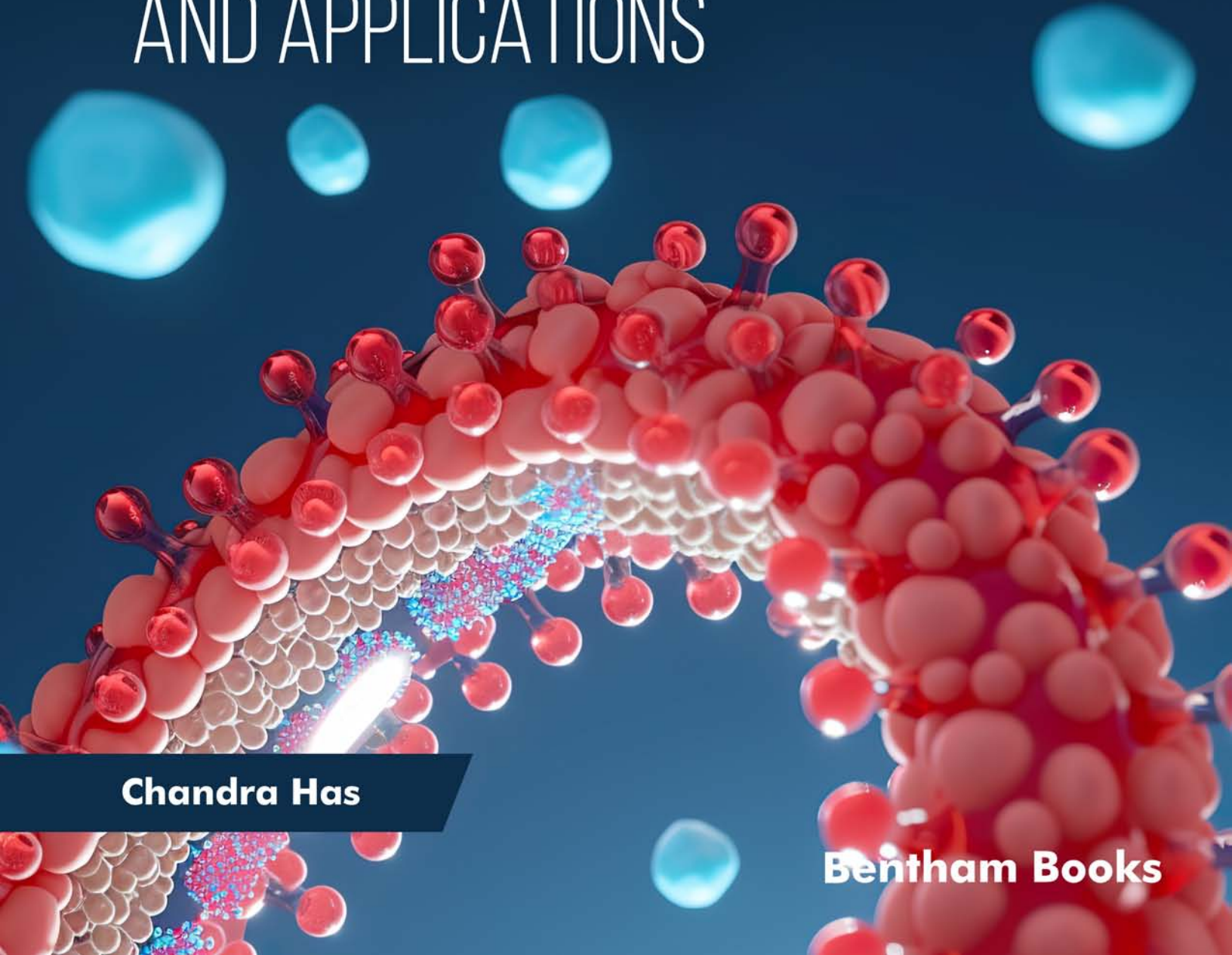
ADVANCED LIPOSOME TECHNOLOGIES

PREPARATION, MECHANISMS, AND APPLICATIONS



Chandra Has

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Advanced Liposome Technologies: Preparation, Mechanisms, and Applications

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PREFACE

Liposome technology has captivated academics and inventors for over five decades, with unrivalled applications in medicine delivery, vaccine development, cosmetics, agriculture, and environmental science. Liposomes, the first nanomedicine to be licensed for human use, are notable for their biocompatibility and biodegradability, originating from their structural similarities to our cell membranes. Today, more than 30 authorized liposomal medicinal solutions address significant health issues, demonstrating the importance of liposomes in modern science and technology. Milestones in liposome technology include the development of PEGylated and cationic liposomes, pH-sensitive systems for gene delivery, and scalable microfluidic production processes, underscoring their continuing impact on modern science and medicine.

This book offers an approachable introduction to liposome technology, connecting fundamental ideas with current advances to appeal to both novice and expert researchers. It covers both traditional and new liposome preparation methods, describing mechanics in a straightforward yet extensive manner. Liposomes are commonly utilised in drug delivery systems for targeted therapy and vaccine development in pharmaceutics, but they also have uses in cosmetics, agriculture, and environmental research. Liposomes serve as excellent model membranes for researching mechanical characteristics, transport properties, curvature dynamics, and pore formation. The book is structured into two comprehensive parts. The first part focuses on lipid chemistry and physics, exploring their classes, advanced extraction techniques, purification methods, and the thermodynamics of bilayer formation, establishing a strong foundation for liposome science. The second part focuses on modern advancements, including microfluidic techniques for liposome synthesis and their applications in medical, non-medical, and model membrane studies.

I believe that this book will be a great resource for researchers, students, instructors, scientists, and academics all across the world, motivating more investigation into the enormous possibilities of liposome technology. I cordially welcome readers to provide feedback and thoughts, which will be useful in improving and refining future versions of this book.

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Dedicated
To
My beloved
Late Grandmother
for her endless love and unwavering support.

LIST OF ACRONYMS

1D	One-dimensional
2D	Two-dimensional
2-MeTHF	2-Methyl tetrahydrofuran
3D	Three-dimensional
ABC	Accelerated blood clearance
ABZ	Albendazole
AC	Alternating current
ADME	Absorption, distribution, metabolism, and excretion
AFM	Atomic force microscopy
AEO	Alcohol ethoxylate
AHs	Amphipathic α-helices
AI	Artificial intelligence
AMPs	Antimicrobial peptides
AOT	Aerosol OT (sodium bis(2-ethylhexyl) sulfosuccinate)
APC	Antigen-presenting cells
API	Active pharmaceutical ingredient
ASE	Accelerated solvent extraction
ASES	Aerosol solvent extraction system
ASO	Antisense oligonucleotide
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
BISHOP	2,3 - Dihexadecyloxyl - propyl-N, N, N-trimethylammonium chloride
BLF	Bilayered lipid fragment
BSA	Bovine serum albumin
BUME	Butanol-methanol extraction
CAC	Critical aggregation concentration
CAS	Continuous anti-solvent
CD	Circular dichroism
CDR	Cumulative drug release
CE	Capillary electrophoresis
CF	Carboxyfluorescein, fluorocarbon

CHAPS	3-(3-Cholamidopropyl) dimethylammonio-1-propanesulfonate
CHEMS	Cholesteryl hemisuccinate
CHOL	Cholesterol
CL	Cardiolipin, cationic liposome
CLSM	Confocal laser scanning microscopy
CM	Carboxymethyl
CMC	Critical micelle concentration
CME	Clathrin-mediated endocytosis
CPME	Cyclopentyl methyl ether
CQAs	Critical quality attributes
Cryo-SEM	Cryo-scanning electron microscopy
Cryo-TEM	Cryogenic transmission electron microscopy
CT	Computed tomography
CTAB	Cetyltrimethylammonium bromide
CU	Curcumin
CVC	Critical vesicle concentration
CVD	Chemical vapor deposition
DAC	Dual asymmetric centrifugation
DAPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DBOT	Dual-beam optical trap
DC	Direct current
DCP	Dicetyl phosphate
DCS	Differential calorimetry scanning
DDS	Drug delivery system
DEAE	Diethylaminoethyl
DELOS	Decompression of an expanded liquid organic solution-suspension
DEPC	1,2-Dierucoyl-*sn*- glycero-3-phosphocholine.
Dex-PEG	Dextran-polyethylene glycol
DG	Diacylglycerol
DGDG	Digalactosyldiacylglycerol
DHF	Double hydrodynamic focusing
DLCA	Diffusion-limited cluster aggregation
DLPC	1,2-Dilauroyl-*sn*-glycero-3-phosphocholine
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid

DOACS	Dioctadecylammonium cumene sulfonate
DODMAB(C)	Dioctadecyl dimethyl ammonium bromide(chloride)
DOMAC	Dioctadecyl methylammonium chloride
DOPE	1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DOPG	1,2-Dioleoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol)
DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane
DOTMA	N-[1-(2,3-Dioleoyloxy) Propyl]-N,N,N-trimethylammonium chloride
DOX	Doxorubicin
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
DPG	Diphosphatidylglycerol
DPH	Diphenylhexatriene
DPhPC	Diphytanoylphosphatidylcholine
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DPPA	1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidic acid (sodium salt)
DPPE	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine
DPPG	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoglycerol (sodium salt)
DPPS	1,2-Dipalmitoyl-*sn*-glycero-3-phosphoserine (sodium salt)
DRIE	Deep reactive-ion etching
DRV	Dehydration–rehydration vesicles
DSC	Differential scanning calorimetry
DSD	Double solvent displacement
DSPC	1,2-Distearoyl-*sn*-glycero-3-phosphocholine
DSPE	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine
DSPE-PEG2000	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (ammonium salt)
DSPG	1,2-Distearoyl-*sn*-glycero-3-phosphoglycerol (sodium salt)
DSPE-mPEG2000	1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)2000]
DSPE-PEG	1, 2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethylene glycol)
DSPS	1,2-Distearoyl-*sn*-glycero-3-phosphatidylserine (sodium salt)
DTA	Differential thermal analysis
ED	Electrodeformation
EDTA	Ethylenediaminetetraacetic acid

EE	Encapsulation efficiency
EGFR	Epidermal growth factor receptor
ELS	Electrophoretic light scattering
ELSD	Evaporative light scattering detector
EM	Electron microscopy
EMA	European medicines agency
EPA	Egg yolk phosphatidic acid
EPC	Egg phosphatidylcholine
EPR	Electron paramagnetic resonance, enhanced permeability and retention
ESI	Electrospray ionization
ESR	Electron spin resonance
ETM	Emulsion transfer methods
EV	Encapsulated volume
EVs	Extracellular vesicles
EYL	Egg yolk lecithin
FAME	Fatty acid methyl ester
FDA	Food and drug administration
FFA	Free fatty acids
FFF	Field-flow fractionation
FID	Flame ionization detection
FL	Fluorescence
FLD	Fluorescent detector
FRAP	Fluorescence recovery after photobleaching
FRET	Förster or fluorescence resonance energy transfer
FRR	Flow rate ratio
ESEM	Environmental scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
GAS	Gas anti-solvent
GC	Gas chromatography
GCh	Glycocholate
Gd-DTPA	Gadolinium with diethylenetriaminepentaacetic acid
GLC	Gas-liquid chromatography
GMP	Good manufacturing practice
GPC	Gel permeation chromatography, glycerophosphorylcholine

GPMVs	Giant plasma membrane vesicles
GSH	Glutathione
GUVs	Giant unilamellar vesicles
H-bonds	Hydrogen bonds
HC	Hydrocarbon
HE	Heat exchanger
HEPES	4-(2-Hydroxyethyl)-1-piperazineethane sulfonic acid
HER2	Human epidermal growth factor receptor 2
HMME	Hematoporphyrin monomethyl ether
HPC	Hydrogenated phosphatidylcholine
HPH	High-pressure homogenizer
HPLC	High-performance liquid chromatography
HS	Hereditary spherocytosis
HSPC	Hydrogenated soybean phosphatidylcholine
ICH	International council for harmonization
IDP	Intrinsically disordered proteins
iLiNP	Invasive lipid nanoparticle production
IPA	Isopropanol
IR	Infrared
ISCRPE	Improved supercritical reverse phase evaporation
ITC	Isothermal titration calorimetry
ITO	Indium tin oxide
IV	Intravenous
LAL	Limulus amebocyte lysate
LC	Loading capacity
LCs	Liquid crystals
LC-MS	Liquid chromatography-mass spectrometry
LDAO	Lauryldimethylamine oxide
LDL	Low-density lipoprotein
LDV	Laser Doppler velocimetry
LLC	Lyotropic liquid crystal
LLE	Liquid-liquid extraction
LNP	Lipid nanoparticle
LPC/LysoPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine

LUVs	Large unilamellar vesicles
OG	Octyl glucoside
OT	Optical tweezers
MALDI	Matrix-assisted laser desorption ionization
MD	Molecular dynamics
MDEI	Modified and derived ethanol injection
MEI	Modified ethanol injection
MG	Monoacylglycerol
MGDG	Monogalactosyldiacylglycerol
MHF	Microfluidic hydrodynamic focusing
ML	Machine learning
MLVs	Multilamellar vesicles
MMP	Matrix metalloproteinase
MMs	Mixed micelles
MPA	Micropipette aspiration
MPS	Mononuclear phagocyte system
MRI	Magnetic resonance imaging
MS	Mass spectroscopy
MTBE	Methyl tert butyl ether
MVF	Microfluidic vortex focusing
MVVs	Multivesicular vesicles
MWCO	Molecular weight cutoff
NaC	Sodium cholate
NaDC	Sodium Deoxycholate
ND	Neutron diffraction
Neu5Ac	N-acetyl-neuraminic acid
NISVs	Non-ionic surfactant vesicles
NLCs	Nanostructured lipid carriers
NLs	Nanosized liposomes
NMR	Nuclear magnetic resonance
NP-HPLC	Normal-phase high-performance liquid chromatography
NPs	Nanoparticles
NTA	Nanoparticle tracking analysis
ODS	Octa decyl silane
OLVs	Oligolamellar vesicles

