.56%
6	148.0	71.6	0.234	4.0/ 82.22%
7	153.5	75.4	0.241	8.1/ 84.44%
8	151.6	78.0	0.265	7.3/ 94.67%
9	152.4	78.1	0.263	7.3/ 95.56%
10	145.2	67.9	0.219	4.5/ 84.00%
Mean	148.7	74.7	0.252	6.5/ 79.16%
Std. Error	1.3	1.3	0.006	1.0/ 4.38
Combined	148.3	74.5	0.253	8.2/ 79.16%

Figure A.3. DLS analysis of cholesterol containing liposome immediately after sonication.



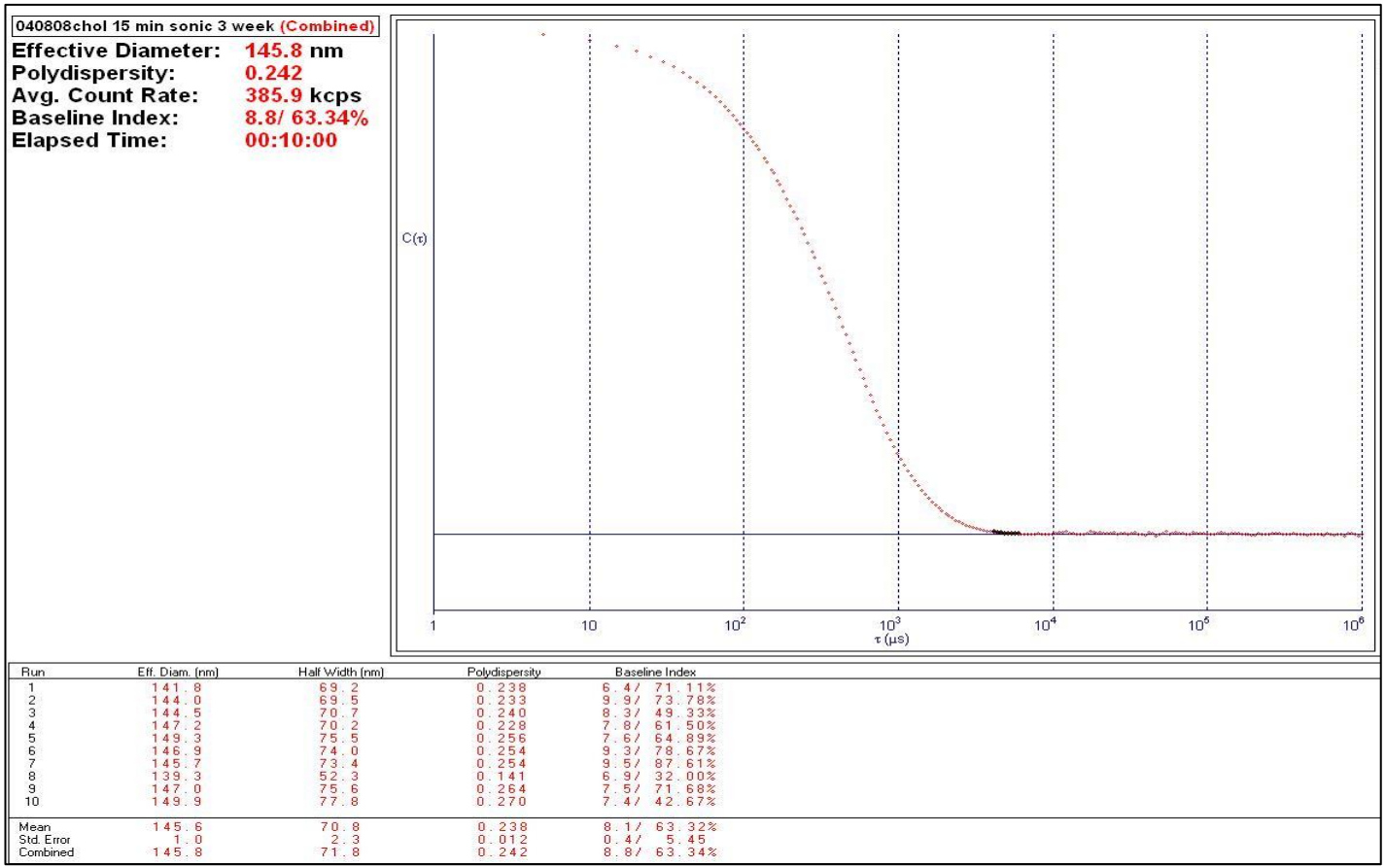
Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	142.2	72.3	0.259	9.9/ 91.56%
2	148.7	75.9	0.260	9.4/ 65.78%
3	147.1	74.6	0.257	9.5/ 55.56%
4	142.8	68.5	0.230	5.7/ 65.78%
5	145.5	77.2	0.282	0.3/ 52.00%
6	143.0	71.2	0.248	7.1/ 62.67%
7	147.0	78.2	0.283	9.3/ 71.24%
8	147.2	75.9	0.266	5.9/ 91.56%
9	144.4	73.8	0.261	1.3/ 58.67%
10	144.1	68.8	0.228	2.7/ 58.67%
Mean	145.2	73.6	0.257	6.1/ 67.35%
Std. Error	0.7	1.1	0.006	1.1/ 4.40
Combined	145.4	73.4	0.255	8.0/ 67.35%

Figure A.4. DLS analysis of cholesterol containing liposome after 1 week of sonication.



Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	135.1	67.6	0.251	4.8/ 81.78%
2	144.6	66.4	0.210	7.7/ 71.68%
3	136.2	65.9	0.234	2.1/ 69.79%
4	142.7	75.7	0.281	9.2/ 69.33%
5	147.0	72.9	0.246	6.7/ 72.89%
6	145.3	74.8	0.265	5.7/ 71.56%
7	143.8	73.1	0.258	9.2/ 76.00%
8	145.9	72.2	0.245	8.8/ 87.11%
9	147.3	75.5	0.263	5.7/ 83.56%
10	143.8	73.3	0.259	6.1/ 74.22%
Mean	143.2	71.7	0.251	6.6/ 75.79%
Std. Error	1.3	1.2	0.006	0.7/ 1.97
Combined	143.3	71.9	0.252	9.4/ 75.79%

Figure A.5. DLS analysis of cholesterol containing liposome after 2 weeks of sonication.



