School of Pharmacy

Are Elastic Liposomes Better for Skin Delivery of Therapeutic Molecules?

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This thesis is presented for the Degree of
Master of Pharmacy
of
Curtin University

January 2011

ACKNOWLEDGEMENTS

I express my wholehearted appreciation to my supervisor Dr. Yan Chen for her excellent guidance and continual support and encouragement throughout my study time.

I want to thank my co-supervisor Assoc. Prof. Heather Benson for providing me valuable guidance throughout the course of my study.

I would like to appreciate the support I received as an international postgraduate student from Prof. Jeff Hughes, the Head of the School.

My sincere gratitude goes to Mr. Mike Boddy for technical support and great help. I am grateful to Mr. Michael Stack for material and equipment supplies. I am thankful to Dr. Rob Hart, Senior Research Fellow, Centre for Materials Research, School of Science for his great help in microscopy. I also appreciate the help I received from Dr. Richard Parsons for statistical analysis.

I wish to thank Basavaraj, and Dr. Sarika Namjoshi for their great help in my research. I wish to acknowledge my co-workers for their help, support and cooperation at various times throughout my study. I am also thankful to the entire staff of School of Pharmacy for their friendly attitude and cooperation.

To whom words cannot express my thankfulness, the Almighty and my parents. Finally I wish to express my thankfulness to my husband for having patience and great cooperation during the study period.

ABSTRACT

The objectives of the present study were to design and evaluate elastic liposomes in comparison to simple drug solution and conventional liposomes, and in delivering a model NSAID, Ibuprofen (IBU) across human epidermis after topical application. The scope of the project included formulation development, characterization, and optimization based on their physical characteristics and determination of IBU penetration when incorporated in conventional and elastic liposomes through human epidermis after topical application, and finally to establish the relationship between the formulation variables and skin penetration of IBU loaded liposomes.

A thin film hydration method was chosen to prepare liposomes where the hydrophilic molecule sodium fluorescein (NaFl) and poorly water soluble drug IBU were selected as molecules of interest for liposome development and evaluation. Conventional liposomes were prepared from phosphatidylcholine (PC) and cholesterol, and elastic liposomes were prepared from PC and surfactant (Tween 80 and sodium cholate) with 7% ethanol in the system. Process variables such as the solvent system, hydration medium, and method of size reduction were carefully selected for initial formulation development. Vesicles were optimized based on their physical characteristics (i.e. particle size and size distribution, zeta potential, entrapment efficiency (EE)) and bilayer elasticity. Morphology of different liposomes was examined by using a Transmission Electron Microscope (TEM). The *in vitro* release of both model drugs from various optimized formulations was investigated by dialysis method. The effects of formulation variables such as, type and concentration of surfactant, and ethanol on transdermal penetration of IBU loaded liposomes were investigated using Franz type diffusion cells under non-occluded condition.

Findings of the current study showed that the formulation variables and type of compound to be entrapped into liposomes have significant effect on the physical characteristics of both conventional and elastic liposomes. For example, inclusion of both cholesterol and surfactant showed a reduction in particle size of liposomes, irrespective of the type of molecule entrapped. However the impact of cholesterol on

drug loading is complex. Cholesterol increased the EE of NaFl loaded conventional liposomes but reduced EE in IBU loaded conventional liposomes. However, the inclusion of surfactants reduced the EE in both NaFl and IBU loaded liposomes. The zeta potential values of different liposomes were largely dependent on the composition of formulation and the type of molecule entrapped. Under TEM, all vesicles were found to be regular and spherical with the exception of those with a higher content of surfactant where vesicular structures were absent. The storage stability of NaFl loaded conventional liposomes was dependent on cholesterol content. Liposomes containing 30% w/w cholesterol, showed maximum stability against particle fusion and aggregation. However all elastic liposomes showed maximum deformability at 15% w/w surfactant. Further increase in the surfactant content reduced elasticity considerably due to the formation of micelles, which lack the propensity for curved structure.

Results of the *in vitro* release study of NaFl and IBU from liposomes showed that the release rate was biphasic in nature and was dependent on liposome composition. In the case of NaFl release, 30% w/w of cholesterol containing liposome had the slowest release rate compared to that of free drug and elastic liposomes. IBU release from 20% w/w cholesterol containing liposome was the slowest compared to all IBU loaded conventional and elastic liposomes.

The skin penetration study *in vitro* showed significantly improved permeation of IBU by elastic liposomes compared to that of the conventional liposome and the control (free drug solution) due to the self penetration enhancing ability of elastic vesicles provided by individual functional components. The improvement in transdermal flux, permeability coefficient, enhancement ratio, and drug deposition in the skin from elastic liposomes indicate that these vesicles are a better delivery system for topical delivery of ibuprofen and may be promising for the delivery of other molecules of interest.

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ABBREVIATION

CI	Confidence interval
EE	Entrapment efficiency
FESEM	Field emission scanning electron microscope
GI	Gastrointestinal
GLC	Gas liquid chromatography
HPLC	High performance liquid chromatography
IBU	Ibuprofen
LOD	Limit of detection
LOQ	Limit of quantification
LUVs	Large unilamellar vesicles
MLVs	Multilamellar vesicles
NaFl	Sodium fluorescein
NMR	Nuclear magnetic resonance
NSAID	Non steroidal anti-inflammatory drugs
P.I.	Polydispersity index
PBS	Phosphate buffer solution
PC	Phosphatidylcholine
REV	Reverse phase evaporation vesicle
RSD	Relative standard deviation
S. Chol	Sodium cholate
SD	Standard deviation
SEM	Standard error of mean
SUVs	Small unilamellar vesicles
T80	Tween 80

T_c	Lipid phase transition temperature
TEM	Transmission electron microscope
TLC	Thin layer chromatography
UV	Ultraviolet-visible

Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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INTRODUCTION

1.1. Skin drug delivery system

The ultimate objective of a successful drug delivery system is to deliver the drug effectively and specifically to the target site at a rate and concentration which will achieve greater efficacy and reduced toxicity than that of the conventional formulation of the same drug[1].

Among various routes of drug administration, drug delivery into and through the skin has established itself as a unique and useful method. Topical and transdermal drug delivery have played a vital role in the history of primitive remedies[2]. Transdermal drug delivery uses the skin as an alternative route for the delivery of systemically acting drugs whereas topical or dermal drug delivery is the topical application of drugs to the skin for treating local skin diseases. Drug delivery into (dermal) or via (transdermal) the skin offers many benefits over other routes of administration, which include providing continuous drug delivery[3] with avoidance of hepatic first pass effect[4], no gastrointestinal (GI) irritation as it bypasses the GI tract[4], and reduced fluctuations in plasma drug levels[2]. Moreover drugs with short biological half-lives can be utilized. Overall dermal drug delivery system targets the drug for a local effect[2], avoids variable absorption and metabolism associated with oral treatment, and enhances and maintains drug concentration in the skin. Furthermore it improves patient compliance[3].