OS	Optical stretcher
OT	Optical tweezer
OSPC	1-Oleoyl-2-stearoyl-*sn*-glycero-3-phosphocholine
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCS	Photon correlation spectroscopy
PDI	Polydispersity index
PDMS	Polydimethylsiloxane
PDT	Photodynamic therapy
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol
PEO	Poly(ethylene oxide)
PET	Positron emission tomography, Polyethylene terephthalate
PFE	Pressurized fluid extraction
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PK	Pharmacokinetics
PL	Phospholipid, phospholipase
PLE	Pressurized liquid extraction
PLVs	Paucilamellar vesicles
PMPs	Peripheral membrane proteins
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PS	Phosphatidylserine
PSMA	Prostate-specific membrane antigen
PTA	Phosphotungstic acid
PTFA	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acid
PUVA	Psoralen plus ultraviolet A
PVA	Polyvinyl alcohol
QELS	Quasi-elastic light scattering
RBC	Red blood cell
RBF	Round bottom flask
RCV	Reverse phase centrifugation

REV	Reverse phase evaporation vesicles
RES	Reticuloendothelial system
RESS	Rapid expansion of supercritical solvent
RI	Refractive index
ROS	Reactive oxygen species
RLCA	Reaction-limited cluster aggregation
RNA	Ribonucleic acid
RP-HPLC	Reverse-phase high-performance liquid chromatography
RS	Raman spectroscopy
RSE	Rapid solvent exchange
RSV	Resveratrol
SANS	Small-angle neutron scattering
SAP	Superabsorbent polymer
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SAS	Supercritical anti-solvent
SAXS	Small-angle X-ray scattering
SCRPE	Supercritical reverse phase evaporation
SEM	Scanning electron microscopy
SC-CO_2	Supercritical CO_2
SCF	Supercritical fluid
SDS	Sodium dodecyl sulfate
SEDS	Solution-enhanced dispersion by SC CO_2
SFM	Scanning force microscopy
SFE	Supercritical fluid extraction
SFS-CFN	SuperFluids phospholipids nanosomes
SHF	Single hydrodynamic focusing
SHM	Staggered herringbone micromixer
siRNA	Small interfering ribonucleic acid
SLB	Supported lipid bilayer
SLiC	Single liposome curvature
SLNs	Solid lipid nanoparticles
SOPC	1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
SPC	Soybean phosphatidylcholine
SPE	Solid-phase extraction
SPECT	Single-photon emission computed tomography

SPG	Shirasu porous glass
SPH	Sphingomyelin
SPI	Stationary phase interdiffusion
sPLA2	Secretory phospholipase A2
SPLVs	Stable plurilamellar vesicles
SQDG	Sulfoquinovosyldiacylglycerol
SSL	Sterically stabilized liposomes
STA	Spontaneous tubulation assay
SUVs	Small unilamellar vesicles
TBA	Tertiary butyl alcohol
TBHQ	*Tert*-Butyl-hydroquinone
TC	Temperature control
TEA	Triethanolamine
TEAE	Triethylaminoethyl
TEM	Transmission electron microscopy
TFR	Total flow rate
TG	Triacylglycerol
TLC	Thin layer chromatography
TMA-DPH	Trimethylammonium diphenylhexatriene
TPA	Tether-pulling assay
TPP	(3-Carboxypropyl)triphenylphosphonium bromide
TR-FS	Time-resolved fluorescence spectroscopy
TRINOV	Trichological innovation (hair treatment)
TR-SANS	Time-resolved light and neutron scattering
US	Ultrasound
USFD	Ultrasonic spray freeze-drying
UV	Ultraviolet
UVB	Ultraviolet B
UV/Vis	Ultraviolet-visible
VFA	Vesicle fluctuation analysis
VFF	Vertical flow focusing
VPGs	Vesicular phospholipid gels
XRD	X-ray diffraction

CHAPTER 1

Introduction

Abstract: The primary purpose of this introductory chapter is to provide a brief overview of the origin of liposomes, the progress in research, and different preparation methods, their characteristics, the mechanism behind their formation, and their diverse applications. Liposomes have received a lot of attention not just for their role in bioactives (drugs and biomolecules) transport, but also as model membranes for biophysical and biological research, making them valuable tools in scientific studies. It is important to have a foundational understanding of the field of liposomal research before exploring its details. This approach ensures that readers can interrelate the various individual topics effectively. A basic knowledge of liposome technology is essential for understanding the various applications and advancements in the field. By starting with an introduction to the history and development of liposomes, readers will appreciate the evolution of this technology and the milestones achieved over the years. Discussing the preparation methods will highlight the technological advancements and the innovative approaches developed. Understanding the characteristics and mechanisms of liposome formation will provide insights into their functionality and the science behind their numerous applications. The chapter also briefly highlights emerging trends such as artificial intelligence (AI)-assisted formulation design, marking a shift toward predictive and data-driven liposome engineering. This comprehensive overview sets the stage for a deeper exploration of specific topics in liposomal research and technology.

Keywords: Liposome technology, targeted delivery, phospholipid bilayers, controlled release, bioavailability, drug delivery, nanocarriers.

1.1. DEFINITION AND SIGNIFICANCE

The term “liposome” originates from the Greek words “lipo,” meaning “fat,” and “soma,” meaning “body.” This name aptly describes its composition, which primarily consists of phospholipids (see Chapters **2–5**) arranged in spherical structures. They are defined by their spherical structures, typically comprising one or more phospholipid bilayers (discussed in Chapter **6**) that encapsulate an aqueous solution (Fig. **1.1 a–d**). Each bilayer comprises two monolayers, the inner and outer monolayers or leaflets. Liposomes were first discovered in the 1960s and have since become a cornerstone in drug delivery systems. The unique structure of liposomes allows them to encapsulate both hydrophilic and hydrophobic therapeutic agents, offering

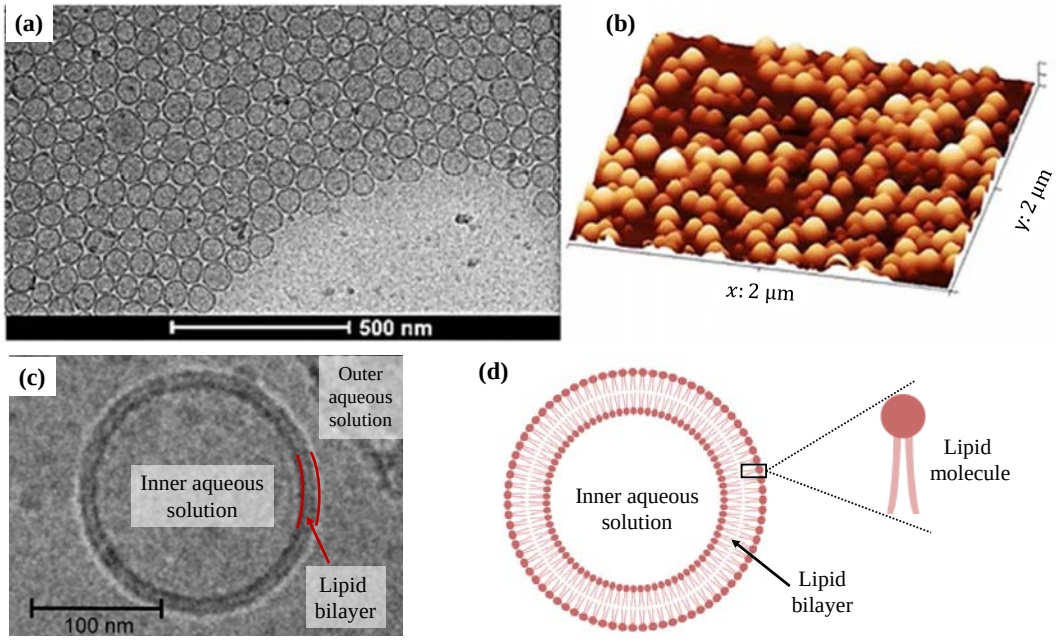

Figure 1.1: Liposome structure. (a) Liposomes revealed by cryoelectron transmission microscopy (Cryo-TEM). (b) Structure of liposomes acquired with atomic force microscopy (AFM). (c) Cryo-TEM image of a single liposome depicting the thickness of the lipid bilayer. (d) Sketch of a liposome along with a lipid molecule. Images (a)–(c) reprinted from ref. [1], under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Schematic (d) created using BioRender.com.

protection from degradation and facilitating targeted delivery to specific cells[1] or tissues[2].

This versatility makes liposomes particularly valuable in enhancing the efficacy and reducing the side effects of various treatments. Over the years, liposomes have been employed in delivering a wide range of therapeutic substances, including anticancer drugs, vaccines, and genetic materials [2]. Their ability to be modified with targeting ligands further enhances their specificity and effectiveness, cementing their role as a vital component in advancing pharmaceutical and biomedical applications.

[1] A cell is the basic structural and functional unit of living organisms, consisting of various organelles enclosed within a membrane.

[2] Tissues are groups of similar cells working together to perform specific functions, such as epithelial tissue covering body surfaces, muscle tissue facilitating movement, nervous tissue transmitting electrical signals, and connective tissue supporting and binding other tissues.

Apart from their prominent medicinal uses, liposomes find applications across diverse fields. In cosmetics and skincare [3], they deliver active ingredients like vitamins and moisturizers effectively into the skin. In the food industry, liposomes enhance the stability and bioavailability of nutrients and flavours. Agriculture explores liposomes for delivering pesticides and growth regulators, aiming to improve efficiency and reduce environmental impact [4]. They serve as an essential tool in biochemical research, aiding in drug delivery studies and membrane modelling. In diagnostics, liposomal formulations are used for targeted imaging and detection in assays. Environmental applications involve using liposomes to encapsulate and deliver remediation agents for cleaning pollutants [5]. Additionally, liposomes have potential uses in industrial settings, such as textile treatments [6] and the production of functional materials, showcasing their versatility beyond medical contexts.

1.2. HISTORICAL BACKGROUND AND DEVELOPMENT

Liposomes were first recognized by researchers studying lecithin[3] dispersions in blood clotting. However, earlier researchers examining lecithin colloidal systems or myelins[4] since the mid-1800s used various names such as lecithin emulsions, sols, micelles, suspensions, or even during studies of dry lecithin film swelling. The first description of lipid swelling came in 1854 by Virchow [7], who observed swelling when he placed a nerve core into an aqueous medium due to external agitation or some crystal defect, some liposomes detached from the hydrating mass. He named these structures "myelin." However, during microscopic observation, he failed to recognize the liposomes and the liquid crystals of myelin figures.

Additionally, in 1911, Otto Lehmann [8] presented an optical micrograph and named the observed structures "artificial cells," which we now understand to be dispersions of multilamellar liposomes (Chapter **7**). Despite this, several researchers continued working on lecithin dispersions without recognizing the structure, characteristics, or system until Alec Bangham and his colleagues published their seminal work in the mid-1960s. In the late 1950s, many researchers studied the physical properties of blood, blood cells, and phospholipases. Bangham was particularly interested

[3]Lecithin is a phospholipid essential for cell membranes and emulsification.
[4]Myelin is a lipid-rich sheath that insulates nerve fibers, ensuring fast electrical signal transmission.

PART I

FUNDAMENTALS OF LIPIDS CHEMISTRY AND PHYSICS

CHAPTER 2

Classification of Lipids

Abstract: This chapter investigates the role of lipids in biological systems, including their structural variety and significance in liposome formation and function. It focuses on a variety of lipids, including fats, oils, sterols, glycerophospholipids, and sphingolipids, with a particular emphasis on phospholipids, sphingolipids, and cholesterol, all of which play important roles in liposome structure. The chapter investigates the structural features of these lipids, their distinct contributions to cellular membranes, and their effects on biological processes. The involvement of cholesterol in lipid bilayer stabilization, the amphiphilic character of phospholipids in membrane synthesis, and the structural importance of sphingolipids in membrane integrity are all highlighted.

Keywords: Lipids, cholesterol, phospholipids, glycerophospholipids, fatty acids, sphingomyelin.

2.1. INTRODUCTION

Lipids are ubiquitous throughout the living world, spanning microorganisms, higher plants, and animals, constituting one of the three major components of biological matter alongside proteins and carbohydrates. As a diverse class of molecules, they exhibit a wide range of structures and biological functions and are found in all cell types. Their primary role in cellular function is to form the lipid bilayer permeability barrier of cells and organelles. Phospholipids, a significant class of lipids, serve as the primary building blocks of cell membranes and liposomes. Additionally, other lipid classes play important roles in biological systems.