Run	Eff. Diam. (nm)	Half Width (nm)	Polydispersity	Baseline Index
1	141.8	69.2	0.238	6.4/ 71.11%
2	144.0	69.5	0.233	9.9/ 73.78%
3	144.5	70.7	0.240	8.3/ 49.33%
4	147.2	70.2	0.228	7.8/ 61.50%
5	149.3	75.5	0.256	7.6/ 64.89%
6	146.9	74.0	0.254	9.3/ 78.67%
7	145.7	73.4	0.254	9.5/ 87.61%
8	139.3	52.3	0.141	6.9/ 32.00%
9	147.0	75.6	0.264	7.5/ 71.68%
10	149.9	77.8	0.270	7.4/ 42.67%
Mean	145.6	70.8	0.238	8.1/ 63.32%
Std. Error	1.0	2.3	0.012	0.4/ 5.45
Combined	145.8	71.8	0.242	8.8/ 63.34%

Figure A.6. DLS analysis of cholesterol containing liposome after 3 weeks of sonication.

APPENDIX B: FLUORECENCE MICROSCOPY ANALYSIS IMAGES

The following pages (121-135) show the fluorescence microscopy images of the samples mentioned in Section 7.2.1, 7.2.2, 7.2.3, 7.3.2, and 7.3.3.

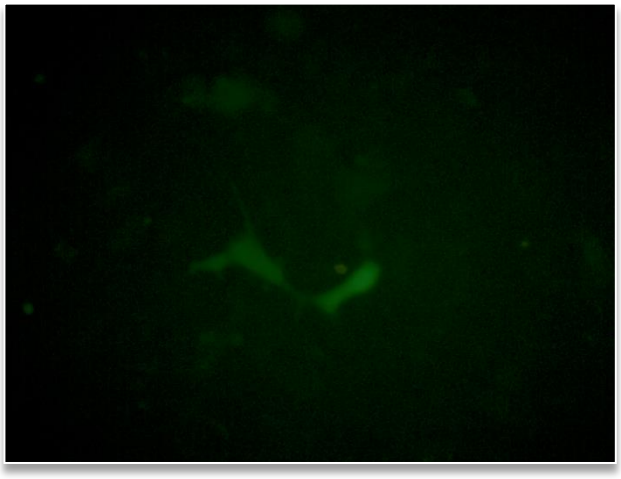


Figure B.1. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%).

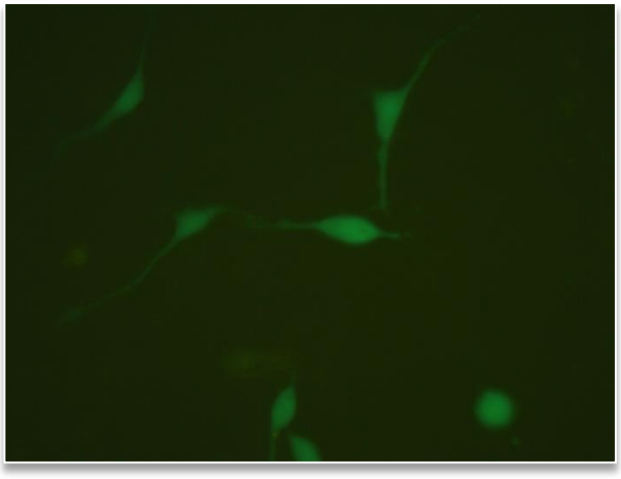


Figure B.2. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%).

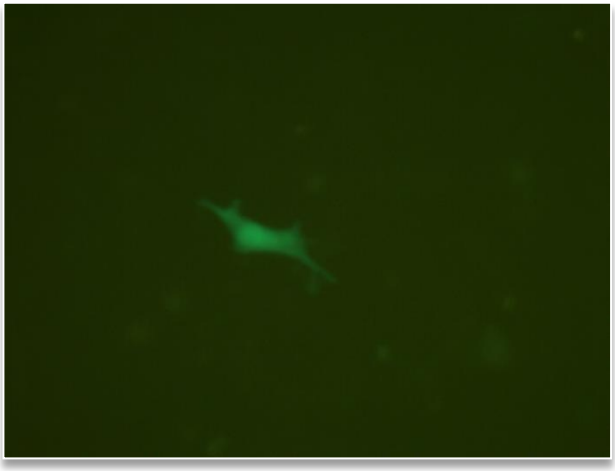


Figure B.3. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded cholesterol containing liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%).

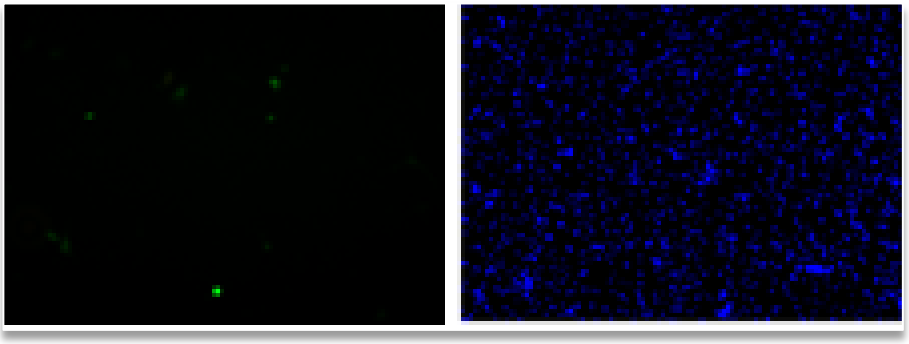


Figure B.4. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded cholesterol containing liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%).