1.1.1. Dermal drug delivery system

Dermal or topical drug delivery system can be defined as the application of a topical drug formulation to the skin to directly treat a dermatological condition (e.g. acne, skin cancer, eczema, and microbial infections) or the cutaneous manifestations of a general disease (e.g. psoriasis) with the intent of providing the pharmacological effect of the drug to the skin surface or within it[5]. This delivery system has received serious attention in the past years due to its influence on enhanced dermal penetration. Dermal drug delivery system contains not only active drug but also other formulation components such as penetration enhancers, stabilizers and

preservatives[6]. These formulation components all together are of utmost importance for successful dermal delivery. In dermal drug administration the primary step is the release and partitioning of the drug from the delivery system into the skin, which to certain extent, is governed by the drug partition coefficient. A vehicle utilized for such delivery usually has high active holding capacity. Penetration enhancers utilized in such vehicle influence drug release, drug diffusion and partition in the skin.

1.2. Skin physiology and barrier function

The skin is the largest organ of human body (up to 10% of body weight), which forms a barrier against the external environment, protecting the body from unwanted influences[2]. It is structured with three layers: epidermis (<100 μm), dermis (<4000 μm), and subcutaneous layer. The pH of the skin varies from 4.5 to 6.0[7]. It is suggested that the acidity of the skin helps in limiting or preventing the growth of pathogens and other organisms[8]. A typical diagrammatic cross-section of human skin is shown in Figure 1.1.

1.2.1. The epidermis

The epidermis, the outermost layer of the skin, is covered with an emulsified lipid film with an acidic pH and is referred to as the "acid mantle"[9]. This acidic pH can be attributed to having glucose as the energy provider for the lower part of epidermis and also lactic acid as the end product of metabolism. These contribute to the skin pH being less than 6 [5]. The epidermis is composed of four strata: the stratum germinativum (the basal layer), the stratum granulosum (the malpighian layer), the stratum lucidum (the granular layer) and the stratum corneum (the horny layer).

1.2.2. The dermis

The dermis lies between the epidermis and the subcutaneous fatty region. It is composed of a network of loose connective tissue principally of collagen fibrils (provide support) and elastic tissue (provides flexibility) embedded in mucopolysaccharides[10]. Numerous structures are embedded within it, for example, nerves, blood vessels, hair follicles, sebaceous and sweat glands. Mast cells,

macrophages, melanocytes, leukocytes, and endothelial cells are also found in this region. However sebum from sebaceous gland is composed of free fatty acids, waxes and triglycerides, which maintain the surface pH around 5. Also eccrine glands secrete sweat, a dilute salt solution which is of pH around 5 [2]. Therefore the overall pH of dermis is around 5. Depending on body area, the dermis can be 10-40 times thicker than the epidermis and is viewed as gelled water layer providing a minimal resistance to most polar drug delivery[2]. The function of the dermis is to nourish the epidermis and to anchor it to the subcutaneous tissue[11].

1.2.3. The subcutaneous tissue/hypodermis

This resides between the dermis and the underlying body constituents[2]. The deeper hair follicles and sweat glands originate in this layer and principal blood vessels and nerves are carried to the skin[12]. The subcutaneous tissue provides a receptacle for the generation and storage of fat which in turn acts as both heat regulator and shock absorber[13]. Dynamic lipid metabolism occurs here[9].

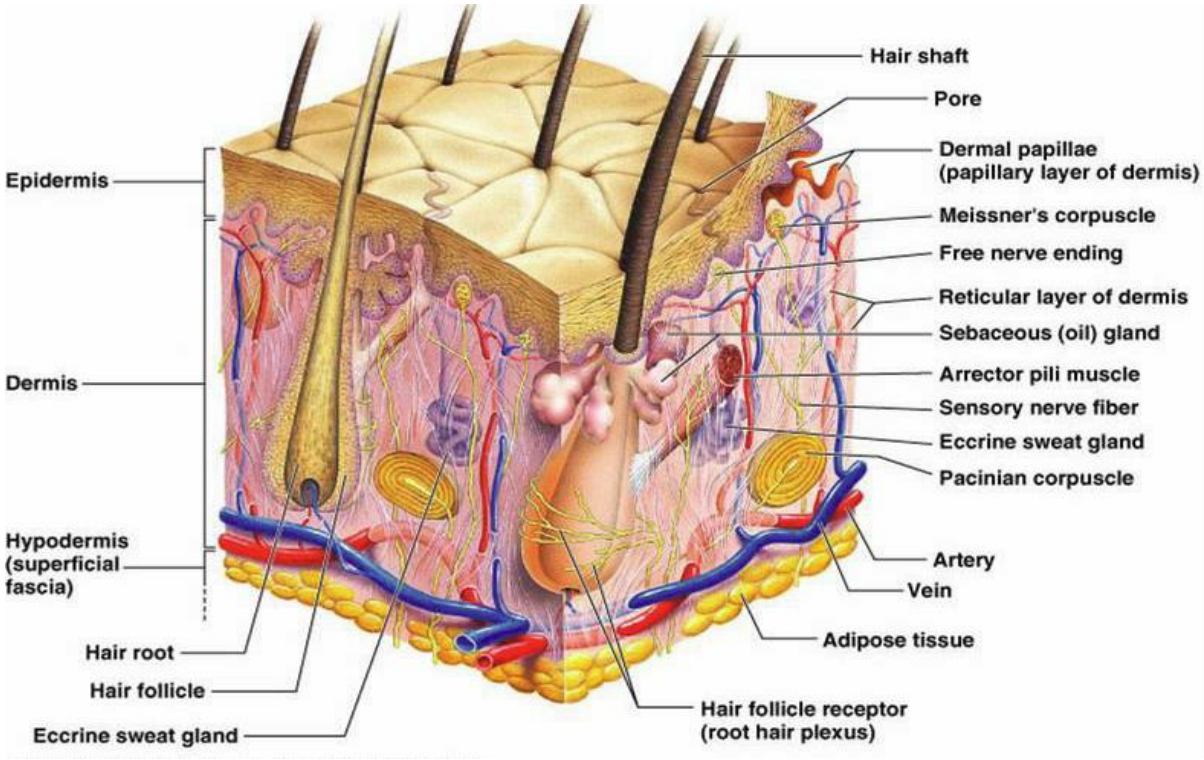


Figure 1.1: A diagrammatic cross-section through full thickness human skin
(adapted from Pearson Education, Inc.;
http://www.osovo.com/diagram/skindiagram.htm[14])

Despite many advantages in skin drug delivery, a limited number of drugs can be designed as topical, transdermal delivery products due to the barrier function of the stratum corneum, which limits the passage of nearly all but smallest lipophilic compounds[15]. Even water trespasses the skin at a rate of 0.4 mg cm^{-2} h^{-1} only[16]. Moreover, achieving a high and constant drug flux through the skin is a difficult task which has little possibility to be successful, unless one compromises the protective skin barrier function[17].