The term “lipids” generally encompasses fats, oils, and waxes. Fats, also known as triglycerides, consist of glycerol triesters and several fatty acids, solidifying at room temperature. Conversely, oils are glycerol esters existing in liquid form at room temperature, while waxes comprise esters of alcohols and acids other than glycerol. The terms “fats,” “oils,” and “lipids” often lead to confusion. Oils typically denote fats with a high proportion of unsaturated fatty acids, remaining liquid at room temperature. In contrast, fats predominantly refer to those solidifying at room

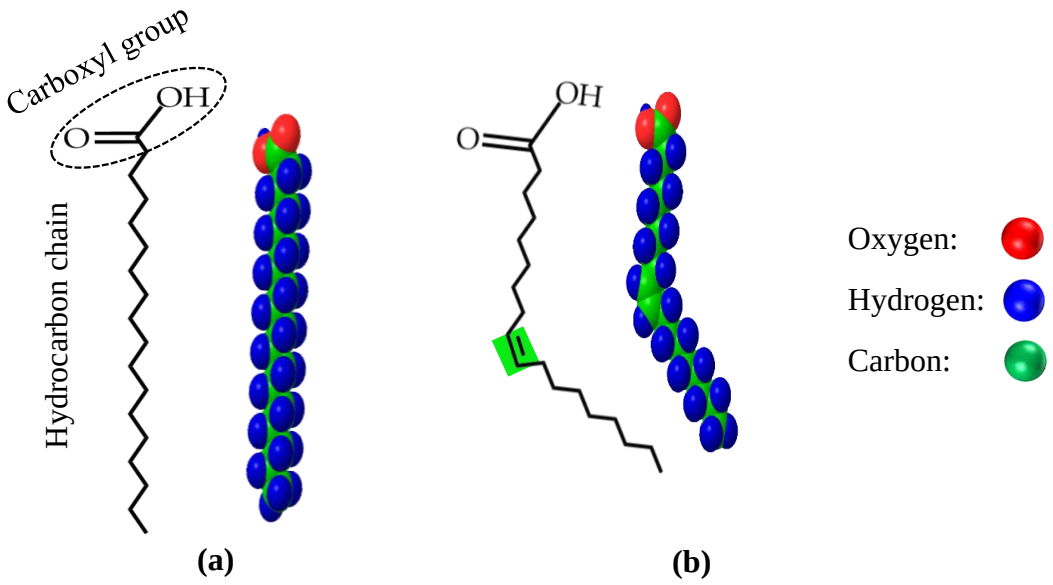

Figure 2.1: Preferred conformations of fatty acids. (a) The 18-carbon saturated fatty acid, stearic acid, appears straight due to only single C−C bonds. (b) The 18-carbon unsaturated fatty acid, oleic acid, bends due to the presence of *cis* double bonds. Drawn from ChemDraw professional 16.0.

temperature due to a high content of saturated fatty acids. Notably, saturated acids exhibit flexible, uniform chains conducive to tight packing. In contrast, unsaturated acids feature rigid kinks at positions containing *cis* double bonds, hindering orderly chain arrangements necessary for solid formation (Fig. **2.1 a, b**). The term "lipids" is a common descriptor, encompassing a broader range of molecules beyond triglycerides, emphasizing their varied roles and structures within biological systems.

There is no exact definition of the term "lipids," but many lipidologists have provided applicable meanings to assist in the chromatographic analysis of these compounds. According to Christie [1], lipids encompass a wide variety of natural products, including fatty acids and their derivatives, steroids, terpenes, carotenoids, and bile acids, which share the common property of solubility in organic solvents like diethyl ether, benzene, chloroform, or methanol. Similarly, Kates [2] defines lipids as compounds composed of extended hydrocarbon chain groups, typically insoluble in water but soluble in organic solvents such as ether, hexane, or chloroform. Kates extensively studied various lipid classes, including hydrocarbons, alcohols, aldehydes, fatty acids, and their derivatives such as glycerols, waxes, phospholipids, glycolipids, and

sulfolipids, along with fat-soluble vitamins, carotenoids, sterols, and their fatty acids. Gurr and James [3] further describes lipids as a chemically heterogeneous group of compounds insoluble in water but soluble in non-polar solvents like hydrocarbons, alcohols, or chloroform. However, defining lipids solely based on solubility presents challenges, as some substances considered lipids, such as very short-chain fatty acids (1–4 carbons), are insoluble in organic solvents but completely soluble in water. While fatty acids with 1–3 carbons are typically excluded from the lipid definition, those with four carbons, found in dairy fats, are often included. Hence, it is evident that lipids are defined based on their solubility properties rather than primarily on their chemical structure. Gunstone and Henslof [4] provide an excellent glossary of lipid terminology, simplifying fatty acid and lipid names, significant oils and fats, analysis-related terms, refining processes, modifications, and notable figures and journals in lipid chemistry.

2.2. MAJOR CLASSES OF LIPIDS

Several classification systems have been suggested based on the biological activity, chemical composition, and physicochemical properties of lipids. Here, we will primarily focus on classifying lipids based on their physicochemical properties, which divides lipids into two basic groups: neutral and polar lipids (see Table **2.1**). Neutral lipids are commonly found in cells as energy storage molecules and are characterized by their lack of net charge at cellular pH, hence their name "neutral." As a result, they are nonpolar and insoluble in water. In contrast, polar lipids exhibit amphiphilic properties and serve as the building blocks of cell membranes and vesicles. Note that *amphiphilic properties* refer to the ability of a molecule to have both hydrophilic (water-attracting) and hydrophobic (water-repelling) regions, enabling interactions with both water and nonpolar substances. Throughout this book, I will refrain from using the term "polar lipids" for brevity, opting instead to use "lipids" to refer to polar or amphiphilic lipids.

Polar lipids consist of both water-soluble and water-insoluble groups. The water-soluble groups, often referred to as polar (or hydrophilic or lipophobic) heads, contrast with the water-insoluble groups, known as nonpolar (or hydrophobic or lipophilic) hydrocarbon chains or tails. The polar groups encompass many function-

CHAPTER 3

Lipid Extraction

Abstract: This chapter thoroughly reviews lipid extraction methods, emphasizing their fundamentals, developments, and real-world uses. With an emphasis on effectiveness, sustainability, and safety, it examines established procedures like the Bligh and Dyer and Folch processes and cutting-edge strategies like solid-phase extraction and supercritical fluid extraction. In order to guarantee high-quality extraction for subsequent studies, the chapter covers important factors such as solvent selection, sample preparation, and lipid preservation. It provides insights into contemporary lipidomics by describing cutting-edge approaches like the Matyash and BUME (butanol-methanol extraction) methodologies, which balance cost-effectiveness, environmental impact, and application across various lipid classes.

Keywords: Lipid extraction, Bligh and Dyer, Folch method, Soxhlet extraction, green solvents.

3.1. INTRODUCTION

Understanding phospholipids' chemistry and biological functions, essential building blocks of biological membranes and liposomes, requires the extraction and analysis of lipids. There is a multitude of methods available, and analysts must select the most suitable extraction method[1] for identifying and quantifying lipids. Solvent extraction methods are widely favoured for extracting lipids from tissue samples. Apart from organic solvent extraction, non-solvent extraction and instrumental methods leveraging the physicochemical properties of lipids are also utilized for content determination. Recently, supercritical fluid extraction and accelerated solvent extraction methods have gained popularity for lipid extraction. The primary objective of all extraction procedures is not only to remove cellular or fluid lipids from other constituents such as proteins, polysaccharides, and small molecules (e.g., amino acids and sugars) but also to preserve these lipids for further analyses. In any lipid extraction technique from tissues, two main objectives are emphasized: firstly,

[1]In chemistry, extraction refers to the separation process of removing a substance from a matrix, including liquid-liquid and solid-liquid extractions.

the effective extraction and solubilization of lipids from the sample using organic solvents, and secondly, the isolation of non-lipid impurities from the extracts.

The most commonly used lipids for liposome preparation include PC, primarily extracted from hen egg yolk or soybeans; PA, PS, PE, and SPH from brain tissue; and CL from bovine heart. Various glycolipids, such as galactocerebroside, are sourced from pig brains, while PG is typically synthesized from PC through chemical conversion. CHOL is predominantly extracted from sheepskin [1].

3.2. SAMPLE STORAGE AND PRE-EXTRACTION STEPS

Proper handling and storage of samples are essential for obtaining valid results in any form of chemical analysis. A sample yields satisfactory results when its properties accurately represent those of the bulk material from which it is derived. Sample preparation for lipid analysis varies depending on the source and nature of the lipids involved, making it impractical to follow a single standard extraction method for all lipid types across different sources.

Extracting lipids as soon as possible after tissue separation is recommended to minimize degradative changes, such as lipid oxidation, which can occur over time. When immediate extraction is not feasible, samples should be stored at very low temperatures (-20 °C or lower) in sealed containers, preferably glass containers with Teflon-lined caps, and stored under a nitrogen atmosphere or on dry ice. Thawing should be avoided during subsequent extraction to prevent lipid degradation. Precautions should also be taken to prevent autoxidation, such as conducting the process in the presence of nitrogen and adding exogenous antioxidants like BHT (butylated hydroxytoluene). Glassware should be kept clean, and the use of plastics should be minimized. For blood samples, it is essential to ensure immediate treatment after collection to prevent sample degradation [2].

Several preparatory steps are followed in all lipid analyses, i.e., removal of water, reduction of particle size, and hydrolysis for separating lipid from the bound proteins and/or polysaccharides. The steps are given below:

- **Sample predrying:** Drying the samples before solvent extraction is often critical because many organic solvents do not easily penetrate samples con-

taining water, leading to inefficient extraction. However, drying at elevated temperatures is problematic as some lipids may bind to proteins and polysaccharides (e.g., carbohydrates), making their isolation with organic solvents difficult. Therefore, vacuum oven drying at low temperatures or lyophilization is preferred, enhancing the sample's surface area and enabling better lipid extraction.

- **Size reduction:** The efficiency of lipid extraction from dried samples depends on particle size, making adequate grinding essential. Grinding provides a more homogeneous sample and increases the surface area of lipid molecules available to the solvent. This step is often performed at low temperatures to reduce lipid oxidation. The sample and solvent are blended in a high-speed comminuting machine, such as a blender, for optimal extraction.
- **Acid/alkali hydrolysis:** Some samples contain lipids bound to proteins (e.g., lipoproteins) or polysaccharides (e.g., glycolipids), making direct extraction with nonpolar solvents difficult. To estimate the concentration of such components, breaking the bonds holding the lipids and other components together is necessary before solvent extraction. Acid hydrolysis[2] is commonly used to release bound lipids into easily extractable forms. Several dairy products, such as butter, cheese, milk, or milk-based products, require alkaline pretreatment with ammonia to break emulsified fat, neutralize any acid, and solubilize proteins before solvent extraction [3].

It has been observed that enzymatic oxidation can lead to the loss of not only unsaturated fatty acids but also intact lipids [4]. Hydroperoxide groups in the oxidized lipids form covalent bonds with membrane proteins, which are released only upon treatment with bacterial proteases. Similar effects are likely observed with autoxidized lipids in tissues. Various pretreatments have been developed to deactivate enzymes, allowing tissues to be stored longer. For instance, lipases in small samples of plant [5] or animal [6] origin can be denatured by briefly plunging them into boiling water, significantly prolonging the shelf life of the treated samples. A similar effect is noted when boiling with a dilute acetic acid solution [7]. However, there is a need for practical re-evaluation of these protocols in light of modern

[2] Acid hydrolysis can break both covalently and ionically bound lipids.

CHAPTER 4

Fractionation of Lipid Extracts

Abstract: Lipid extraction and fractionation are essential methods for understanding varied and complex composition of natural lipids. This chapter emphasizes the need for the precise separation procedures for isolating and analyzing distinct lipid classes. Column chromatography, thin-layer chromatography, and high-performance liquid chromatography are investigated, emphasizing their concepts, advances, and practical applications. Modern technologies, such as solid-phase extraction and chromatographic procedures combined with modern detection systems such as liquid chromatography-mass spectrometry, are emphasized for their efficiency and high resolution. These approaches are beneficial for isolating and characterizing specific lipids required to form stable and functional liposomal structures, hence facilitating the development of optimized formulations for liposome-based applications.

Keywords: Lipid extraction, fractionation, thin-layer chromatography, high-performance liquid chromatography, solid-phase extraction, lipid analysis.

4.1. INTRODUCTION

Total lipid extracts from natural sources often contain a diverse mixture of lipid classes, making the complete separation, identification, and estimation of each component complex. While some lipid components may dominate proportionally, minor lipid classes are exciting due to their unique properties. Conversely, the presence of these minor lipid classes can sometimes obscure the characteristics of major lipid classes. In such cases, it is essential to separate each lipid class for individual analysis. After extracting lipids from tissue samples, the extracts are typically fractionated into various lipid classes, followed by the separation and analysis of each component.

Several techniques are available for lipid fractionation. However, the ideal method should be rapid, convenient, and cost-effective, ensuring high yield and purity of each lipid class while maintaining the integrity of the original lipid composition. The choice of fractionation method often depends on the properties of the extracted lipids. For example, lipid extracts from animal sources are likely to have a high

proportion of polar lipids, with the remainder being neutral or nonpolar. In contrast, lipids from plant seeds typically exhibit the reverse composition.

Generally, two main techniques are used to separate lipid components: solvent-based fractionation, which leverages the specific chemical properties and solubility differences of certain lipid classes, and chromatographic methods (including column, paper, or thin-layer chromatography), which separate lipids based on differences in polarity. These methods provide a means to isolate and analyze individual lipid classes effectively.

4.2. SOLVENT FRACTIONATION

Solvent fractionation is the simplest and often the most efficient method for separating a specific group of lipids when other lipids are not of interest. Single-solvent fractionation using different adsorbents is commonly employed to separate triacylglycerols (TGs) from more polar lipids. In this method, the least polar solvent capable of eluting all TAGs from the adsorbent is determined experimentally. The lipid extracts are then applied to an adsorbent column, and total triglycerides and less polar lipids are readily eluted with the designated solvent. Alternatively, a slurry of lipid extracts, solvent, and adsorbent can be mixed, filtered, and followed by additional washing with fresh solvent. In either case, the eluate contains triglycerides and fewer polar lipids, whereas more polar lipids remain bound to the adsorbent [1].