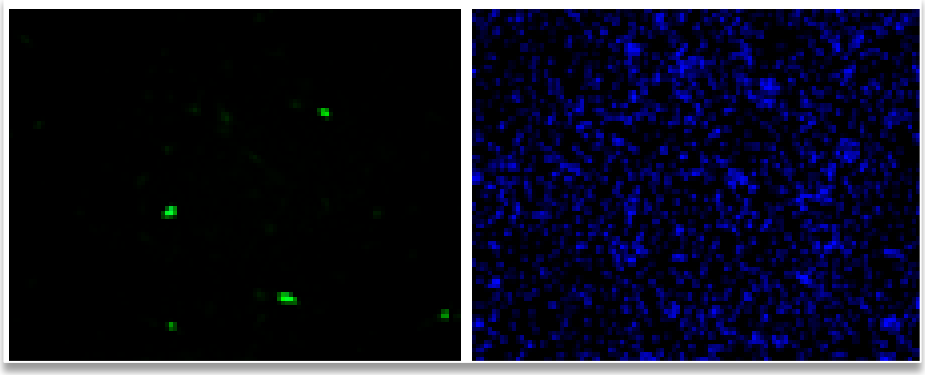


Figure B.5. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated cholesterol containing liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 5%).

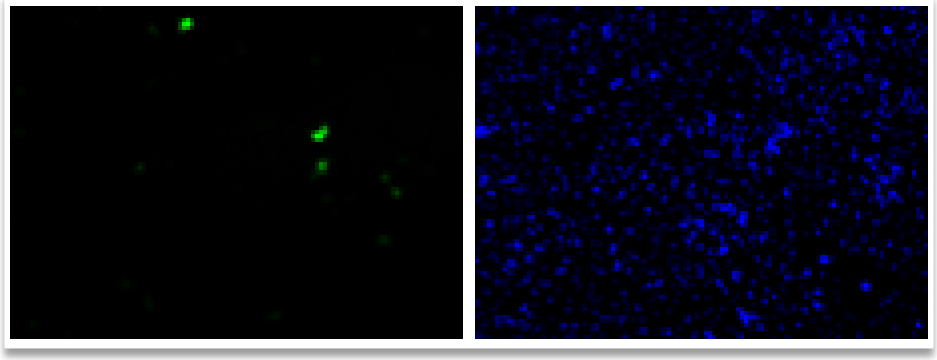


Figure B.6. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of sonicated liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%).

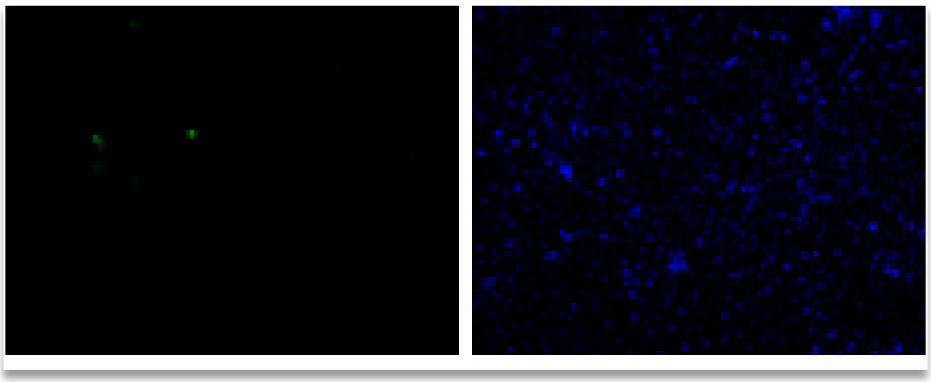


Figure B.7. Fluorescence microscopy images of transfected MIO-M1 cells in the presence of extruded liposome (5 μL) and SIK2-GFP vector (transfection efficiency less than 1%).

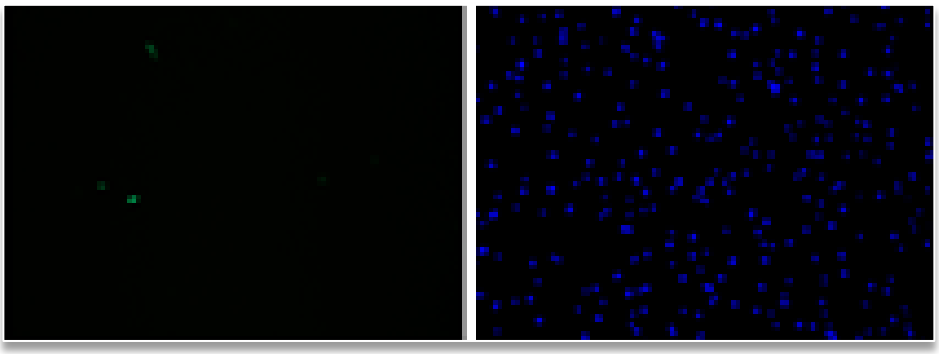


Figure B.8. Fluorescence microscopy images for 20 minutes incubation time (5% transfection efficiency).

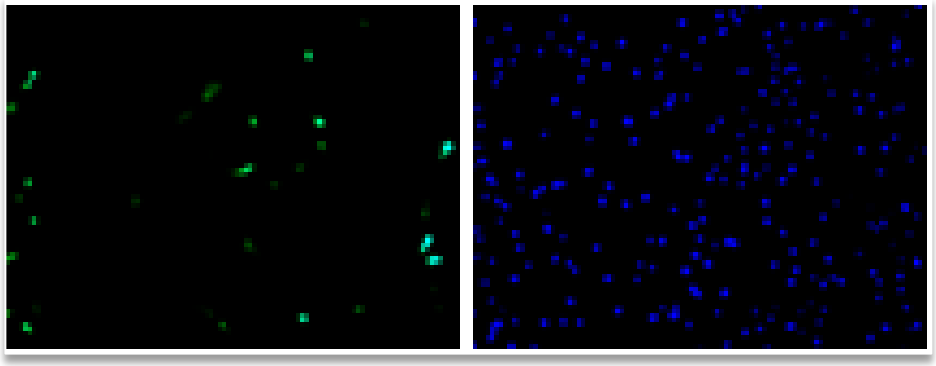


Figure B.9. Fluorescence microscopy images for 30 minutes incubation time (12% transfection efficiency).