1.2.4. The stratum corneum as a permeability barrier

The stratum corneum, the outermost layer of the epidermis is a quasi-passive and very thin anatomical barrier with a thickness between 5 and 8 µm, exceptionally up to just above 20 µm[18] and when hydrated up to 40 µm[19]. It consists of 10-15 layers of keratin-filled dead flattened cells, the corneocytes[20]. The stratum corneum is a "brick and mortar"-like structure of corneocytes. "Brick" represents corneocytes and "mortar" is the intercellular matrix[21]; corneocytes are more than 90% of all cells in the stratum corneum [17].

Keratin is a fibrous protein, which is produced by the epidermis beneath. The cell boundary, the cornified envelope, runs perpendicular to the skin surface and is entirely surrounded by crystalline lipid lamellar regions[20] which is not only abundant but also highly organized and thus acts as an extra intercellular "glue" sealing the empty spaces between the cells[22]. Such architecture of the stratum corneum is extremely impermeable but to the smallest compounds.

The thickness of the average mammalian corneocyte is approximately 0.3µm[23, 24] but values up to 0.8 µm have also been reported[25]. The average number of corneocytes per square centimetre of stratum corneum is ~2×10^6 cm^{-2} [26]. Each flat superficial corneocyte covers 1100-1200 μm^2 of the skin, but deep in the dermis, the cells are typically thicker and smaller, covering ~700-750 μm^2 [26]. Groups of up to a dozen corneocytes columns in the stratum corneum form clusters which represent the basic skin permeation resistance unit[17].

The stratum corneum is neither continuous, nor homogenous. Some regions (especially the finger tips, bottom of the toes, the palmar and plantar surfaces) show extensive lines and ridges or dermatoglyphics, whereas the rest of the skin is comparatively smooth[20]. Again the resistance to molecular diffusion varies according to quality, crystallinity and total quantity (<15 wt %) of lipids in the stratum corneum, body sites, gender and species and is higher in human or pig than in rodent[17]. Although some researchers have suggested that sweat pores and hair ducts penetrating through the stratum corneum could be utilized to traverse the barrier, it seems that the cross sectional area of the pores is so small as to be negligible[20]. Moreover the outward movement of the sweat or sebum would tend to flush out everything which did penetrate[20].

The major lipid classes in the stratum corneum are long chain ceramides (40%), cholesterol (25%), free fatty acids (FFA; 25%), triglycerides, cholesterol sulphate, and sterol or wax esters[21,27,28]. These are called 'skin lipids' and are arranged in bilayer sheets filling the intercellular space in the stratum corneum creating a compact structure. Lipid synthesis occurs in the corneocytes during epidermal differentiation, and they are extruded into the extracellular domains and form extracellular lipid enriched layers[29]. Altogether the stratum corneum provides a greater resistance unit to drug penetration.

1.3. Skin penetration pathways

A penetrant that is applied to the skin surface has three potential pathways by which it may traverse intact stratum corneum: via appendages (shunt routes), through the intercellular lipid domain, or via a transcellular route.

1.3.1. Shunt route

The pores of the appendages (hair follicles, sweat ducts) bypassing the barrier of the stratum corneum occupy only around 0.1% of the total skin surface[19]. Though eccrine sweat glands are numerous in palms and soles, their openings onto skin surface are also very small. Moreover, beyond this surface area, they either evacuate or secrete sweat that is expected to reduce inward diffusion of topically applied substances. The hair follicular pore openings are considerably larger than that of the

eccrine glands but are less numerous. The sebaceous gland ducts are also filled with sebum which is lipoidal[2]. Hence at a pseudo-steady state, penetration through the appendages makes a negligible contribution to total drug flux[30]. Shunt routes were found to be minor contributors at steady state flux of steroids through excised human skin[31]. Still, it can be utilized to contribute in the early time period between drug application and the establishment of steady state flux[32]; in the application of finite doses of medicine to skin in vivo for short time; the passage of large polar molecules and ions[2], and in the delivery of vesicular structures targeting pilosebaceous units[33].

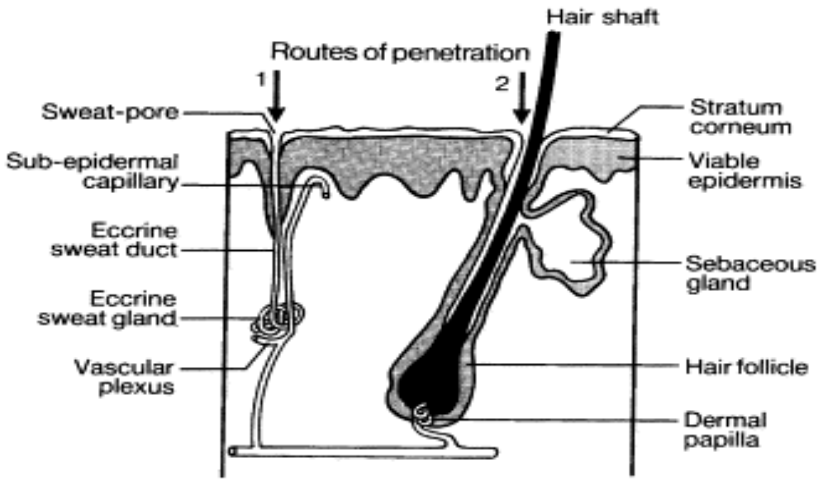


Figure 1.2: A typical diagram representing macro-routes of drug penetration: (1) via the sweat ducts; (2) through the hair follicles with their associated sebaceous glands (Figure adapted from Barry[3] and slightly modified)

1.3.2. The intercellular route

The only continuous domain within the stratum corneum is formed by the intercellular lipid matrix[34, 35] which is generated by corneocytes in the mid to the upper part of the stratum granulosum, discharging their lamellar contents into the intercellular space[36]. This route is highly tortuous where penetrants pass through the continuous lipid domains between the corneocytes. The pathway the molecule has to traverse is considerably greater (ranging from 150-500 μm)[2]. Yet this route is considered to show much faster absorption due to the high diffusion coefficient of most drugs within the lipid bilayers as this route provides both hydrophilic and lipophilic regions. Such junctions between corneocytes clusters often reach several micrometers into the skin and each junction in the stratum corneum is a “hot spot”

for dermal and transdermal drug delivery[17]. Therefore this route is the principal pathway by which most small and uncharged molecules diffuse along the lipid lamellae[37, 38].