Precipitation of polar lipids (e.g., phospholipids and glycolipids) from all neutral or nonpolar lipids (e.g., triglycerides, cholesterol, some pigments) using acetone is one of the simplest one-step methods. In this procedure, an aliquot of crude lipid extract is evaporated to dryness under nitrogen, and approximately 20 to 30 volumes (around 5 mL/100 g lipid extract) of acetone is mixed by vortexing for 1 min. The mixture is then left on ice for about 1 h. After centrifugation, the supernatant is collected, and the procedure is repeated. The acetone extracts contain nonpolar lipids such as glycerides, cholesterol, sterol esters, and lipid-soluble vitamins, while the pellets contain phospholipids and glycolipids [2].

4.3. CHROMATOGRAPHIC FRACTIONATION

Chromatography is a powerful technique where components are separated based on slight differences in their distribution within a two-phase system, typically gas-liquid or liquid-liquid (similar to adsorption where gas/liquid-solid systems are utilized). Table **4.1** outlines the various terminologies used in chromatography. In 1952, the first gas-liquid chromatography (GC or GLC) was introduced by James and Martin [3], and a few years later, Stahl [4] developed thin-layer chromatography (TLC). TLC was the earliest chromatographic technique used to analyze phospholipids and remains in use today.

Table 4.1: Common terminologies used in chromatography.

Term	Definition
Analyte (or eluite)	Component to be separated during chromatography
Bonded phase	A stationary phase covalently bonded to support particles or the inside wall of the column
Chromatograph	Equipment for sophisticated separations, such as gas or thin-layer chromatography
Chromatography	Method where mixture components are distributed between stationary and mobile phases
Chromatogram	Visual output of the chromatograph; different peaks indicate different sample components
Detector	Instrument for qualitative and quantitative detection of analytes post-separation
Eluent	Solvent carrying the analyte
Eluate	Mobile phase leaving the column
Elution	Process of washing out a component through a column using a suitable solvent
Mobile phase	Solvent that migrates through the column
Stationary phase	Substance immobilized on support particles or the inner wall of the column tubing
Retention time	Characteristic time for an analyte to pass from the column inlet to the detector
Sample	Matter analyzed in chromatography, consisting of single or multiple components
Solute	Synonymous with components in a sample
Solvent	Substance capable of solubilizing another substance; the liquid mobile phase in liquid chromatography
Zone (or band)	Region in the chromatographic bed where one or more sample components are located

CHAPTER 5

Lipid Oxidation and Hydrolysis

Abstract: This chapter discusses lipid hydrolysis and oxidation, which are critical for liposome stability. Liposomes are vulnerable to these processes due to their lipid-based nature, which might compromise their structural soundness and functioning. Fatty acids and LPCs are produced during hydrolysis, which destabilizes liposomes. Enzymatic hydrolysis by lipases speeds up this breakdown even more, especially in inflammatory environments. Conversely, oxidation results in hazardous compounds that harm bilayer membranes. These processes, which are affected by temperature, pH, and metal ions, can be stopped by antioxidants or proper storage. This chapter also covers strategies for minimizing degradation in stable liposomal formulations and the effects of pH, temperature, and buffer concentration on alkaline hydrolysis.

Keywords: Lipid degradation, oxidation, hydrolysis, liposome stability, antioxidants.

5.1. INTRODUCTION

Lipids, essential for liposome preparation, are inherently prone to chemical instability, primarily due to functional groups in their structure that are susceptible to hydrolysis and oxidation. Unsaturated bonds in hydrocarbon chains are very reactive, rendering them more prone to oxidation than saturated sites. Similarly, hydrolysis breaks down fatty acid chains, producing byproducts, including glycolipids, free fatty acids, and glycerol.

Using unsaturated lipids, such as those obtained from biological sources like egg or soybean lecithin, significantly reduces the shelf life of liposomes due to this chemical instability. When exposed to air, metal ions, or peroxides, these polyunsaturated fatty acids are more likely to undergo oxidative destruction, even though they are essential for proper operation. On the other hand, because saturated lipids do not contain reactive double bonds, they provide superior chemical stability.

To address these issues, several analytical techniques, such as liquid chromatography, thiobarbituric acid assays for lipid oxidation, and spectroscopy approaches, are used to monitor lipid breakdown. Understanding and reducing lipid hydrolysis

and oxidation is very important for improving the chemical stability of liposomal formulations, which is required for their effective medicinal use.

5.2. LIPID OXIDATION

Lipid oxidation has long been a significant topic in biological and food sciences. When lipids are exposed to oxygen, their unsaturated moieties rapidly oxidize, necessitating manipulation in an oxygen-free environment, often achieved using nitrogen. Lipid oxidation occurs similarly to other organic compounds through a free radical chain mechanism. Free radicals, formed when weak bonds split, are highly unstable and react rapidly to achieve stability by capturing electrons from nearby stable molecules, initiating a chain reaction. This radical reaction can result in chain breakage or cyclic peroxide formation in lipids with adjacent double bonds.

Lipid oxidation yields many chemicals, including aldehydes, ketones, alcohols, hydrocarbons, and fatty acids. These chemicals are distinguished by their particular UV-vis spectra, which may be identified instrumentally or by their distinct odours caused by volatile molecules interacting with olfactory receptors. Lipid oxidation, known as peroxidation or autoxidation, can have negative consequences if not stopped soon. It can harm cell membranes (*in vivo*), prevent cell reproduction, and cause toxicity in cell membranes (*in vitro*). For example, treating red blood cells (RBCs) with vesicles containing oxidative products might result in haemolysis and vesicle shedding from erythrocytes [1]..

In addition to polyunsaturated fatty acids (PUFAs), the free amino groups of phospholipids (e.g., PE, PS) can oxidize, leading to a brownish-yellow discolouration and a fishy smell. It has been reported that free amino groups of PE disappear in proportion to the oxygen absorbed during oxidation [1]. PUFA and their esters are particularly susceptible to free-radical mediated oxidation, while cholesterol is more resistant. For instance, cholesterol oxidation in low-density lipoprotein (LDL) occurs only after most PUFAs esters have oxidized. However, cholesterol oxidative products, such as 7-hydroxycholesterols and 7-ketocholesterols, can be toxic, necessitating careful analysis of liposome formulations containing cholesterol [2].

Table 5.1: Factors affecting lipid peroxidation.

Accelerating factors	Inhibiting factors
High temperature	Refrigeration
Light (UV and blue)	Opaque or colored containers/wrappers
Radiation (α, β, γ)	Exclusion of oxygen
Peroxides	Blanching
Lipoxygenases	Antioxidants
Organic iron catalysts	Metal deactivators
Trace metal catalysts	

Lipid oxidation is influenced by various physical and chemical factors (Table **5.1**). Factors accelerating lipid oxidation include oxidants, sonication, and radiation-induced free radicals. To minimize oxidation, sonication should be performed at low temperatures (above the lipid's transition temperature) in an inert atmosphere. Prolonged sonication can also promote lipid oxidation. Additionally, antioxidants and radical scavengers, such as tocopherol (vitamin E), butylated hydroxyanisole (BHA), BHT, and mono-*tert*-butyl-hydroquinone (TBHQ), can be added to prevent oxidation. These compounds inhibit oxidation by delocalizing the π electron produced during the free radical reaction.

5.2.1. Mechanism of Lipid Oxidation

PUFAs are more sensitive to oxidation than saturated fatty acids due to their multiple double bonds, which create activated methylene bridges with highly reactive hydrogen atoms. The presence of a double bond adjacent to a methylene group ($-CH{=}CH-CH_2-$) weakens the methylene C$-$H bond, making the hydrogen atom more susceptible to abstraction. Lipid oxidation, like other radical reactions, proceeds through three significant steps: initiation, propagation, and termination. Once initiated, lipid oxidation is autocatalytic; the reaction becomes self-propagating and self-accelerating.

5.2.1.1. Initiation

Lipid peroxidation proceeds so rapidly that it is often considered a spontaneous process [3]. However, it does not occur spontaneously from a thermodynamic perspective, as it cannot happen independently. Ground-state oxygen (O_2) exists

CHAPTER 6

Understanding Lipid Self-Assembly

Abstract: In this chapter, the structural dynamics and properties of liposomes, composed of amphiphilic lipid molecules with liquid crystal-like features, are examined. The self-assembly of lipids into aggregates such as micelles and bilayers is driven by weak non-covalent interactions, including hydrophobic forces. Factors such as temperature, pH, and lipid concentration influence the size and shape of these aggregates, while the packing parameter provides insights into the geometry of lipid structures. The chapter also discusses lipid bilayer permeability, highlighting the effects of temperature and hydrophobicity on the movement of molecules like ions, water, and nonpolar gases. Investigating packing constraints and permeability mechanisms is critical for advancing knowledge of liposome behavior, particularly in biomedical applications.

Keywords: Amphiphilic lipids, self-assembly, packing parameter, bilayer structure, permeability, drug delivery, lipid dynamics.

6.1. INTRODUCTION

As the main structural and functional component in liposomes, phospholipids play a vital role in their formation. Their amphiphilic nature, with hydrophilic headgroups and hydrophobic tails, drives the organization into bilayered vesicles with unique structural and dynamic properties. Liposomes, which may form bilayer structures in aquatic conditions, have many features in common with liquid crystals. Liquid crystals are an unusual state of matter that combines organized molecular structures akin to solids with the fluid-like movement of liquids. The dual nature of liposomes, which resemble liquid crystals, is critical to their structural dynamics and usefulness. Understanding liposomes in the context of liquid crystals helps to explain their capacity to undergo modifications, which is critical for their application in numerous fields, such as controlled medication release, gene therapy, and cosmetic formulations.

The process by which amphiphilic lipids self-assemble into ordered aggregates is the first step in the production of liposomes. Weak, non-covalent interactions, including electrostatic, van der Waals, hydrophobic, and hydration forces, are the main drivers

in this self-assembly. Because the nonpolar tails of lipid molecules group together to minimize disturbance to the hydrogen-bonding network of water, hydrophobic interactions are particularly prominent among them. Entropically favourable stable structures are formed as a result of this clustering. The precise arrangement of the hydrophilic and hydrophobic components determines the size, form, and stability of the resultant aggregates. The structure of lipid aggregates, which can be spherical, cylindrical, or bilayers depending on factors like temperature and lipid concentration, is largely determined by the balance between repulsive interactions between polar headgroups and attractive hydrophobic forces.

The packing parameter, which defines the geometry of lipid aggregates, is a key factor in determining the formation and stability of liposomes. The area of the headgroup, the critical chain length of the lipid molecules, and the adequate volume of the hydrophobic chains are some variables that affect the packing parameter. Lipid aggregation shape is mainly determined by the packing parameter, whose value may be used to forecast whether a given lipid will form bilayers, spherical micelles, or cylindrical micelles. Lipids with packing parameter values between 1/3 and 1/2, for instance, create cylindrical micelles, whereas those with values around 1 form bilayer phases. Spherical vesicles, widely utilized in liposome-based drug delivery systems, are generally formed when lipids possess a packing parameter between 1/2 and 1.

In addition to their structural characteristics, liposomes have certain dynamic characteristics that are critical to their operation. These include their permeability to different chemicals, stability in aqueous solutions, and capacity to encapsulate hydrophilic and hydrophobic compounds. The fundamental building block of liposomes, lipid bilayers, show preferential permeability to various compounds according to their charge, size, and hydrophobicity. The permeability of lipid bilayers is poor for charged molecules like ions, but very easy for nonpolar gases like oxygen, carbon dioxide, and nitrogen. Temperature and cholesterol content both affect lipid bilayer permeability; cholesterol can decrease permeability by rearranging the lipid chains and making the bilayer more stiff.

The study of liposome dynamics and behaviour, particularly the processes that control their shape and permeability, is critical for furthering their use in drug

administration. Understanding lipids' packing limitations and permeability processes within liposomes gives important information about how these structures work and how their characteristics might be optimized for specific purposes. Furthermore, the ability to control lipid aggregation and bilayer properties enables the development of liposome formulations that can adapt dynamically to environmental changes, increasing their flexibility in medicinal and cosmetic applications.

6.2. LIQUID CRYSTALS

Matter exists in three primary states: solid, liquid, and gas. Gases are characterized by widely spread particles that move freely, lacking a fixed shape or volume. In contrast, solids and liquids are more condensed forms, with particles closer together and interacting significantly. This particle arrangement and interaction difference leads to distinct behaviors and properties across these states. To explore these properties, condensed matter physics focuses on understanding and manipulating the physical properties of matter in its solid and liquid states. This field employs principles from quantum mechanics, electromagnetism, and statistical mechanics to study these interactions.

Within condensed matter physics, soft condensed matter, or soft matter, constitutes a specialized sub-field that deals with materials existing on an intermediate scale between atomic and macroscopic sizes. Soft matter physics investigates complex fluids and systems such as polymers, colloids, liquid crystals, and biological substances. These materials exhibit unique properties that lie between those of traditional solids and liquids. As temperature rises, materials transition from crystalline solids to isotropic liquids, leading to the emergence of intermediate, liquid crystalline phases. These phases exhibit diverse mechanical, optical, electrical, and structural properties, distinguishing them from traditional solids and liquids. Materials in these phases are termed "liquid crystals (LCs)", characterized by a certain degree of anisotropy where molecules exhibit liquid-like order along specific axes.