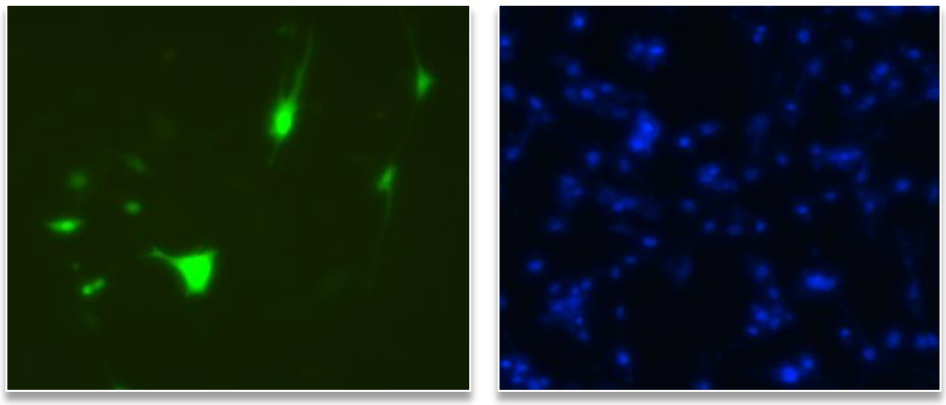


Figure B.10. Fluorescence microscopy images for 2 μg SIK2-GFP with 20 mM Ca^{2+} at 90 minutes incubation time (15% transfection efficiency).

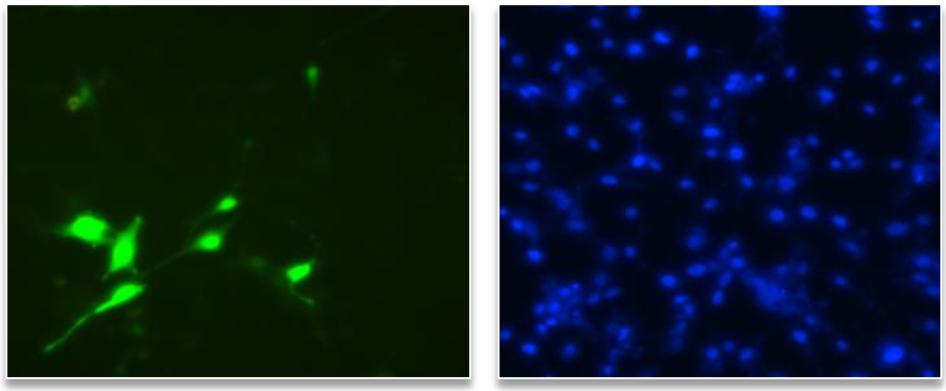


Figure B.11. Fluorescence microscopy images for 4 μg SIK2-GFP with 20 mM Ca^{2+} at 90 minutes incubation time (15% transfection efficiency).

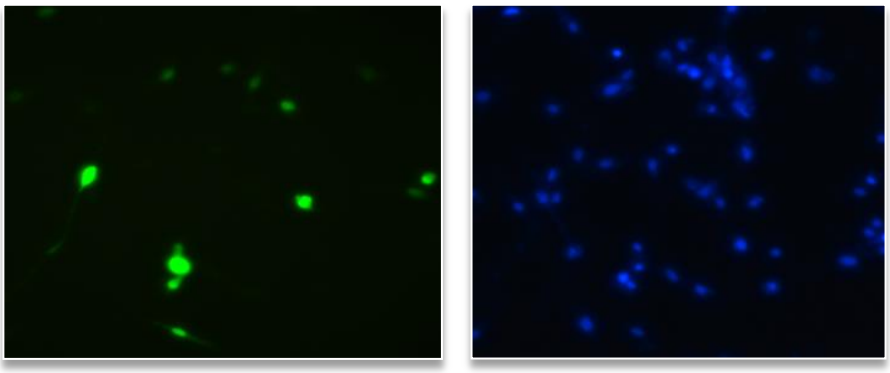


Figure B.12. Fluorescence microscopy images for 2 μg SIK2-GFP with 30 mM Ca^{2+} at 90 minutes incubation time (30% transfection efficiency).

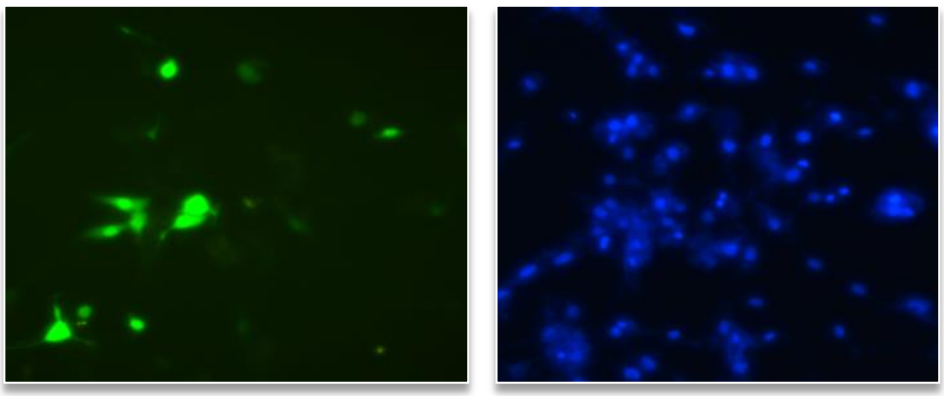


Figure B.13. Fluorescence microscopy images for 4 μg SIK2-GFP with 30 mM Ca^{2+} at 90 minutes incubation time (33% transfection efficiency).

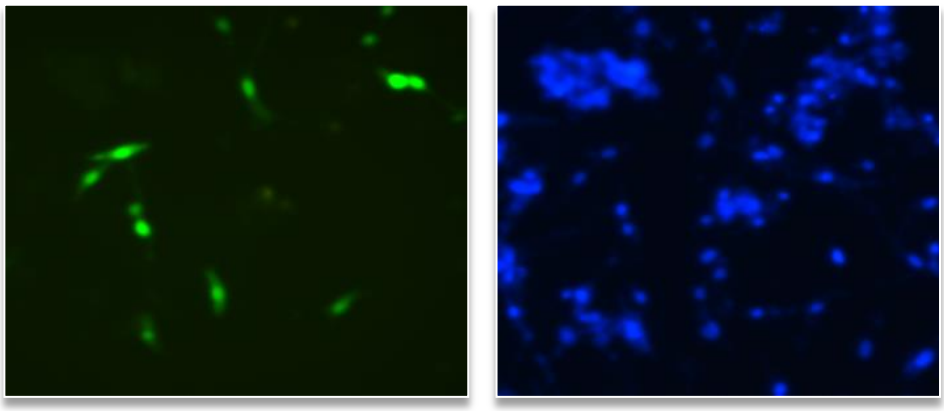


Figure B.14. Fluorescence microscopy images for 2 μg SIK2-GFP with 40 mM Ca^{2+} at 90 minutes incubation time (26% transfection efficiency).