1.3.3. The transcellular route

The transcellular route is often regarded as a polar route and is suitable for highly hydrophilic compounds at a pseudo-steady state[2]. A penetrant traversing the intact stratum corneum via this route faces many hurdles. Firstly, it has to partition into the corneocytes, then diffuse through the hydrated keratin and then again partition into the lipid bilayer to reach the next corneocyte. In this traversing through multiple lipid bilayers, the molecule has to partition into and diffuse across the hydrophobic chains and hydrophilic head groups of the lipids[20]. With this route, the pathway is directly across the stratum corneum and hence the path length for permeation is usually regarded as the thickness of the stratum corneum. Considering all these factors, this route is usually not considered as a preferred route for dermal invasion. However when a penetration enhancer (for example, urea) was used, this route gained importance in increasing permeability by altering the keratin structure[39].

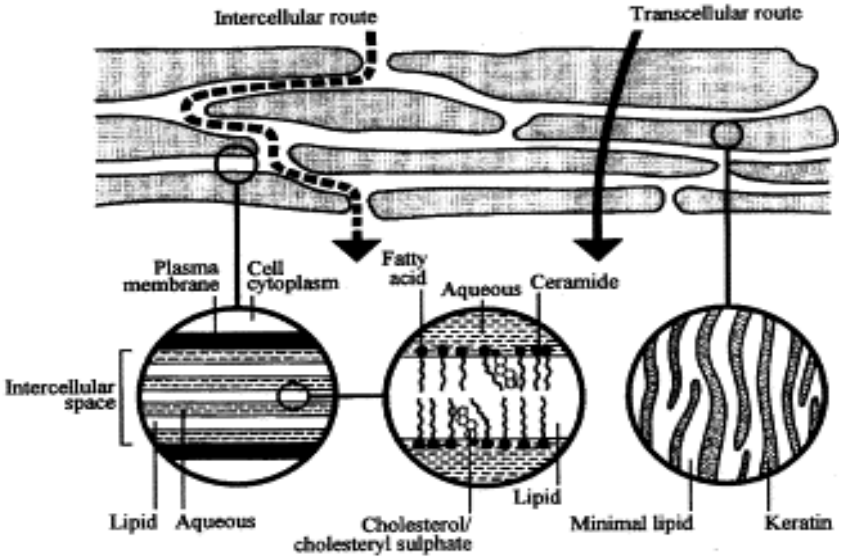


Figure 1.3: Simplified diagram of stratum corneum and two micro-routes of drug penetration (Figure adapted from Barry[3])

1.4. Penetration enhancement techniques

The main difficulty in topical application of drug is to cross the stratum corneum, the major permeability barrier. Many strategies have been investigated to overcome this barrier which can be achieved chemically, physically or by use of vesicular components[40]. Combination of both chemical and physical methods or by combination of several physical methods can often work simultaneously[41-43].

1.4.1. Chemical penetration enhancers

Chemical penetration enhancers basically enhance skin permeability by reversibly altering the physicochemical nature of the stratum corneum [44, 45]. They disrupt the highly ordered structure of stratum corneum lipid, interact with intercellular proteins, and improve permeant partition into it with a view to reduce its diffusional resistance[46]. Solvents and surfactants are suitable examples of this. Good solvents are water, methanol, ethanol, dimethyl sulfoxide, alkyl homologs of methyl sulfoxide, dimethyl acetamide, dimethylformamide, glycerol, propylene glycol, isopropyl palmitate, Azone, N-methyl-2-pyrrolidone, pyrrolidones-2-pyrrolidone[47]. Surfactants that act as chemical enhancers can be either anionic (e.g. dioctyl sulphosuccinate, sodium lauryl sulphate, decodecylmethyl sulphoxide), cationic (e.g. cetyltrimethyl ammonium bromide) or non-ionic (e.g. Pluronic F127, Pluronic F68)[2, 48]. Cationic surfactants damage human skin and are therefore not suitable for skin penetration[48]. Besides surfactants, bile salts (e.g. sodium taurocholate, sodium deoxycholate, sodium tauroglycocholate), binary systems (e.g. propylene glycol-oleic acid, 1, 4-butane diol-linoleic acid) and miscellaneous chemicals (e.g. urea, N, N-dimethyl-m-toluamide, calcium thioglycolate) are found to be useful for this purpose[47].

1.4.2. Physical methods

There are a few physical techniques which are applied to enhance skin permeation of topically applied agents. Iontophoresis, electroporation, ultrasound, microfabricated microneedle techniques cover the majority of approaches[32]. Iontophoresis works by electrically ($\leq$ 0.4 mA/cm^2) opening the stratum corneum by nanoporating the skin on a time scale of an hour by enlarging the pre-existing pores (pores of appendages,

already described under section 1.3.1) with an initial diameter of 0.5 nm[49]. The drug is briefly, applied under an electrode of the same charge as the drug, and a counter electrode is positioned elsewhere on the body. The active electrode effectively repels the drug and forces it into the skin[50]. Electroporation (or electropermeabilisation) is quasi-catastrophic electrical skin break-through, giving rise to relatively wider pores in the skin organ. These wide pores induce a dramatic and reversible increase in transmembrane transport. A relatively high potential (150 to 600 V) is applied to the membrane for a short time (micro to milli seconds)[51] leading to transitory structural perturbation of lipid bilayer membranes. The skin resistance drops by several orders of magnitude during high voltage pulsing. Electroporation is widely used in introducing genetic material into bacterial cells and transcutaneous flux for small fluorescent tracers up to 10^4 fold[52]. Electroporation, however, when applied repeatedly, will result in strong skin damage, becoming an irreversible process and compromising the skin's function as barrier protection. In microneedles, needles (with an array of 20 x 20 needles with height of 150 µm, spaced 100 µm apart[53]) are applied on skin and then removed to form pores within the stratum corneum where the drug solution is to be added later. Alternatively drug loaded microneedle tips can be used for immediate delivery where the drug is delivered by application of pressure or electrical means[2]. Sonophoresis is the application of ultrasound to the skin on which a drug, most preferably proteinaceous in nature, alone or in combination with a carrier, has been applied. It significantly enhances the transdermal transport of drugs. Sonophoresis produces an effect made up of a combination of high-frequency heat and motion energy, which is generated by sound waves at millions of cycles a minute (up to 3 million hertz). This method enhances transdermal transport in two ways: (i) enhanced diffusion through structural alterations of the skin (i.e, formation of transient pores in skin) and (ii) convection induced by ultrasound. Transdermal transport enhancement induced by this type of sonophoresis decreases after ultrasound is turned off, therefore the process is reversible. Charged and larger biomolecules are generally transported by the physical enhancement techniques: Iontophoresis and Electroporation respectively[49, 50].

1.4.3. Formulation approaches

Penetration enhancement with special formulations is mainly based on the use of colloidal carriers where submicron-sized particles are intended to transport entrapped active molecules into the skin. Such carriers include traditional liposomes, elastic liposomes, ethosomes, niosomes, nanoemulsions, and solid-lipid nanoparticles[54]. The first two types of liposomes will be discussed in this chapter in detail. Ethosomes are one sort of liposomes composed of mainly phospholipid, relatively high concentration of alcohol, sometimes glycerol and water[55]. Niosomes are vesicles composed of nonionic surfactants that have been evaluated as carriers for a number of drug and cosmetic applications[56].