LCs flow similarly to liquids but possess a molecular orientation reminiscent of crystals. Fig. **6.1** illustrates these three states: solid, liquid crystal, and liquid. Another synonymous term for LCs is mesogens, and their various phases are collectively termed mesophases. A "mesophase" is an intermediate phase between the solid (crys-

PART II

LIPOSOME PREPARATION, MECHANISMS, AND APPLICATIONS

CHAPTER 7

Liposome Classification

Abstract: This chapter is important as it provides a comprehensive understanding of the various classifications of liposomes, which are essential for designing effective drug delivery systems. Size and lamellarity liposome, composition, and preparation methods are the factors that determine the classification of liposome. The chapter emphasizes how these classes affect the size of liposomes, stability, and function, with particular attention to their use in drug administration.

Keywords: Liposome size, lamellarity, PEGylation, ligand-targeted liposomes, multifunctional liposomes, drug delivery.

7.1. INTRODUCTION

In the first chapter, a brief introduction was given to give readers an idea of liposomes and their uses. However, it might be challenging to comprehend the different terms, including lipids, liposomes, and self-assembly. These ideas become more apparent following the in-depth talks in chapters 2 through 6. In Chapter **2**, the fundamental role of double-chain lipids, particularly phospholipids, as the building blocks of biological membranes and liposomes is briefly introduced. Understanding the structure and physicochemical characteristics of lipids is essential for working with liposomes. To this end, the preceding chapters provide an in-depth overview of lipids, addressing their definition, structure, extraction and purification methods, degradation mechanisms, and the theory of lipid self-assembly (Chapters **2–6**). The final chapter in this series (Chapter **6**) discussed how phospholipids in the fluid phase aggregate into a lamellar phase (bilayer). Under specific conditions, such as temperature, concentration, and the ionic strength of the lipid-water mixture, these planar bilayers transform into spherical bilayers, known as spherical vesicles or liposomes.

This chapter explores the classification of liposomes, which is essential for understanding their diverse applications. Liposomes can be classified based on their

structural properties (size and lamellarity), preparation methods, composition, and application.

7.2. CLASSIFICATION BASED ON THE LAMELLARITY AND SIZE

Based on lamellarity (the number of lipid bilayers present), liposomes can be classified into two main categories: unilamellar and multilamellar vesicles [1]. Unilamellar vesicles have a single lipid bilayer separating the internal and external aqueous solutions. They are further categorized based on size into small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs), and giant unilamellar vesicles (GUVs). Multilamellar vesicles (MLVs) contain multiple concentric lipid bilayers, which trap aqueous solutions between them. MLVs are typically polydisperse and exhibit spherical onion-like structures, oblong forms, or tubular-like configurations. When MLVs have a smaller number of bilayers, they are known as oligolamellar vesicles (OLVs) or paucilamellar vesicles (PLVs), typically comprising 2–5 bilayers. Sometimes, a single bilayer vesicle encapsulates many smaller vesicles in a non-concentric arrangement. These structures, known as multivesicular vesicles (MVVs), efficiently trap multiple smaller vesicles within them. The size, number of vesicles formed, and arrangement of liposome bilayers can vary depending on the lipid type used, preparation methods, and specific operating conditions. The average size (diameter) and number of bilayers (lamellarity) of these vesicles are depicted in Table **7.1**.

Unfortunately, the nomenclature for liposomes lacks precise standardization. For instance, definitions of SUVs and LUVs can vary among researchers. SUVs are often referred to as vesicles at the lower limit of size, constrained by lipid packing considerations that prevent them from being smaller. The radius of curvature in SUVs is so pronounced that the ratio of lipid molecules between the outer and inner monolayers is approximately 2:1. This high curvature imposes geometric constraints that distinguish SUVs from LUVs. The exact size limit can vary slightly depending on factors like the ionic strength of the aqueous phase and the lipid composition, typically around 15 nm for EPC liposomes and 25 nm for DPPC liposomes.

LUVs are frequently employed in carrier studies due to their larger encapsulation volume of the aqueous solution compared to SUVs and MLVs, though they tend to be less stable than MLVs [2]. GUVs, which can reach sizes of a few tens of micrometres,

Table 7.1: Classification of liposomes based on size and lamellarity.

Liposome type	Liposome size (nm)	Lamellarity	Structure
SUVs	20 – 100	1	
LUVs	> 100	1	
GUVs	> 1000	1	
MLVs	> 500	> 5	
OLVs	100 – 1000	2 – 5	
MVVs	> 1000	1	

CHAPTER 8

Traditional Liposome Preparation Methods

Abstract: This chapter provides a complete overview of traditional (or conventional or classical) liposome preparation methods, including their concepts, benefits, and limits. It explains the fundamental procedures, such as thin-film hydration, solvent injection, and emulsification, required to produce liposomes with specified sizes, lamellarity, and encapsulation efficiencies. Furthermore, the chapter establishes the framework for comprehending advanced and unique approaches addressed in later chapters, making it an invaluable resource for new and seasoned liposome researchers.

Keywords: Liposome preparation, thin-film hydration, solvent injection, emulsification, encapsulation efficiency, lipid bilayer.

8.1. INTRODUCTION

Currently, several methods exist for liposome preparation, including traditional, modified conventional, and novel methods. Regardless of the technique, liposomes must be produced using methodologies that ensure reproducible products regarding composition, size, and size distribution while minimizing lipid oxidation and hydrolysis. Researchers should select the appropriate liposome type, such as SUVs, LUVs, GUVs, MLV, or MVVs, based on their specific study needs.

MLVs, with their higher lipid content, are ideal for membrane research, particularly in investigating geometrical, dynamic, and thermodynamic characteristics where sensitivity is critical. On the other hand, unilamellar liposomes, especially LUVs, are preferred for studying membrane permeability and reconstituted proteins due to their larger entrapment volume compared to SUVs and MLVs. GUVs offer significant advantages as model membranes for studying mechanical properties such as elasticity, tensile strength, and transport properties such as permeability under an optical microscope [1]. Additionally, they are valuable for investigating curvature sensing and generation by proteins or peptides [2, 3], as well as pore formation kinetics [4]. When encapsulating substances, MLVs are suitable for hydrophobic materials, whereas LUVs are better for hydrophilic substances [5]. SUVs are the

preferred choice for applications requiring smaller liposomes. MVVs, produced with DepoFoam® technology, have gained significant attention in recent years due to their ability to provide sustained release of encapsulated drugs over a period of 1 to 30 days, without altering the drug's molecular structure. These liposomes ensure prolonged therapeutic effects, reducing the frequency of drug administration and enhancing patient compliance. MVLs are currently used in the treatment of neurological diseases, cancer, and post-surgical pain, with ongoing development for other applications such as reducing surgical bleeding. Note that liposomes of the same type prepared by different methods may exhibit varying properties, including encapsulation efficiency, leakage rate of the encapsulant (membrane permeability), and stability.

As discussed in Chapter **1**, researchers in the late 1950s investigated various characteristics of phospholipid dispersions in aqueous solutions. It was Bangham in the mid-1960s who made a pivotal observation about liposomes, highlighting their ability to encapsulate aqueous solutions, their osmotic activity, and their distinct permeability properties for different molecules and ions. Following Bangham's groundbreaking work, numerous new methods for preparing liposomes were explored, taking into account factors such as size distribution, lamellarity, charge, encapsulation efficiency, and membrane permeability. Concurrently, advancements in physical, chemical, and biological methods were developed to characterize liposomes. These developments in liposome research for drug delivery systems evolved with these advancements.

To enhance understanding, it is helpful to categorize liposome preparation methods (classical and novel) in a clear and structured manner. These methods can be broadly classified into two main categories, as shown in Table **8.1**. All these methods either start with preformed lipid bilayers and involve subsequent energy or power input through various steps, or they may originate from non-bilayer lipid systems such as mixed micelles (oil-in-water type, O/W), organic solutions, and micro-emulsions (water-in-oil type, W/O), each with differing solubility conditions. Essentially, vesicles are formed either by rearranging pre-existing bilayers (hydration methods) or by introducing lipid solutions in organic solvents into an aqueous phase (bulk

Table 8.1: Major classification of liposome preparation methods.

Category	Method	Description
Hydration methods	Direct dissolution	Lipids in the form of powders or cakes are dissolved directly into water.
	Thin-film hydration	Thin lipid films are deposited onto a glass substrate and then hydrated with an aqueous solution.
	Electroformation	Lipid films are hydrated with an aqueous solution in the presence of an electric field.
Bulk/solution methods	Lipid-solvent replacement	Lipids dissolved in an organic solvent are replaced by an aqueous solution.

methods). In both cases, bilayer formation precedes liposome generation. The mechanism of liposome formation has been discussed in Chapter **10**.

The fundamental requirement for forming liposomes in all techniques is the contact of lipid molecules with an aqueous phase at temperatures above the lipid's phase transition temperature (T_g) (see Section **6.7**). Temperatures below T_g cause phospholipid chains to become rigid, preventing them from bending and folding into the closed spheres necessary for liposome formation. Consequently, the preparation of liposomes generally requires a temperature higher than T_g of the chosen lipids. Below this critical temperature, liposome formation becomes challenging, and any resulting aggregates tend to have a fragile surface with roughness and uneven edges [6]. The values of T_g of various PCs have been provided in Table **6.4**.

Adding dry powdered phospholipids directly into water rarely leads to effective liposome formation in hydration methods. In such cases, most lipids remain incompletely hydrated, trapped within structures where only the outermost lipid bilayers hydrate. Subsequent application of heat and mechanical treatments (e.g., sonication, homogenization, extrusion) can help alleviate this issue, especially for single-component systems. However, achieving uniform mixing of various lipids and cholesterol in multicomponent mixtures remains challenging under these conditions. The addition of cholesterol complicates these treatments further. While potentially effective, prolonged heat and mechanical treatments can also degrade lipids significantly, which is a critical consideration in liposome preparation. Enhancing hydration effectiveness can be achieved by increasing the surface-to-volume ratio. This is typically done by forming a thin lipid film through solvent removal (e.g., chloroform) on a rotary evaporator under reduced pressure. Lipids in powder form can also be dissolved in

CHAPTER 9

Advances in Liposome Preparation Methods Since 2000

Abstract: This chapter explores recent advancements in liposome preparation techniques, including the adoption of innovative technologies such as electrohydrodynamics and microfluidics, alongside an improved understanding of lipid assembly mechanisms. These developments have made liposome production more efficient, reliable, and scalable, supporting both laboratory studies and industrial applications. The chapter also discusses modifications to traditional methods, highlighting their specific advantages and limitations. Furthermore, it distinguishes between methods suitable for large-scale industrial production and those designed for laboratory-scale research. It provides a valuable guide for researchers in choosing the most appropriate approach for liposomal formulations.

Keywords: Liposome production, hydration, microfluidics, electroformation, supercritical fluid.

9.1. INTRODUCTION

In Chapter **8**, various traditional methods for liposome synthesis were discussed. Majority of the methods were bulk processes where the local environment is not well controlled, leading to significant chemical and mechanical variations that result in a heterogeneous population of liposomes [1]. Liposome size and its distribution play a pivotal role in determining their effectiveness for *in vivo* applications. These parameters directly affect drug loading capacity, circulation time, biodistribution, cellular uptake, and overall therapeutic performance. While liposomes can vary in size from a few nanometers up to several micrometers, drug delivery systems generally require vesicles within the 50–150 nm range to ensure optimal performance [2, 3]. To control size and distribution, conventional methods often require additional processing steps (e.g., sonication and extrusion), which increase batch processing time and may lead to the inactivation or loss of active pharmaceutical ingredients (e.g., DNA) and degradation of lipid molecules [4]. In addition, scaling up these traditional preparation methods for industrial use comes with several challenges—such as high consumption

of organic solvents, poor EE, limited stability, poor consistency between batches, difficulty in incorporating new bioactive compounds, and the need for complex, multi-step procedures.

In the last two decades, there have been numerous developments in liposome production technology. These advancements are mainly aimed at producing liposomes on a large scale while achieving high EE of bioactive compounds. However, most of these techniques remain suitable only for laboratory-scale applications, with very few being appropriate for large-scale production. While the last chapter covered methods developed before the year 2000 under conventional techniques, this chapter explores novel methods and modifications made to conventional methods over the last two decades. Much of this discussion builds upon and extends the insights presented in our previously published comprehensive review on recent liposome preparation methods [5]. This chapter aims to cover a broad range of these recent preparation techniques, highlighting both their merits and demerits for both laboratory and industrial scales. Similar to conventional methods, recently developed methods will also be described in two categories: hydration of dry lipid and bulk methods.

9.2. HYDRATION METHODS

9.2.1. Heating Method

To overcome the limitations of conventional liposome production, such as the need for high shear or pressure and the use of toxic organic solvents like diethyl ether, acetone, methanol, or chloroform, a novel approach involving controlled heating has been developed. While posing questions regarding the stability and safety of the product, these problems also impede large-scale production. Dialysis, vacuum and gel filtration are examples of post-processing methods that might lessen toxicity, but they are time-consuming and impractical for industrial applications. The heating approach, which does away with the need for hazardous solvents and streamlines the liposome manufacturing procedure, was developed by Mozafari and his group [6, 7] to address these problems.