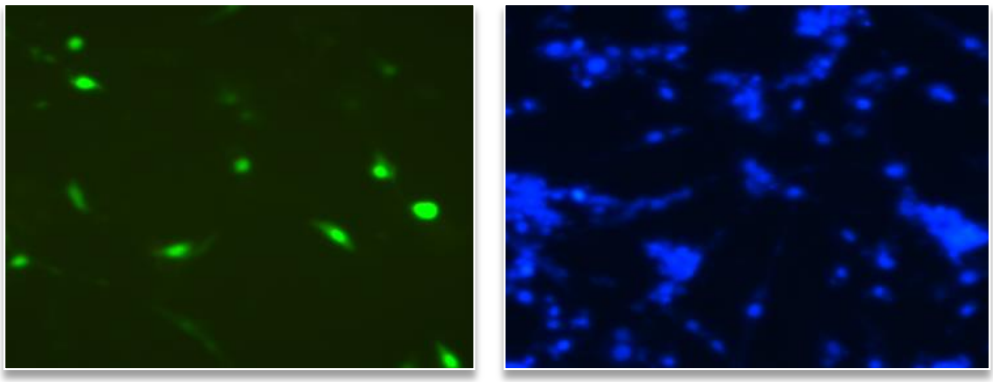


Figure B.15. Fluorescence microscopy images for 4 μg SIK2-GFP with 40 mM Ca^{2+} at 90 minutes incubation time (25% transfection efficiency).

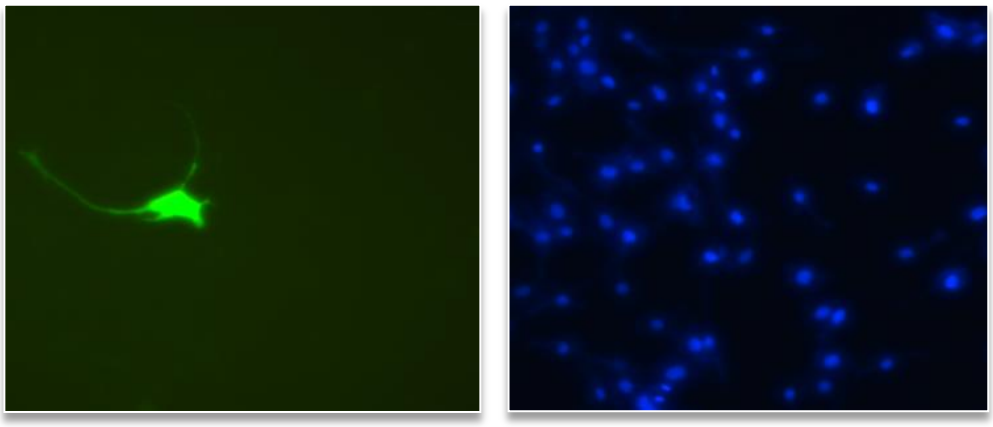


Figure B.16. Fluorescence microscopy images for 2 μg SIK2-GFP with 10 mM Ca^{2+} at 180 minutes incubation time (2% transfection efficiency).

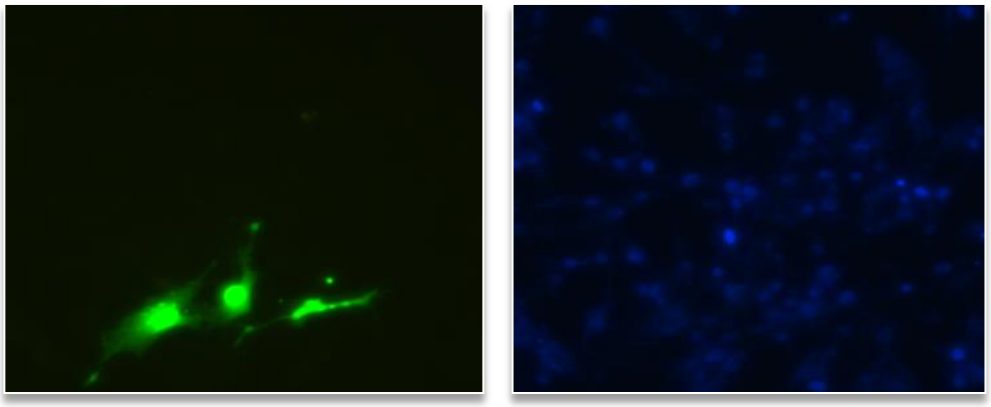


Figure B.17. Fluorescence microscopy images for 4 μg SIK2-GFP with 10 mM Ca^{2+} at 180 minutes incubation time (2% transfection efficiency).

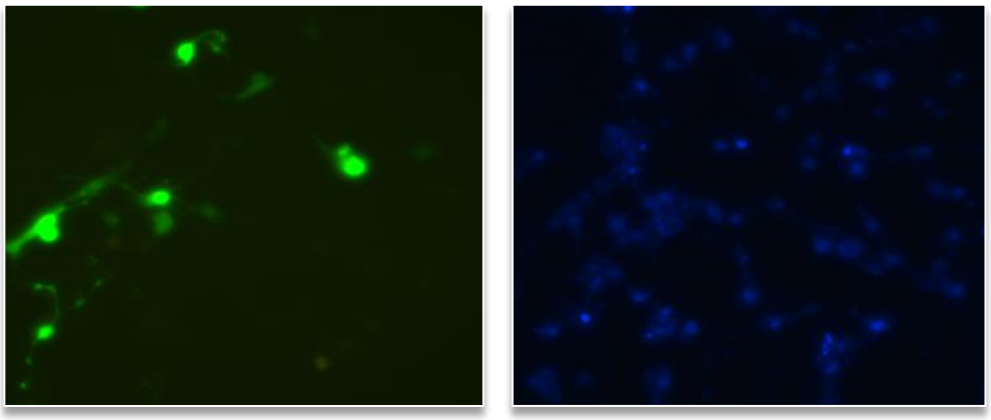


Figure B.18. Fluorescence microscopy images for 2 μg SIK2-GFP with 20 mM Ca^{2+} at 180 minutes incubation time (26% transfection efficiency).