1.5. Liposomes for skin drug delivery system

In order to deliver drugs into or through the skin, numerous methods have been investigated. One of the most studied approaches to enhance penetration of drugs is the use of vesicular systems such as liposomes[57]. Liposomes were first invented by a British physician, Alec D. Bangham in 1965[58]. He discovered phospholipid vesicles, which he initially called tiny fat bubbles, and later named liposomes. However the use of vesicles for skin delivery was first introduced by Mezei and Gulasekharam, who reported a four to five-fold greater permeation of topical application of liposomal lotion of triamcinolone acetonide as compared with an ointment containing same drug concentration[59]. Thus they concluded that liposome use might be beneficial for increased local activity while diminishing systemic absorption.

Liposomes literally mean “fat body” are nano- and micro lipoidal vesicles[58] (tiny spheres), which have diameters ranging from 50 nm to few microns and are formed as concentric bimolecular layers separated from its aqueous environment[32]. They are biocompatible and capable of incorporating both hydrophilic (in the aqueous zone) and lipophilic drugs (in the lipid domain). Liposomes may show significant improvement in apparent aqueous solubility of a lipophilic drug, consequently making possible delivery of a dose much higher than its water solubility[60]. Moreover drugs can be encapsulated within liposomes without further chemical modification that could interfere with the drugs’ therapeutic activity. Improved stabilization of unstable drugs by liposome encapsulation is possible[61]. Furthermore, liposomes are

non-toxic at any concentration of usual administered doses, biodegradable and can be richly biofunctionalized with antibodies, protein receptors, and other biosensor molecules [62]. Therefore liposomes have been investigated as parenteral drugs and antigen carrier systems and more recently have been extensively researched as topical and transdermal drug delivery systems[63-67].

Liposomes were found to be markedly superior to conventional dosage forms in a few areas, especially for intravenous and topical administration of drugs[28]. The rationale for using liposomes for skin delivery lies in the unique structural and functional properties of liposomes by which they can encapsulate and store drugs of widely varying polarities including various cargoes, such as enzymes, proteins, and DNA[60]. Their functional properties is such that they facilitate their entry into the skin where they form depots to enhance and extend the pharmacodynamic effects of the incorporated drug[68]. They may act as penetration enhancers (especially deformable liposomes), which aid in the penetration of the individual lipid components into the stratum corneum and subsequently alter the intercellular lipid lamellae[69]. Moreover topically applied liposomes, especially those prepared with skin lipids may provide an effective delivery system for skin diseases. These vesicles can act as a drug reservoir and have been shown to help sustain regular release of dermally active compounds into the skin[70], even to the deeper strata[28], releasing encapsulated drugs of half-lives ranging from 0.6 to 11 days[71]. Prolonged release from liposomal delivery systems can be useful in the treatment of a variety of skin lesions[45]. Furthermore liposomes are easy to apply on the skin, enhance the local therapeutic index[72] and reduce serious side effects and incompatibilities that may arise from undesirable high systemic absorption of drugs[28]. However a suitable modification in barrier function of the stratum corneum can be brought by a favourable interaction between liposomal phospholipids and lipid bilayers of the skin[73]. Vesicular deformability, size, shape, lamellarity, lipid composition and surface charge facilitate to form a wide range of liposomes which modify the barrier function[74].

1.5.1. Classification of liposomes

Liposomes are classified by their lamellarity and size. Liposomes can be subdivided by lamellarity into multilamellar and unilamellar vesicles. They can also be subdivided by size into small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs).

Multilamellar vesicles (MLVs) (size from 100-1000 nm) form spontaneously when an excess volume of aqueous medium is added to dry lipid film[75] and under gentle agitation (by mechanical dispersion or by hand shaking). Each vesicle generally consists of five or more concentric lamellae in an onion-like arrangement and are more suitable for lipophilic drugs. They are very simple to prepare, can easily be scaled up and are usually mechanically stable upon long storage period[76]. Due to the slow degradation of the concentric lamellae, MLVs give a more gradual and sustained release of entrapped material[76]. However MLVs were found not suitable for hydrophilic compounds as the percentage captured by aqueous components is usually low (~5-10%)[77] for 0.2µ MLVs with lipid concentration of 30 mg/mL.

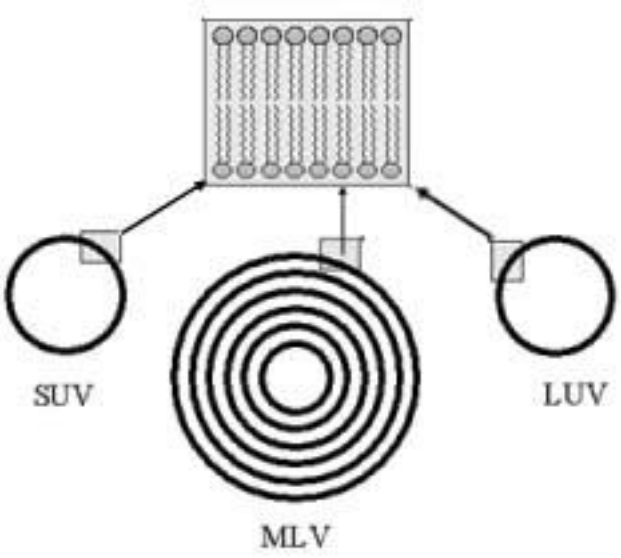


Figure 1.4: Schematic illustration of liposomes of different size and number of lamellae (Figure adapted from Daniels[78] and modified).

Unilamellar vesicles however are single bilayer liposomes. SUVs reviewed by Bangham[79], are at the lowest limit of size (25-50 nm; according to some authors up

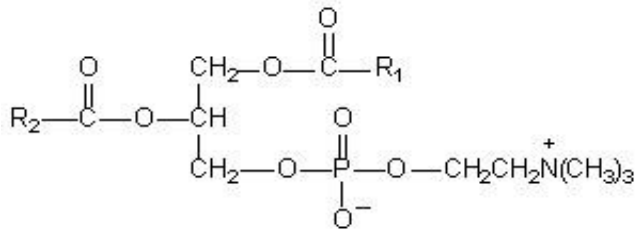
to 100 nm) possible for phospholipid vesicles. However size differs slightly according to the ionic strength of the aqueous medium and the lipid composition of the membrane[60]. Usually MLVs are subjected to sonication to form SUVs. They are the only well characterized vesicles homogenous in size and lamellarity[76]. But these vesicles also have some disadvantages. Their production usually requires a high energy input (for example, sonication or homogenization power) and they are thermodynamically unstable and are susceptible to aggregation and fusion on storage, particularly below the phase transition temperature (T_c, the temperature at which the lipid passes from gel state to liquid state). In addition to this, the entrapped volume is small, and thus the percentage entrapment of water soluble molecules is correspondingly low[76]. LUVs however are a very heterogeneous group of vesicles having the diameters ranging from 100 nm to cell size[80]. They can be formed by the reverse phase evaporation (REV) technique and by detergent dialysis[81]. Unlike SUVs, they have a high aqueous-lipid compartment ratio; thus a high entrapment for hydrophilic solutes can often be achieved with very economical use of membrane lipids. They also show reproducible drug release rates[76]. However the presence of only a single bilayer makes the vesicles not highly mechanically stable and retention of entrapped compound is not high[60].