In this method [7], lipids are hydrated in an aqueous solution containing additives such as PEG or glycerol while continuously stirring for 1 h under carefully regulated

heating settings, usually between 60 and 120 °C. The mixture is heated, cooled, and then centrifuged for 15 min at 4000 rpm to separate the liposomes. Glycerol enhances liposome stability and does not need to be removed from the final product. TLC analysis confirmed that no lipid degradation occurred at this elevated temperature. There is less need for further sterilization procedures since the high temperature promotes lipid hydration and is a sterilizing agent. This process facilitates more stable liposomes while preserving the structural integrity of phospholipids. Bioactive compounds can be entrapped to liposomes at different points in the process—either right at the start when the temperature is above the transition point, or later on, once the liposomes have already formed at room temperature. Interestingly, Mortazavi et al. [8] managed to load plasmid DNA (pCMV-GFP) into liposomes with impressive efficiency (81%) by using calcium ions. This method also achieved a 71% EE for a liposomal formulation of isoniazid-hydrazone-phthalocyanine conjugate, with a particle size of 240 nm, a zeta potential of −57 mV, stability for up to 5 weeks at 4 °C, and a controlled drug release of 40% at pH 7.4 [9].

An upgraded version of the heating method (i.e., modified heating method), the Mozafari method [10], further simplifies the process by eliminating organic solvents entirely. Lipid components such as cholesterol, DPPC, DSPC, and phospholipon 90H are hydrated in an aqueous solution and heated to temperatures below 70 °C. This approach has advantages, such as good storage stability and homogeneous particle size distribution. For example, a liposomal formulation of nisin Z produced using the Mozafari technique had an encapsulation effectiveness of 54% and a particle size of less than 300 nm. It remained stable at 4 °C for 14 months [11]. Furthermore, the approach has been effectively utilized in various applications, including bioactives administration and developing de-bittering agents such as flavourzyme [12].

The heating method is a simple and fast process that avoids contamination from organic solvents, eliminating the need for sterilization. It can be adopted from small to industrial scale, offering scalability. However, it has some disadvantages, such as low EE, the requirement for high temperatures, and the potential degradation of phospholipids and drugs during the process.

CHAPTER 10

Liposome Formation Mechanism

Abstract: This chapter provides a comprehensive review of the mechanism underlying liposome formation, with a focus on the kinetic and thermodynamic factors that drive the process. It explores various pathways, such as lipid transfer into an aqueous medium and the formation of liposomes from pre-existing bilayers, while also examining the influence of kinetic parameters on the final structure. A thorough understanding of these mechanisms is essential for optimizing liposome size, stability, and drug release properties, ultimately enhancing their efficacy in applications such as drug delivery, diagnostics, cosmetics, and food science.

Keywords: Liposome formation, bilayer deformation, bending energy, line tension, critical radius.

10.1. INTRODUCTION

Understanding the mechanism of liposome formation is essential for optimizing their design and functionality in various applications. Even though amphiphilic compounds and liposomes are widely available, the exact process of how they form remains a scientific mystery, making it a subject for ongoing research. No single principle can explain the formation mechanisms across all systems, as these processes vary with the type of amphiphilic compound involved. For instance, the assembly process of phospholipid-based liposomes is quite different from those formed using detergents or block co-polymers. To better understand the thermodynamic stability and size distribution of various lyotropic structures (such as micelles, reverse micelles, microemulsions, and vesicles), researchers have explored several theoretical models and conducted numerous computer simulations. However, only the detergent depletion method has adequately explained the energy of intermediate structures in liposome generation processes.

Gaining a more profound understanding allows for better control over liposome size, stability, and drug release properties, thereby enhancing their effectiveness in targeted drug delivery and reducing side effects. Additionally, this knowledge aids in developing scalable and reproducible manufacturing processes, ensuring consis-

tency and quality in production. Such advancements also contribute to diagnostics, cosmetics, and food science, where liposomes are increasingly utilized.

This chapter provides the necessary background information on the factors governing liposome formation. The thermodynamic and kinetic aspects of the liposome formation process are discussed in detail. The self-assembly of lipids into well-organized structures like planar bilayers or spherical vesicles is primarily governed by three factors: (1) the interaction free energy between lipid molecules, (2) their molecular geometry, and (3) the elastic characteristics of the resulting lipid bilayers. While aspects such as interaction energy and molecular shape have been explored in Chapter **6**, there remains limited understanding about the precise conditions that cause bilayers to bend and close into vesicular forms. To understand these conditions, the elastic properties of lipid bilayers are discussed in the present chapter, followed by an examination of the conditions necessary for bilayer bending and closure into vesicles.

10.2. STATISTICAL THERMODYNAMICS OF LIPID BILAYERS

Among the various models developed to describe cell membrane deformation, the fluid mosaic model proposed by Singer et al. [1] is widely accepted. This model portrays the cell membrane as a 2D, fluid-like lipid bilayer in which lipid molecules can move freely. The behavior of synthetic lipid bilayers shares significant similarities with that of natural biological membranes [2]. Helfrich proposed that the elasticity of lipid bilayers can be interpreted as a special case of the broader theory of thin elastic shells developed by Naghdi [3]. In Helfrich's model, the bilayer is treated as a 2D surface, where the deformation energy is solely a function of surface curvature. Building on this, Safran demonstrated that such a 2D framework can effectively represent a 3D bilayer, as long as the radius of curvature is considerably larger than the bilayer's thickness [4]. A bilayer membrane, such as the lipid bilayer in cell membranes, can deform in two main ways due to its elastic properties:

1. Stretching/compression and
2. Bending

Note that "shearing effect" is not considered in lipid membranes because, like water, these membranes lack a shear modulus. This means that static shear does not cause energy-costing deformation in a fluid membrane; such deformation cannot even be defined. The fluid nature of the lipid bilayer in the membrane allows lipid molecules to move freely, making the concept of static shear irrelevant for these membranes.

10.2.1. Bilayer Stretching/Compression

Lipid bilayers resist area deformation due to lipid molecules' tight lateral packing. However, when sufficient force is applied, the membrane's area can still be deformed by stretching or compressing. Membrane stretching and compression were initially explored using lipid monolayers at the lipid-water interface and later extended to studies on GUVs. In both cases, the membrane, whether composed of a single monolayer or a bilayer, exhibits a high surface area-to-volume ratio, since monolayers are only one molecule thick and bilayers consist of just two molecular layers. As previously described, these membranes can be approximated as 2D films with negligible thickness. For such 2D systems, the concept of pressure is replaced by surface pressure, which represents the tangential or lateral stress acting within the membrane plane. Unlike conventional 3D pressure, which acts over an area, this 2D surface pressure operates along a line, and is thus expressed in force/length or equivalently in energy/area. This surface pressure or tangential stress is also known as membrane surface tension. The deformation generated in the membrane due to membrane tension is called "area strain" α (similar to bulk or volumetric strain). For a monolayer, it can be expressed as:

$$\alpha = \frac{\Delta A}{A_0}, \tag{10.1}$$

where $\Delta A = A - A_0$ is the area change with A and A_0 being the surface area of a monolayer membrane under stressed and rest states (Fig. **10.1 a, b**). Rest state means no external force is applied to the material, so it is the case of the unstressed or original state of the material.

Evans [5] showed that membrane tension increases linearly with how much a monolayer is deformed, even until it breaks. This indicates that a normally deformed elastic

CHAPTER 11

Liposomes Characterization Methods

Abstract: This chapter discusses the key factors influencing liposome formation, including size, stability, lamellarity, and encapsulation efficiency, and highlights their importance across diverse applications. It investigates cutting-edge technologies, such as TEM, SEM, and AFM, for assessing the size, shape, and structure of liposomes. The chapter also discusses spectroscopic methods such as NMR and UV/Vis for analyzing liposomes' composition and behaviour. Furthermore, DLS is described as a quick approach for determining size and stability, with a focus on optimizing its application in liposome characterization. These technologies give critical insights for optimizing liposomal formulations in a wide range of medicinal applications.

Keywords: Liposome formation, encapsulation efficiency, microscopy techniques, spectroscopy techniques, dynamic light scattering.

11.1. INTRODUCTION

The physicochemical properties of liposomes determine their *in vivo* and *in vitro* behavior [1]. For the assessment of the quality of liposomal products and to achieve quantitative measures that allow comparison between different batches of liposomes, several parameters should be monitored, such as mean diameter, polydispersity index, encapsulation efficiency, lamellarity, and the encapsulating agent/lipid ratio [2]. Other common parameters like surface properties, phase transition, physical and chemical stability, and quantification of the residual solvents also require monitoring [2, 3].

Size and size distribution are critical factors for *in vivo* applications, particularly size because liposome size influences drug loading, biodistribution, drug clearance rate from the body, targeting efficacy to specific organs, and therapeutic efficiency. Stable vesicle formulations preserve liposome size distribution, whereas physical instability may lead to aggregation or fusion of liposomes, forming larger particles. Several methods for assessing these factors are available, including microscopic techniques, size-exclusion chromatography, field flow fractionation, and dynamic light scattering.

Lamellarity assessment is essential as it affects EE and drug release kinetics. Vesicle lamellarity can be evaluated using electron microscopy or spectroscopy methods. EE can be estimated in several ways, such as using dialysis membranes, ultracentrifugation, filtration, chromatography, and field flow fractionation. The stability of liposomes can be measured by zeta potential using dynamic light scattering. Alternatively, isothermal calorimetry can also be employed to study vesicle stability.

11.2. MICROSCOPY TECHNIQUES

Various microscopy techniques are available to visualize liposomes, broadly categorized into light/optical, electron, or atomic force microscopy. Each technique has its own pros and cons, which should be considered when studying liposomes. Conventional light or optical microscopy commonly uses visible light and a system of lenses to magnify the field of view. Although such microscopes cannot provide detailed information about the bilayer, they can be used for quick imaging of liposomes using essential lab equipment. In general, optical microscopy helps gather significant information on GUVs, which range in size from single to hundreds of micrometers (Fig. **11.1 a**). Introduction of polarization, fluorescent, and confocal microscopy techniques can provide more detail on the 3D structure and lamellarity of liposomes [4].

Polarization microscopy provides an alternative way to observe vesicles, mainly to confirm the formation of liposomes. However, it does not give any conclusive visualizations related to vesicle lamellarity and is mainly helpful for micrometer-range vesicles with optimal clarity. Fluorescent (or epifluorescent) microscopy is widely used in biophysics to investigate essential details about the structure and dynamics of membrane components [5]. This technique allows for evaluating more than one parameter, as fluorescence probes can be placed in the inner aqueous core and the bilayer. GUVs and sufficiently large MLVs can be employed with this technique. Despite similar limitations related to magnification as conventional light microscopes, fluorescent microscopy enables the identification of regions of interest within the liposome constructs through fluorescent labelling.

Confocal laser scanning microscopy (CLSM), an advancement in fluorescence microscopy, can observe liposomes using 3D projections. For GUVs, this technique

can visualize the internal structure of lipid systems (Fig. **11.1 b**), which is not possible with other microscopy techniques [6]. CLSM can also be used to study various mechanical properties of bilayers. For instance, elastic moduli of bilayers can be determined by combining confocal microscopy with micropipette aspiration techniques [7]. However, both epifluorescent and confocal microscopy experiments involve dye photobleaching due to prolonged exposure, and the fluorescent probe can influence membrane properties such as bending elasticity and phase rate. Therefore, these effects should be considered when performing fluorescence microscopy experiments.

For greater magnification (up to 2,000,000x), electron microscopy (EM) can be used to investigate the accurate size of submicrometer liposomes, lamellarity, and homogeneity (Fig. **11.1 c**). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are the two important electron microscopes used for

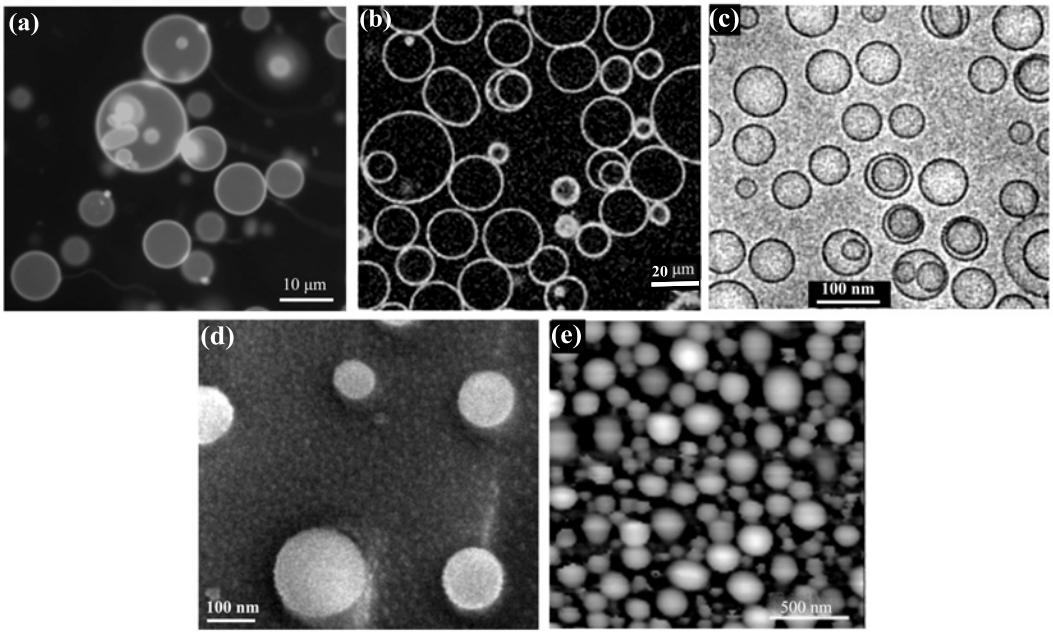

Figure 11.1: Liposomes morphology imaged by various microscopes. (a) GUVs obtained by optical microscopy (IIT Palakkad, India). (b) Confocal fluorescence micrograph of GUVs Reprinted with permission from ref. [8]. Copyright 2021 Elsevier B.V. (c) Cryo-TEM image of SUVs/LUVs. Reprinted with permission from ref. [9]. Copyright 2005 Wiley-Liss, Inc. Published by Elsevier Inc. (d) Cryo-SEM image of SUVs/LUVs (IIT Bombay, India). (e) AFM image of SUVs/LUVs. Reprinted from ref. [10], under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

CHAPTER 12

Medical and Nonmedical Applications of Liposomes

Abstract: This chapter investigates the several uses of liposomes, from medicine to nonmedical fields. Liposomes are important drug delivery methods in medicine because they improve therapeutic efficacy, reduce adverse effects, and allow for tailored drug release. Their uses in cancer therapy, vaccine administration, and antimicrobial therapies are particularly emphasized. The chapter discusses the mechanics of liposome drug loading, including active and passive loading strategies and parameters such as liposome size, surface charge, and lipid composition that impact their effectiveness. It also dives into ways to measure drug encapsulation efficiency and loading capacity, emphasizing sophisticated techniques like ion gradient methods and transition metal complexation. Beyond medicine, liposomes are utilised in cosmetics, food, textiles, and agriculture to regulate the release of active chemicals, improve product efficacy, and increase sustainability. In research, liposomes are used as model membranes to examine biological processes. The chapter also discusses the importance of liposomes in scientific developments such as biosensors, diagnostic imaging, and nutraceuticals, demonstrating their wide range of uses.