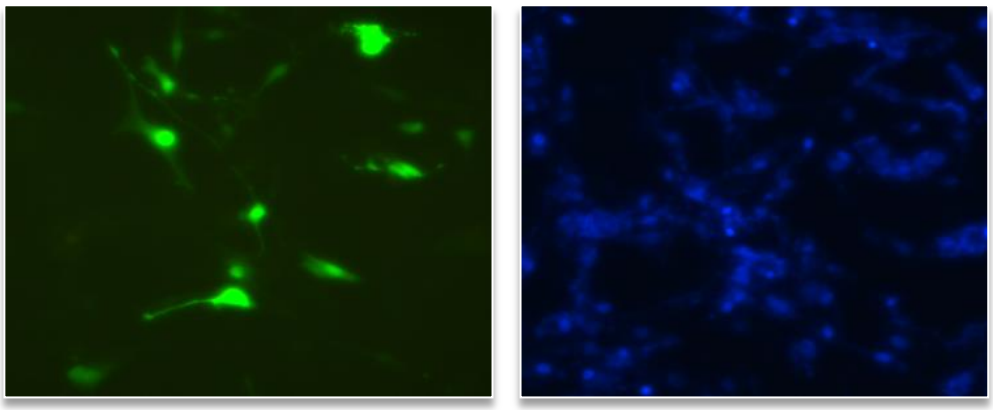


Figure B.19. Fluorescence microscopy images for 4 μg SIK2-GFP with 20 mM Ca^{2+} at 180 minutes incubation time (33% transfection efficiency).

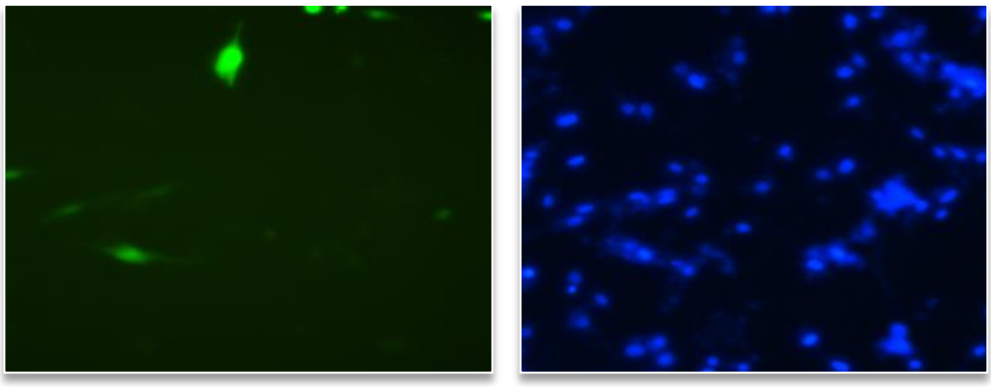


Figure B.20. Fluorescence microscopy images for 2 μg SIK2-GFP with 30 mM Ca^{2+} at 180 minutes incubation time (14% transfection efficiency).

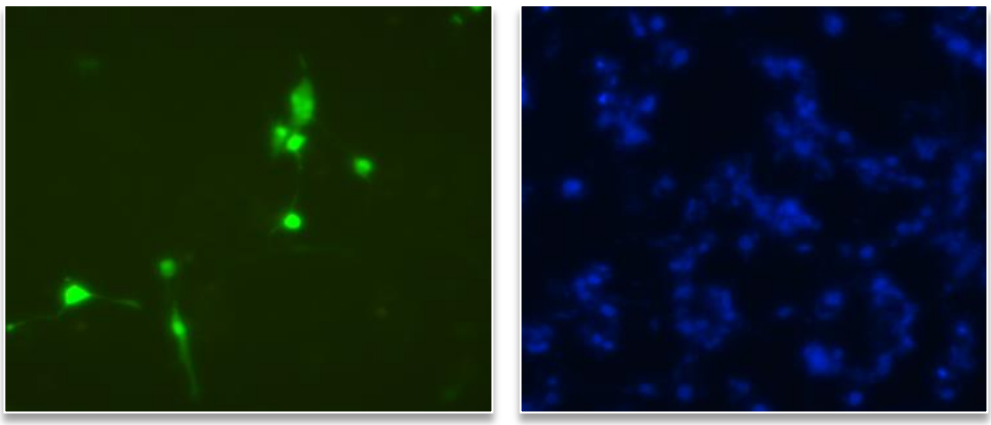


Figure B.21. Fluorescence microscopy images for 4 μg SIK2-GFP with 30 mM Ca^{2+} at 180 minutes incubation time (15% transfection efficiency).

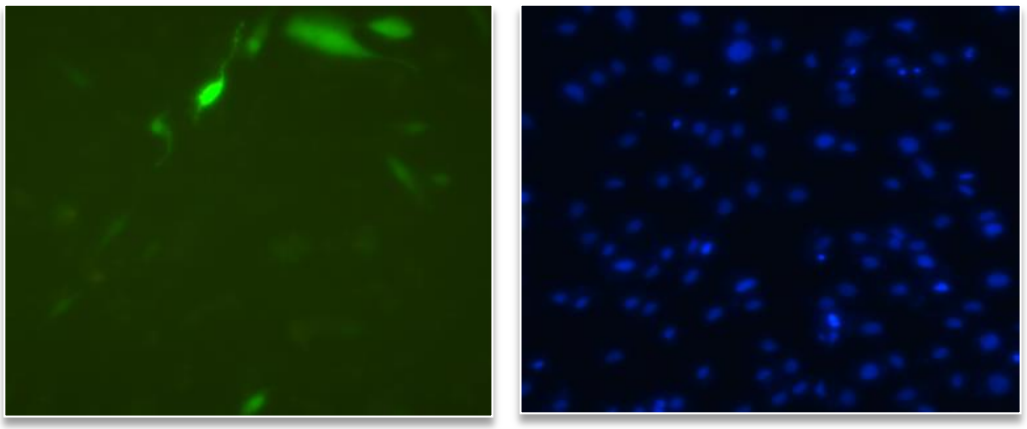


Figure B.22. Fluorescence microscopy images for 0.5 µg SIK2-GFP with 2 µL X-tremeGENE HP (positive control) (20% transfection efficiency).