1.5.2. Conventional liposomes

Conventional liposomes can be prepared from many amphiphilic ingredients. The most common liposome composition is phosphatidylcholine (PC), a phospholipid from soybean or egg yolk[82] with or without cholesterol. Conventional liposomes can also be prepared from other phospholipids to impart a charge, for example phosphatidylserine and phosphatidylinositol (for positive charge) and phosphatidylglycerol and phosphatidic acid (for negative charge)[76]. The type of lipid utilized in a liposome is very important. For example, the presence of net charged lipids (for some bioactive compounds) not only prevents spontaneous aggregation of liposomes but also influences the effectiveness of solute entrapment into the vesicles[1]. The two primary components (phospholipid and cholesterol) of conventional liposomes are discussed below in brief.

1.5.2.1. Phospholipids

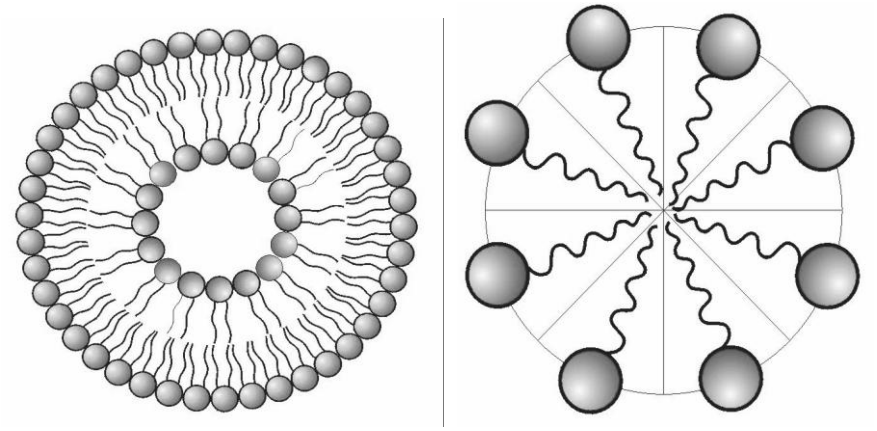
Phospholipids are amphiphilic molecules which are mostly constructed from the combination of a polar head group (negatively charged phosphate group) and a glycerol backbone moiety substituted with either one or two acyl or alkyl chains (PC), or an N-acylated sphingoid base (sphingomyelin) which is hydrophobic in nature. Phospholipids from natural sources are classified into phosphodiglycerides and sphingolipids[60]. PCs are the major phosphodiglycerides that can be obtained from natural (egg or soybean) and synthetic sources, and when extracted from plant and animal sources, they are known as "lecithins" (unpurified form).



R_1 and R_2= Fatty acid residues

Figure 1.5: Chemical structure of Phosphatidylcholine (PC)

The reasons for choosing PC in liposome formulations are many. In the corneocytes of the stratum corneum, cellular membranes account for 50% of the dry mass and consist mainly of PC and sphingomyelin. The intercorneocyte matrix is rich in phospholipids[83]. As phospholipids share a high structural similarity with skin lipids and have many advantages such as strong tissue affinity, biodegradability, and very little toxicity they are widely used in topical and transdermal delivery systems[83]. Moreover, PC is comparatively cheap and chemically inert. Due to the double chain tail, PC molecules have an overall tubular shape, and form lamellar bilayer vesicles (Figure 1.6(a)) in the presence of aqueous medium, in contrast to most other amphiphilic molecules (e.g. detergents and lysolecithin) whose geometry (a conical shape with single tails) lead to the formation of micelles[60] (Figure 1.6(b)).



(a) **(b)**

Figure 1.6(a): Double chain Phosphatidylcholine (PC) turned into liposome; (b): Single chain surfactant turned into micelle (Figure adapted from Kulkarni[84])

1.5.2.2. Penetration enhancement effect of phospholipid

Phospholipids containing unsaturated fatty acids are strong permeation enhancers for percutaneous delivery of some topically applied drugs[85]. There are few concepts which are thought to be applied in the penetration enhancement effect of phospholipid. Phospholipids possess the property of surfactants. They may penetrate the intercellular lipid bilayers of the stratum corneum and reduce its crystallinity and increase the permeability of drugs[86]. Moreover phospholipids may occlude the skin surface and thus can increase tissue hydration which in turn can increase drug permeation[44].

Phospholipid vesicles can either disrupt the bilayer fluidity in the stratum corneum or fuse with the stratum corneum lipids. The latter will collapse the structure and liberate the permeant from the phospholipid vehicles. If the drug is poorly soluble in the vehicle the thermodynamic activity will be raised which will result in facilitated permeation[2]. Moreover, phospholipids may mix with the stratum corneum lipids creating a lipid-enriched environment. This lipid depot is preferred by lipophilic drugs, resulting in enhanced skin uptake[87].

Few studies, however, have used phospholipids in a non-vesicular form as penetration enhancers. For instance, theophylline penetration was enhanced through hairless mice skin by 1% egg PC in propylene glycol (a concentration at which liposomes did not form)[88]. On the other hand, various studies demonstrated that phospholipids can exhibit their enhancing effect on skin in the presence of organic solvents such as propylene glycol, tetraglycerol and ethanol[89,90].

1.5.2.3. Cholesterol

Cholesterol and its derivatives are the most important and predominant sterols in animal tissues. Incorporation of cholesterol into liposome bilayers was found to bring major changes in membrane properties[60]. However cholesterol does not form a bilayer structure of liposomes by itself. As it is amphipathic in nature; it can be incorporated into phospholipid membranes at very high concentrations (up to 2:1 molar ratios of cholesterol to PC). In phospholipid liposomes, cholesterol enters the membrane by orienting its hydroxyl group towards the aqueous surface; the tricyclic ring sandwiched between the first few carbons of the fatty acyl chains, into the hydrocarbon core of bilayer, with the aliphatic chain aligned parallel to the acyl chains in bilayer centre[81].