Keywords: Liposomes, drug delivery, cancer therapy, vaccine delivery, antimicrobial treatments, drug encapsulation, biosensors, controlled release, encapsulation efficiency, nonmedical applications.

12.1. INTRODUCTION

Liposomes have a broad range of applications in both medical and nonmedical fields. In medical applications, liposomes are primarily used as drug delivery systems, offering targeted delivery of therapeutic agents to specific tissues or cells, which enhances the efficacy and reduces the toxicity of treatments, especially in cancer therapy. They are also employed in vaccine delivery, where they encapsulate antigens to enhance immune responses, and in treating bacterial and fungal infections by delivering antimicrobial agents directly to the infection site, increasing effectiveness while reducing side effects.

Liposomes also serve as biosensors in diagnostics, where they can be designed to detect specific biological markers, aiding in the early detection and monitoring of diseases.

In nonmedical applications, liposomes are utilized in the cosmetic industry to deliver active ingredients such as vitamins and moisturizers deeper into the skin, improving product efficacy. In the food industry, they encapsulate flavors, nutrients, and preservatives, enhancing stability and controlled release. In the textile industry, liposomes encapsulate and release fragrances or antimicrobial agents in fabrics, providing long-lasting effects and improving textile functionality.

As model membranes, liposomes are invaluable in scientific research for studying the properties of cell membranes, investigating membrane-bound proteins, and understanding mechanisms of membrane fusion and permeability, offering insights into fundamental biological processes. This chapter extensively discusses the applications of liposomes in medical and nonmedical fields. The use of liposomes as model membranes will be addressed in the next chapter.

12.2. DRUG DELIVERY SYSTEMS

Drug delivery systems (DDSs) are methods to administer pharmaceutical drugs to achieve therapeutic effects in humans or animals. DDSs can be categorized into conventional DDSs and novel DDSs. Conventional DDSs involve formulating drugs into tablets, capsules, and syrups for oral administration or liquids for intravenous administration. These forms often result in higher required dosages, frequent dosing, lower efficacy, and adverse side effects due to the immediate release of the entire drug dose into biological fluids, with little control over the release rate (e.g., hypotension induced by oral administration of nifedipine tablets [1]). Many drugs have a narrow therapeutic index, meaning the therapeutic drug concentration is close to the toxic concentration, making conventional DDSs less suitable.

To address the aforementioned issues, novel DDSs based on carriers have been developed. These carriers, also known as vehicles, can deliver drugs in submicron sizes, forming colloidal or particulate drug delivery systems. Unlike conventional DDSs, novel DDSs release the drug dose slowly and steadily, maintaining consistent drug levels in circulation, enhancing drug efficacy at the intended sites, reducing

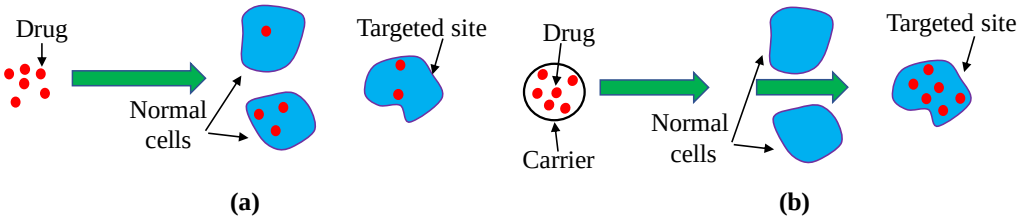

Figure 12.1: Drug delivery systems (DDSs). (a) Conventional DDSs showing only a few drug molecules reach the targeted area, while some can adversely affect healthy cells. (b) Novel DDSs (nanoparticle carriers loaded with drugs) showing all drug molecules reach the targeted area, thereby avoiding interaction with healthy cells.

dosing frequency and total drug usage, and minimizing side effects and toxicity (Fig. **12.1**). However, the higher cost of unit doses in controlled therapeutic systems is a disadvantage. Despite the cost, these carriers can be used not only for novel drugs but also for existing drugs that are efficacious but difficult to use due to high toxicity. Therefore, novel DDSs offer significant advantages over conventional systems.

DDSs must navigate a series of anatomical, cellular, and subcellular barriers to reach the target site of the parent drug. The active drug must be released selectively at the target site with a desired, predetermined rate and duration. Therefore, the primary goals of DDSs are to enhance specificity toward target cells or tissues, improve drug bioavailability at the target site by increasing membrane diffusion, and protect the drug against enzyme inactivation. Typical carriers include lipid-based nanoparticles, such as micelles, mixed micelles, (micro)emulsions, and liposomes; lipid nanoparticles with a solid matrix, including solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs); and polymeric nanoparticles, such as nanospheres and nanocapsules. Other notable carriers include erythrocytes, lipoproteins, and iscoms. Among these, liposomes are particularly popular and successful due to their ability to deliver the encapsulated agents to target sites with controlled release rates. It is important to note that a suitable DDS can alter the temporal and spatial delivery of the drug, affecting its pharmacokinetics (PK) and biodistribution. As already discussed in the last chapter, PK investigates drug absorption, distribution, metabolism, and excretion, whereas biodistribution monitors drug localization in tissues, which is critical for determining therapeutic and diagnostic efficacy.

CHAPTER 13

Applications of Liposomes as Membrane Models

Abstract: This chapter explores the versatile application of liposomes as model membranes for studying the fundamental properties of biological cell membranes. By closely mimicking the structural and functional aspects of natural membranes, liposomes provide an invaluable platform for investigating membrane mechanics, including elasticity, tension, and transport properties such as permeability. A key focus is their role in understanding membrane curvature sensing and generation by proteins/peptides, which is essential for biological processes like endocytosis and vesicular trafficking. Additionally, liposomes serve as effective models for studying membrane pore formation by peptides, offering critical insights into the development of antibacterial and anticancer therapies. As model membranes, they also play a critical role in evaluating drug-membrane interactions, helping to optimize stability, permeability, and controlled release in drug delivery systems. Beyond these applications, liposomes are widely employed in investigating biomolecular interactions and membrane-associated processes, including cell adhesion, membrane-polyphenol interactions, peptide-membrane translocation, lipid peroxidation, and membrane rafts. Their ability to provide controlled experimental conditions makes them indispensable tools for elucidating lipid organization, membrane dynamics, and signaling pathways.

Keywords: Liposomes formation, membrane mechanics, curvature sensing, curvature generation, pore formation, drug delivery.

13.1. INTRODUCTION

Liposomes are extensively used as model membranes to study the fundamental properties and behaviours of biological cell membranes. The architecture of biological cells, which undergo significant changes involving cell membranes, is essential for processes such as cell division, neuron growth, endocytosis, and the morphological functions of organelles like the endoplasmic reticulum and Golgi apparatus. Understanding cell membranes' deformability, elasticity, and permeability is essential for these processes. Researchers use model membrane vesicles like liposomes to simplify the complexity of biological membranes. These synthetic membranes, which

mimic the lipid bilayer structure of cell membranes, allow for controlled studies of membrane properties such as elasticity, tension, permeability, and pore formation.

Liposomes can be finely tuned by varying their composition, making them versatile tools for characterizing the physical properties of cell membranes. Their ability to self-assemble into bilayers and form vesicular structures provides insights into membrane dynamics, mechanics, and drug interactions. Liposomes are also instrumental in studying membrane pore formation by peptides, which is important for developing antibacterial and anticancer therapies. Additionally, the depth of membrane insertion by various molecules and the interactions between drugs and the lipid bilayer can be precisely analyzed using liposomes. Various biophysical techniques, such as assessing drug hydrophobicity, membrane binding, drug location within the bilayer, and the effects of drugs on membrane properties, are employed to explore these interactions, contributing to a better understanding of cellular processes and the development of effective drug delivery systems.

Moreover, liposomes play a critical role in studying membrane curvature sensing and generation, which is essential for various cellular functions. Membrane curvature sensing refers to the preferential binding of proteins to membranes with specific curvatures, aiding processes like cell signalling and vesicular trafficking. Curvature generation involves the induction of membrane curvature by proteins or lipid compositions, facilitating the formation of vesicular structures necessary for intracellular transport and endocytosis. By providing a simplified and controllable environment, liposomes allow researchers to investigate these complex interactions and mechanisms, offering valuable insights into cellular membrane dynamics and their implications for health and disease.

In this chapter, I discuss the application of liposomes as model membranes to assess important membrane mechanical properties, such as stretching/compressibility, bending moduli, membrane tension, and transport characteristics like permeability and membrane insertion depth by peptides. For instance, in designing effective vesicular drug delivery systems, optimizing attributes such as high bending modulus and low permeability leads to more excellent vesicle stability and reduced leakage of encapsulated materials [1]. Additionally, the stiffness of vesicle membranes plays a significant role in cellular uptake through endocytosis, with softer vesicles

potentially enhancing processes like skin delivery [2]. These studies are essential for understanding the biophysical properties of membranes and their roles in various cellular functions. The chapter also provides an overview of membrane curvature sensing and generation, which are critical for cellular processes such as endocytosis, vesicular trafficking, and cell signalling [3], along with an examination of the role of membrane pore formation by peptides. Finally, various biophysical techniques used to study membrane-drug interactions are highlighted, emphasizing their importance in understanding drug behaviour within biological membranes.

13.2. LIPOSOMES IN MEMBRANE MECHANICS AND TRANSPORT

Liposomes play a central role in studying the mechanical and transport properties of membrane-bound objects, including cells and natural vesicles. Various experimental and computational methods have been developed for this purpose, such as vesicle fluctuation analysis (VFA), atomic force microscopy (AFM), micropipette aspiration (MPA), tether-pulling assay (TPA), electrodeformation (ED), optical stretcher (OS), and molecular dynamics (MD) simulations. GUVs, in particular, are favoured due to their fully hydrated state and lack of constraints from neighbouring membranes or surfaces, prepared using methods like gentle hydration, electroformation, microfluidics, or other suitable techniques, as discussed in Chapters **8** and **9**. This section extensively explores the mechanical and transport properties of membranes, highlighting techniques applied to GUVs and examining factors like membrane composition (e.g., cholesterol, charged lipids, polymers) and solution conditions (e.g., salts, pH, buffers).

13.2.1. Vesicle Fluctuations Analysis

Measuring the bending rigidity $\kappa_c^{(b)}$ of membrane bilayers through the observation of thermally-induced shape fluctuations (or undulations) of vesicles is a well-established experimental technique. This method was pioneered almost 50 years ago by Brochard and Lennon [4] on RBCs and by Servuss et al. [5] on long cylindrical vesicles. It was later applied to GUVs [6], with theoretical descriptions of membrane fluctuations provided by Helfrich, and Milner et al. [7, 8], and others [9–11]. This technique is

POSTFACE

Liposome technology has advanced dramatically over the last few decades, transitioning from a conceptual framework to a revolutionary instrument in a variety of scientific and industrial fields. Liposomes have proved their versatility and influence in a variety of applications, including nanomedicine, cosmetics, agriculture, and environmental science. Their contributions to targeted drug delivery, vaccine development, and model membrane studies continue to influence present research, integrating fundamental lipid science with cutting-edge technology breakthroughs.

This book provides a thorough overview for researchers, students, and professionals by examining the fundamental ideas as well as the most recent advancements in liposome technology. The book attempts to offer a structured yet approachable resource by going into detail into lipid chemistry, self-assembly, and bilayer thermodynamics in the first section, and then moving on to contemporary fabrication methods and interdisciplinary applications in the second.

Looking ahead, artificial intelligence (AI) is set to revolutionize liposome research. AI-driven models can predict formulation parameters such as size, stability, and polydispersity, streamlining quality control and large-scale production. Integrated with microfluidics, AI enables rapid optimization of liposomal formulations, enhancing precision, efficiency, and cost-effectiveness in therapeutic applications.