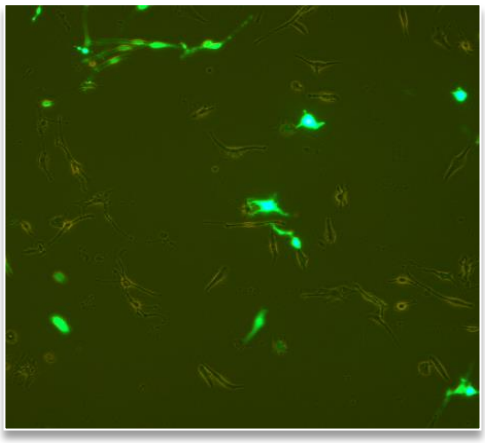


Figure B.23. Fluorescence microscopy image of experiment 1 in Table 7.7.

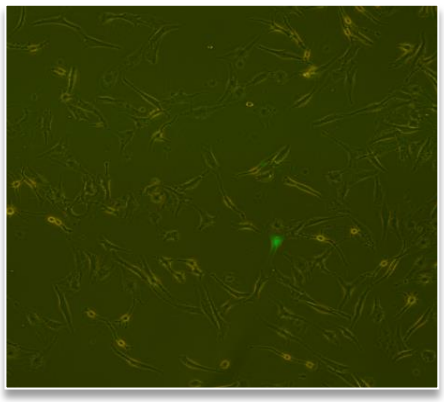


Figure B.24. Fluorescence microscopy image of experiment 2 in Table 7.7.

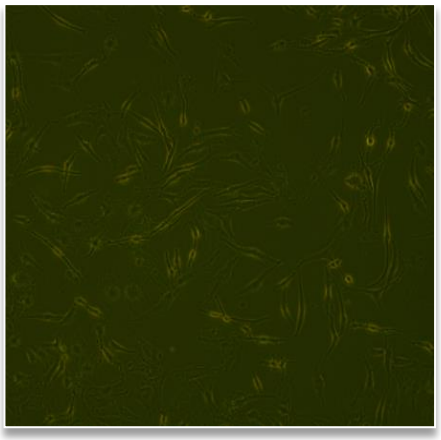


Figure B.25. Fluorescence microscopy image of experiment 3 in Table 7.7.

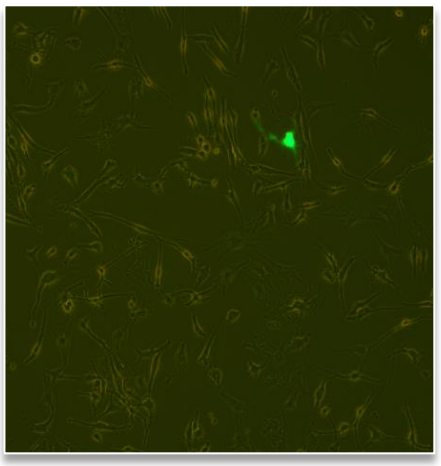


Figure B.26. Fluorescence microscopy image of experiment 4 in Table 7.7.

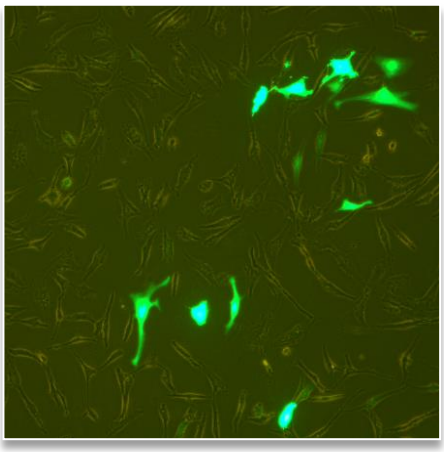


Figure B.27. Fluorescence microscopy image of experiment 5 in Table 7.7.

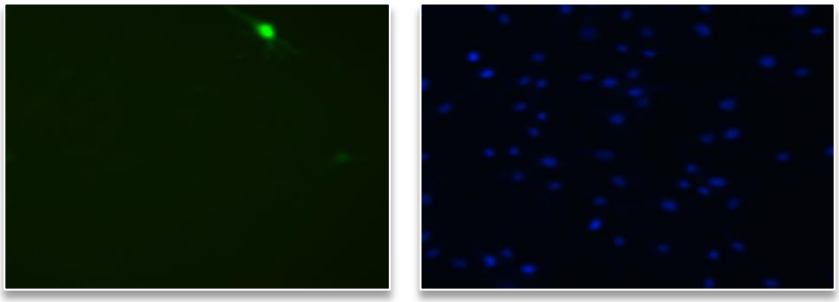


Figure B.28. Fluorescence microscopy image for 4 µg SIK2-GFP, 2 µL liposome with 20 mM Ca^{2+} at 90 minutes incubation time (1.8% transfection efficiency).

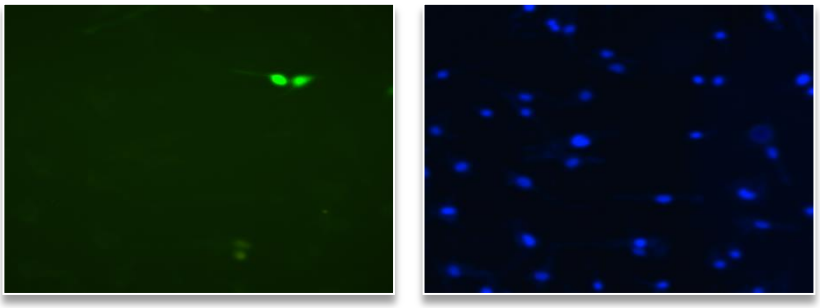


Figure B.29. Fluorescence microscopy image for 4 µg SIK2-GFP, 5 µL liposome with 20 mM Ca^{2+} at 90 minutes incubation time (7.5% transfection efficiency).