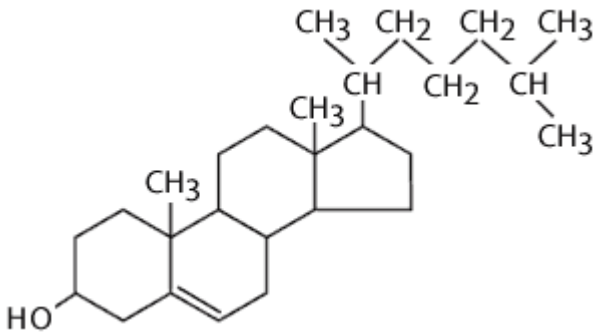


Figure 1.7: Chemical structure of cholesterol

Cholesterol acts as a fluidity buffer (both in biological membranes and in synthetic vesicles) due to their ability to interact with the phospholipid head groups and tail groups by hydrogen bonding and hydrophobic interactions respectively[60]. Cholesterol can broaden the bilayer transition considerably, and at high concentration (50 mol %) may completely abolish the heat of transition[91]. In the presence of cholesterol, above T_c, the freedom of molecular motion of the bilayer is

increased thus suppressing the tilts and shifts in membrane structure specifically at phase transition. Consequently at ambient temperature, cholesterol fluidizes bilayers containing saturated phospholipids, but decreases the fluidity of bilayers containing unsaturated phospholipids[60]. Thus the overall effect of cholesterol is to moderate the difference between gel and liquid crystalline phases.

Cholesterol in liposomes improves bilayer characteristics[81] by increasing micro viscosity of the bilayers by filling empty spaces among the phospholipid molecules, by reducing permeability of the membrane to water soluble molecules, and by stabilizing the membrane[92]. Moreover it increases the rigidity of the vesicles and helps to form smaller and more uniform liposomes. Lastly it prevents phase separation.

1.5.2.4. Mode of action of conventional liposomes on skin penetration

There has been a large amount of extensively reviewed research work carried out with conventional liposomes to improve skin delivery of drugs[2, 93-97]. Four possible mechanisms by which conventional liposomes may enhance skin delivery of drugs are the intact vesicular skin penetration, the penetration enhancing effect, the adsorption and fusion effect and the penetration of liposomes through the transappendageal route[98].

The intact vesicular skin penetration concept was first suggested by Mezei and Gulasekharam[59,99] who showed that intact liposomes penetrated up to the vascular dermis. Being large, intact liposomes were unable to enter the capillary circulation and thus acted as a drug reservoir at the site of action. However it was unexpected that large vesicles could penetrate the densely packed stratum corneum in great numbers[97]. Therefore many attempts were taken to find out the actual scenario. Foldvari *et al*[100] showed the presence of intact unilamellar liposomes in the dermis after application of MLVs, concluding that MLVs lost their external bilayers during epidermal penetration. They added that these liposomes could be adsorbed intact on the skin surface before penetration, with a possibility of some vesicle rupture. SUVs produced better input of aqueous radiolabelled inulin and radiolabelled lipid bilayer components[101] into deeper skin strata compared with large MLVs, suggesting that

vesicle size affects skin deposition. This all in turn supported the concept of intact vesicular penetration as a possible mechanism for improved skin accumulation.

While Du Plessis *et al* investigated the effect of vesicle size on skin deposition of cyclosporin, they showed that intermediate size and not the small size vesicles resulted in higher skin deposition. This indicated that intact liposomes did not penetrate the skin because greater flux is expected from smaller vesicles in case of intact vesicular penetration[102]. Similar improvement in stratum corneum penetration was observed when a fluorescent marker was applied onto skin after pre-treatment with empty vesicles and vesicles encapsulating the marker[95]. This suggested that improved delivery was not due to intact vesicular penetration, and suggested a possible second mode of action, the penetration enhancing effect, proposed by Kirjavainen *et al*[94], who suggested that liposomes penetrated the stratum corneum by adhering onto the surface of the skin and subsequently destabilizing, fusing or mixing with the lipid matrix. Therefore, conventional liposomes may act as penetration enhancers by altering the lipid structure of stratum corneum and promoting impaired barrier function of these layers to the drug. This ultimately creates a less well-packed intercellular lipid structure with subsequent increased skin partitioning of the drug. However the extent of interaction between liposomes and skin is highly dependent on the lipid composition of liposomes revealing that dermal delivery with skin-lipid liposomes is more effective than delivery with non-skin lipid vesicles[94]. Du Plessis *et al* studied the influence of skin pre-treatment with liposomes on topical absorption of inulin and hydrocortisone. They found that pre-treatment did not give advantage of encapsulated drug, showing no penetration enhancement for both. Therefore they concluded that liposome-stratum corneum interaction hypothesis was invalid, suggesting that liposomes must at least be applied along with the drug or the drug must be encapsulated within them[103].

The third mechanism, adsorption and fusion of liposomes onto the skin surface was demonstrated by Hofland *et al*[104] and Abraham and Downing[105], who showed that lamellae and rough structures formed on the top of the outermost layers of stratum corneum. This probably increased the driving force for permeation of liberated molecules. However, when the vesicles collapse on the skin surface they may form an additional barrier, which could reduce the permeation of hydrophilic molecules

encapsulated in the aqueous core. This mechanism also cannot account for the increased delivery of macromolecules[2]. Mechanism of enhancement via the transappendageal route was suggested by Du Plessis[106]. Recently this route has been identified suitable for liposomal skin targeting rather than through hair follicles, i.e. transdermal effect[97, 107].

Although some authors have suggested conventional liposomes as suitable carriers for skin delivery of some drugs[108-110], after intensive research it has become evident that traditional liposomes are of little or no importance as dermal drug carriers, because they do not deeply penetrate the skin, but rather remain accumulated in the upper layers of the stratum corneum[94]. The widest pores in the stratum corneum are around 30 nm in diameter or less[111] and inhibit the entry of conventional liposomes with a minimum diameter of 100 nm[22]. To push large entities across the intact skin, one must therefore depend on the 'penetration' mechanism, rather than on simple 'permeation' (diffusion-based transport). In order to squeeze through the small pores, the membrane elasticity of liposomes becomes important[22]. As membrane elasticity is lacking in conventional liposomes, a second type of liposome has emerged called elastic liposomes which have successfully improved dermal drug delivery[112].

1.5.3. Elastic liposome

Elastic liposomes, also called deformable or ultra flexible vesicles, were introduced by Cevc and Blume in 1992[112] who termed them as 'Transfersomes'. In composition, elastic liposomes differ from conventional liposomes as they contain an edge activator (10-25%) as well as a low concentration of ethanol (3-10%). An edge activator is often a single chain surfactant with a high radius of curvature that destabilizes vesicular bilayer and increases bilayer elasticity[22, 69, 113] by lowering the interfacial tension[114]. Various surfactants which were employed to formulate elastic liposomes are sodium cholate, sodium deoxycholate, Span 60, Span 65, Span 80, Tween 20, Tween 60, Tween 80 and dipotassium glycyrrhizinate[115-119].

Due to the self adaptability and high deformability of elastic liposomes, it is suggested that they can efficiently penetrate even through the pores of stratum

corneum that would be difficult for other particulates of same size[22]. The concomitant presence of stabilizing (phospholipid) and destabilizing molecules (surfactants) in bilayers of elastic liposomes and their tendency to redistribute in the non-uniformly stressed bilayer structure allow these vesicles to be several orders of magnitude more elastic than conventional liposomes and thus are suited for skin penetration[22]. However to be elastic, an optimal balance between the amount of lipid and surfactant is required. If surfactant level is too low, vesicles still remain rigid, if the level is too high, vesicles turn into micelles[3].