Despite tremendous progress, obstacles persist in refining liposomal formulations, increasing scalability, and broadening their applicability across industries. Liposome-based treatments will continue to evolve as new lipid compositions and production techniques emerge, unlocking their promise in medicine and beyond. As scientific study expands the frontiers of lipid-based systems, fresh discoveries will determine the field's future.

To improve future editions, I kindly ask for helpful criticism and thank the readers for their participation. The development of liposome technology is still in its early stages, and I am excited to see how it develops further.

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APPENDIX A

Soft Matter Physics

A.1. SOFT MATTER CHARACTERISTICS

Matter exists in three primary states: solid, liquid, and gas. While gases consist of widely dispersed particles that move freely without a fixed shape or volume, solids and liquids are more condensed, with particles that interact significantly. Unlike gases, where entropy dominates, the stability of solids and liquids is governed by interparticle forces, including attraction and repulsion. These interactions are studied within condensed matter physics, a field that applies principles from quantum mechanics, electromagnetism, and statistical mechanics to understand and manipulate the physical properties of matter in its solid and liquid states.

A specialized sub-field, soft condensed matter or soft matter, focuses on complex fluids and materials that exhibit behaviors intermediate between atomic and macroscopic scales. Many soft matter systems exist as solutions, where solute interactions depend on factors such as temperature and composition. This field is essential for understanding fundamental physical principles and has broad applications across disciplines, including materials science and biophysics.

Soft matter encompasses polymers, colloids, liquid crystals, and biological substances such as proteins, lipids, and nucleic acids, all of which contribute to the dynamic properties of living systems. These materials share defining characteristics such as their macroscopic length scales, where interactions between supramolecular entities dictate behavior rather than atomic-scale forces. Their properties often exhibit universality, meaning behaviors like membrane elasticity or polymer conformation are determined by structural features rather than specific chemical compositions.

Thermal fluctuations play a significant role in soft matter dynamics, as their energy scales are comparable to thermal energy, driving continuous motion, Brownian dynamics, and structural reorganization. Soft matter also exhibits viscoelasticity, combining fluid-like and elastic behavior, leading to non-Newtonian flow properties, as seen in polymer melts. Polydispersity, where systems contain varying sizes or compositions, influences self-assembly and crystallization. Many properties follow scaling laws, where system size dictates behavior rather than exact values. Notably, soft matter tends to self-assemble, with solvent-mediated interactions organizing structures like lipid bilayers, micelles, and polymeric nanostructures, making them essential in biological and synthetic applications.

A.2. INTERPARTICLE FORCES IN SOFT MATTER

The existence of condensed phases (liquids and solids) indicates the presence of attractive forces between the building blocks of matter. For instance, a liquid film minimizes its surface area at a constant volume to reduce the number of molecules exposed at the surface, which are less strongly bound than those in the bulk. Condensed matter also resists compression due to repulsive intermolecular forces at short distances. These repulsive forces arise from electron-electron interactions and the Pauli exclusion principle, preventing atoms and molecules from penetrating each other. A common approximation for short-range repulsion is the hard-core potential. Several types of long-range interactions exist in soft matter[1]:

- **Van der Waals forces:** These forces arise from interactions between instantaneous dipoles in neutral atoms. An instantaneous dipole in one atom induces a dipole in another, resulting in an attractive force. The strength of these forces decreases with the sixth power of the distance between particles and has magnitudes around 1×10^{-20} J, comparable to thermal energy (k_BT).
- **Electrostatic force:** Electrostatic interactions in soft matter often occur in solutions with dissolved ions. In these environments, the basic Coulombic interaction is more complex due to the presence of counterions. These counterions screen the interactions, causing them to decay exponentially with distance. The strength of electrostatic interactions in a crystal can be much higher than in a solution due to this screening effect.
- **Covalent bonds:** Covalent bonds are strong bonds that hold atoms together in molecules, with energies around 50–500 k_BT, much higher than thermal energy (k_BT). These bonds are not significantly affected by thermal motion and include both covalent and metallic bonds.
- **Hydrogen bonds:** Hydrogen bonds occur when a partially positive hydrogen atom is attracted to an electronegative atom in a neighboring molecule. A common example is the bond between hydrogen and oxygen in water. The energy of hydrogen bonds can be up to 5–10 k_BT.
- **Hydrophobic interaction:** Hydrophobic interactions occur when nonpolar molecules or parts of molecules come together in water, driven by the exclusion of nonpolar substances by water molecules. The interaction energy is typically around 1×10^{-20} J. This interaction energy plays a crucial role in driving self-assembly processes in both biological and non-biological systems.

[1] Various interparticle forces are extensively discussed in Chapter 2 of *Soft Condensed Matter* by Richard Jones (Oxford University Press, 2002).

A.3. VISCOELASTIC BEHAVIOR IN SOFT MATTER

A hallmark feature of soft matter is the ambiguous distinction between solids and liquids. In simple solids and liquids, the difference is clear-cut. Solids, such as crystals, follow Hooke's law where an applied shear stress leads to a proportional shear strain, inversely related to the material's Young modulus. Liquids, like water, adhere to Newtonian fluid dynamics where shear stress induces flow, proportional to the stress and inversely related to the viscosity.

However, many soft matter materials, such as polymers and biological substances like biomembranes, exhibit a viscoelastic response, which is a more complex behavior that encompasses both elastic (solid-like) and viscous (liquid-like) properties. The behavior of viscoelastic materials depends on the time scale or frequency of the applied shear stress. At short times or high frequencies, these materials behave more like solids, resisting deformation. Conversely, at long times or low frequencies, they flow like liquids.

A.4. VESICLES AND CELL MEMBRANES AS SOFT MATTER

Vesicles and cell membranes are classified as soft matter due to their elastic nature, responsiveness to external stimuli, and complex non-equilibrium behaviors. Liposomes, which are artificially prepared vesicles composed of lipid bilayers, serve as model membranes for studying biophysical properties and drug delivery applications. Both natural vesicles and liposomes can undergo shape transformations in response to environmental conditions or external forces, mimicking the adaptability of biological membranes.

Cell membranes, as flexible barriers, experience continuous deformation to facilitate cellular processes such as endocytosis, exocytosis, and signaling. Their structural integrity and function are governed by dynamic interactions between lipids and proteins, leading to processes such as membrane fusion, fission, and remodeling. These interactions allow membranes to respond to changes in temperature, pH, ionic strength, and the presence of bioactive molecules, altering their physical and functional properties.

Vesicles, liposomes, and cell membranes occupy an intermediate length scale, between microscopic and macroscopic systems, situating them in the domain of soft condensed matter physics. This scale enables the emergence of collective phenomena, such as phase transitions, membrane undulations, and lipid domain formation, which influence their overall properties and dynamics. As essential components of soft matter systems, vesicles, liposomes, and cell membranes provide fundamental insights into the behavior of deformable, responsive materials with complex biophysical characteristics.

APPENDIX B

Regulations for Liposomal Drugs

B.1. GLOBAL REGULATORY LANDSCAPE

The liposomal drug delivery market was projected to surpass $4.7 billion by 2022 and is expected to grow at a compound annual rate of 8.5% from 2023 to 2031. Liposomal drug formulations are frequently utilized clinically in cancer treatment, fungal infections, vaccinations, ophthalmic therapies, and anesthesia [1, 2]. Generic versions of liposomal drugs have been slower to appear than anticipated because of regulatory and technological complications after the first approval of the drug (Doxil^{TM}) in 1995 [3] and the patent expiration in 2009. Regulatory policies significantly influence the development, approval, and market trends of liposomal drug formulations, particularly in antifungal and anticancer therapies. Agencies like the US FDA and Health Canada promote liposomal formulations, while Indian government focuses on price reductions for essential drugs to encourage investment.

Given the complexity of liposomes, regulatory agencies continuously refine guidelines to ensure therapeutic equivalence and safety. The FDA classifies liposomes as complex generic drugs, posing challenges in asserting bioequivalence. To address this, the FDA has introduced product-specific guidances (PSGs) [4], harmonizing regulatory processes for complex drug products. Similar initiatives exist globally, including efforts by the European Commission's Joint Research Centre (JRC) and the International Pharmaceutical Regulators Programme (IPRP) [5, 6].

Liposomes fall under non-biological complex drugs (NBCDs), where manufacturing variability can impact drug performance [7]. The 2018 FDA draft guidance refined previous liposome regulations, covering chemistry, manufacturing, and controls but not clinical efficacy or toxicology [8]. Regulatory frameworks for generic doxorubicin liposomes, such as Lipodox (US), demonstrate differing global approaches. While the FDA approved Lipodox in United States, the EMA rejected it due to bioequivalence concerns with the European Caelyx [9].

In the EU, EMA lacks specific liposome guidelines, relying on centralized procedures and a reflection paper for follow-on liposomes [5]. Regulatory comparisons between the US and EU focus primarily on chemistry, manufacturing, and controls requirements, with both agencies emphasizing that even minor process changes post-approval require thorough re-evaluation. For manufacturers, continuous communication with regulatory agencies is essential for navigating approval pathways, ensuring compliance, and mitigating risks associated with formulation changes.

B.2. MANUFACTURING AND ANALYTICAL REQUIREMENTS

Careful formulation design, adherence to regulations, and reliable analytical techniques are necessary for optimizing the development of liposomal drugs. The integrity of liposomes is maintained via sterile filtration and process validation, while the use of excipients on the FDA list guarantees a more seamless approval process. Comprehensive characterization, including *in vitro* drug release tests, zeta potential, particle size, and encapsulation efficiency, is required by regulatory bodies.

The focus of drug product specifications is on *in vitro-in vivo* correlations, coating stability, and ligand orientation. To guarantee consistency, process development requires stringent control measures, sterilizing procedures, and scale-up validation. With in-use stability testing conducted in clinical settings, stability studies concentrate on coating integrity and lipid degradation. The principles of Quality by Design (QbD) direct the process of identifying critical quality attributes (CQAs), design space, and process parameters in order to guarantee consistent product quality. Preclinical research necessitates accurate measurement of both free and encapsulated drug levels while taking *in vivo* release processes into account. To guarantee safety, effectiveness, and regulatory approval, the FDA and EMA both place a strong emphasis on comprehensive validation, lifecycle monitoring, and ongoing optimization.

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APPENDIX C

Units and Constants

Table C.1: International system (SI) basic units.

Quantity	Unit	Symbol	Dimension
Length	Meter	m	L
Mass	Kilogram	kg	M
Time	Second	s	T
Electric current	Ampere	A	I
Temperature	Kelvin	K	θ
Amount of substance	Mole	mol	N
Luminous intensity	Candela	cd	J

Table C.2: Units derived from SI base units.

Quantity	Name basis SI unit	Symbol	Other SI units	SI base units
Angle	Radian	rad	-	-
Frequency	Hertz	Hz	-	s^{-1}
Force	Newton	N	-	$kg \cdot m/s^2$
Pressure, stress	Pascal	Pa	N/m^2	$kg/m \cdot s^2$
Energy, work, heat	Joule	J	$N \cdot m$	$kg \cdot m^2/s^2$
Power	Watt	W	J/s	$kg \cdot m^2/s^3$
Electric charge	Coulomb	C	-	$A \cdot s$
Electrical potential	Volt	V	W/A	$kg \cdot m^2/s^3 \cdot A$
Electric capacitance	Farad	F	C/V	$kg^{-1} \cdot m^{-2} \cdot s^4 \cdot A^2$
Electrical conductance	Siemens	S	A/V	$kg^{-1} \cdot m^{-2} \cdot s^3 \cdot A^2$
Magnetic flux	Weber	Wb	$V \cdot s$	$kg \cdot m^2/s^2 \cdot A$
Magnetic field	Tesla	T	Wb/A	$kg \cdot m^2/s^2 \cdot A^2$

Table C.3: Mathematical constants.

$\pi = 3.14159\ldots$
$e = 2.1728\ldots$
$\log_e 10 = 2.30259\ldots$
$\log_{10} 10 = 1$

Table C.4: Physical constants.

Constant	Value	Unit
Acceleration of gravity (g)	9.80665	$\mathrm{m/s^2}$
Avogadro's number ($\mathrm{N_A}$)	6.02214×10^{23}	molecules/gmol
Atomic mass unit (amu)	1.66×10^{-27}	kg
Boltzmann constant (k_B)	1.38066×10^{-23}	J/K
Electronic charge (e)	1.6022×10^{-19}	Coulomb (C)
Electron mass (m_e)	9.1096×10^{-31}	kg
Electron-volt (eV)	1.6022×10^{-19}	J
Faraday constant (F)	96485.3	C/mol
Gas constant (R)	8.31451	$\mathrm{J/mol \cdot K}$
Gravitation constant (G)	6.67×10^{-11}	$\mathrm{Nm^2/kg}$
Molar volume (v)	22.4138	$\mathrm{m^3}$/kmol [0 °C, 1 atm]
Planck constant (h)	6.62608×10^{-34}	J/s
Proton mass (m_p)	1.6726×10^{-27}	kg
Stefan constant (σ)	5.67051×10^{-8}	$\mathrm{W/m^2 \cdot K^4}$
Speed of light in vacuum (c)	2.9972×10^{8}	m/s

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The advent of liposomes in nanomedicine innovation has revolutionized pharmaceuticals, transforming delivery of chemical & biological therapeutic agents with precision to organs, tissues, membranes or cells, rendering untargeted medicines obsolete. The complexity of liposomes needs comprehension to exploit potential applications. This book fulfils a critical need for a reference that bridges theory, preparation, and clinicals of lipidic pharmaceutical nanotechnology-based nano-drug delivery systems (NDDS).

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