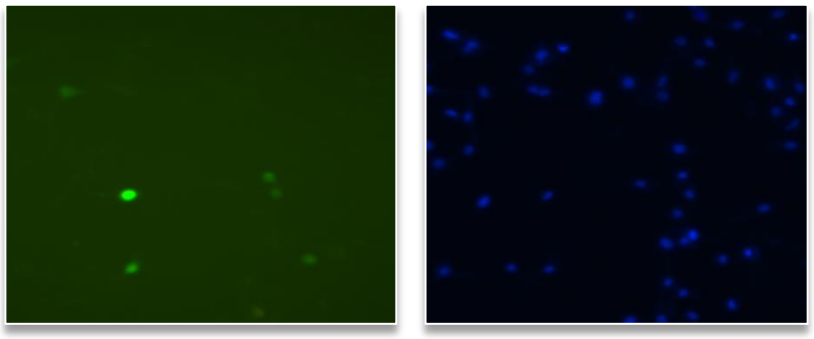


Figure B.30. Fluorescence microscopy image for 4 µg SIK2-GFP, 8 µL liposome with 20 mM Ca^{2+} at 90 minutes incubation time (11% transfection efficiency).

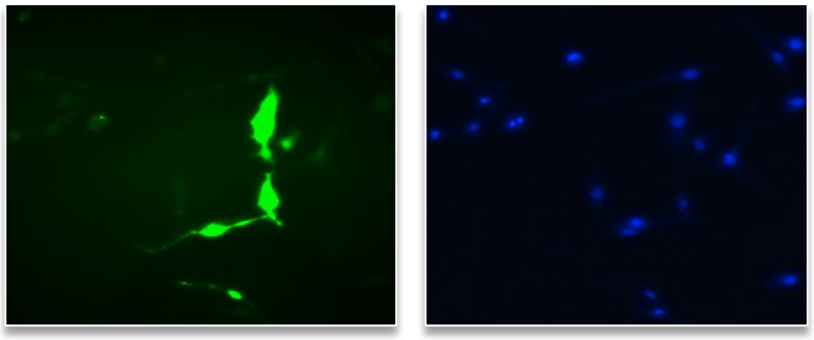


Figure B.31. Fluorescence microscopy image for 4 µg SIK2-GFP, 2 µL liposome with 30 mM Ca^{2+} at 90 minutes incubation time (22.7% transfection efficiency).

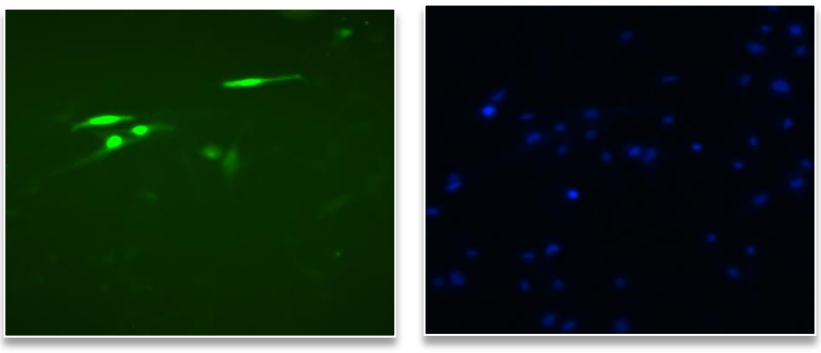


Figure B.32. Fluorescence microscopy image for 4 µg SIK2-GFP, 5 µL liposome with 30 mM Ca^{2+} at 90 minutes incubation time (17.4% transfection efficiency).

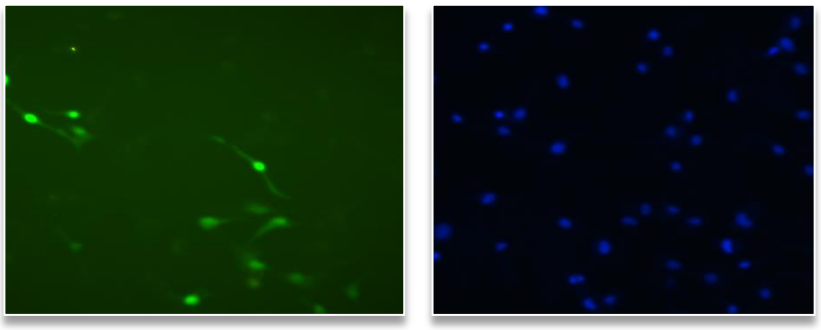


Figure B.33. Fluorescence microscopy image for 4 µg SIK2-GFP, 8 µL liposome with 30 mM Ca^{2+} at 90 minutes incubation time (30% transfection efficiency).

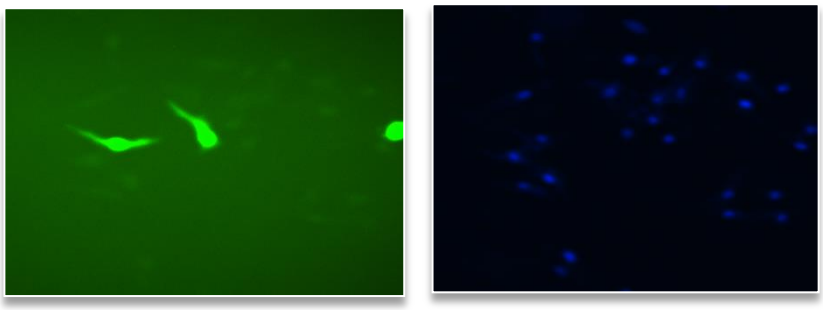


Figure B.34. Fluorescence microscopy images for 0.5 µg SIK2-GFP with 2 µL X-tremeGENE HP (positive control) (11% transfection efficiency).

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