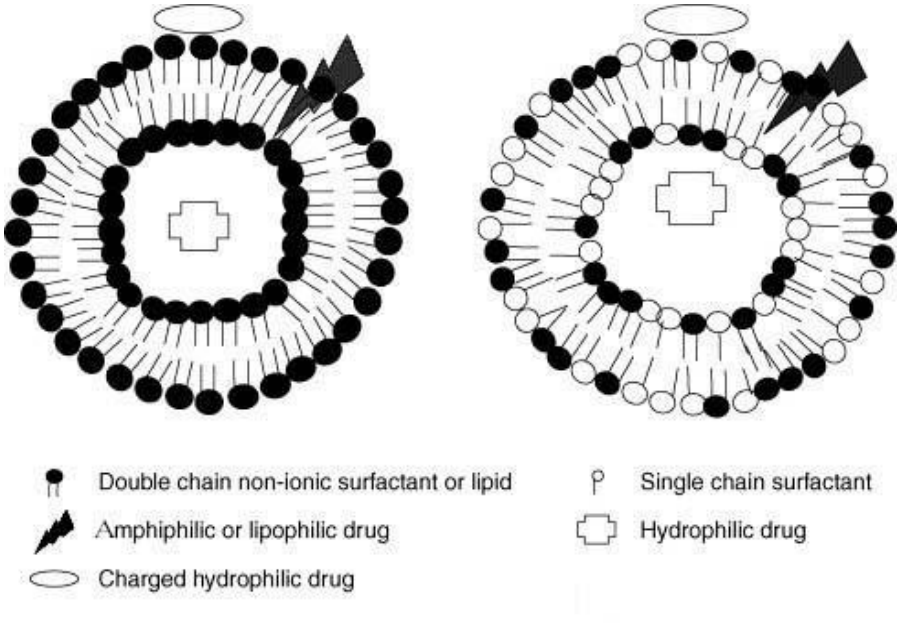


Figure 1.8: Location of drug molecules inside liposome; charged hydrophilic, amphiphilic and lipophilic drug molecules can be associated with the bilayer, whereas hydrophilic compounds can also be entrapped in the aqueous core of liposomes. Rigid vesicles may consist of double chain lipids in the presence or absence of cholesterol (left image). Elastic vesicles may consist of double chain lipid and a single chain edge activator (right image) (Figure adapted from Honeywell-Nguyen and Bouwstra[69])

1.5.3.1. Penetration enhancement effect of ethanol and surfactants

Elastic liposomes show enhanced dermal penetration because of the presence of surfactants and ethanol in their structure. Therefore these two components are described in brief here.

1.5.3.1.1. Ethanol

Ethanol is a known efficient penetration enhancer and is commonly believed to act by affecting the intercellular region of the stratum corneum[120, 121]. The penetration enhancing effect of ethanol can be attributed to two main factors: (a) "push effect" and (b) "pull effect" in which penetration of drug molecule is increased due to reduction in barrier property of stratum corneum[86, 122]. When a finite dose of drug is applied on the skin, due to the evaporation of ethanol, drug concentration reaches beyong its saturation solubility which possibly modifies the thermodynamic activity of the drug. This leads to a supersaturated state which allows greater driving force for penetration. This is how the "push effect" works. The "push effect" is negatively correlated with the solubility parameter of the donor phase. The second mechanism is the "solvent drag" effect or "pull effect". In this effect ethanol rapidly permeates across the skin carrying the permeant[123] altering the solubility properties of the tissue, thereby reducing the barrier property of the stratum corneum with a consequent improvement of drug partitioning into the membrane. It is reported that ethanol permeates rapidly through human skin with a steady state flux of approximately 1 mg/cm^2/h[124].

Ethanol has been reported to enhance the permeability of doclofenac[125], levonorgestrel and estradiol[126] through rat skin and estradiol through human skin in vivo[127]. However the enhancement effect of ethanol is concentration dependent. For example salicylic acid diffusion across human epidermis was promoted up to ethanol/water ratio of 0.63, higher level of ethanol decreased permeation[128].

1.5.3.1.2. Surfactant

Surfactants are generally composed of a lipophilic alkyl or aryl fatty chain, together with a hydrophilic head group. They are often defined in terms of the nature of the hydrophilic moiety[129]. Many traditional topical formulations contain ionic

surfactants that enhance skin penetration of drug molecules in a variety of ways. For example, they are widely known to interact with proteins, thus inactivate enzymes and bind well within the stratum corneum[2]. Anionic surfactants (for example, sodium laury sulphate; SLS) swell the stratum corneum possibly by uncoiling the keratin fibers and altering the α-helices to a β-sheet conformation[2]. They are also able to expose more water binding sites to the stratum corneum, are able to extract lipids from it and disrupt the lipid bilayer packing[130].

While surfactants usually possess low chronic toxicity[2] cationic surfactants were reported to have potential to damage skin; SLS is a powerful irritant and increased the transepidermal water loss in human volunteers in vivo[48]. However cationic surfactants are still widely used in cosmetic formulations with no apparent side effects[131]. Non-ionic surfactants (nonoxynol series) and anionic surfactants are regarded as safe. The polysorbates were shown to enhance the permeation of hydrocortisone, lignocaine and methanol[132, 133]. On the contrary naloxone and naproxen permeation were not improved by non ionic surfactants[134]. Polyoxyethylene alkyl ethers and esters were shown to be more effective permeation enhancers than the polysorbates[134]. In order to provide enhancement by altering the lipid region of stratum corneum, the surfactants have to permeate, a phenomenon strongly dependent on the partition coefficient and solubility of the surfactants[135]. The more lipophilic is the surfactant, the greater the enhancement potential.

1.5.3.2. Mode of action of elastic liposome

Two mechanisms were proposed by which elastic vesicles can improve skin delivery of drugs[114]. They are 1) as drug carrier systems; 2) as penetration enhancers.

1.5.3.2.1. As drug carrier system

This mechanism was first put forward by Cevc and his colleagues[112, 136-138] where vesicles were proposed to act as drug carrier systems. In this system, intact vesicles enter the stratum corneum by transdermal hydration gradient[3] which exists across human skin in vivo from around 20% at the outer surface to nearly 100% within the skin at the epidermal/dermal junction[2]. The osmotic force for the vesicles entering the skin is called xerophobia (the tendency to avoid dry surroundings)[112]. Therefore

when elastic liposomes are applied on the skin surface non-occlusively, phospholipid tends to avoid dry surroundings and requires hydration to remain in maximum swollen state. The gradient operating from the skin towards water-logged viable tissues drives them to squeeze between the cells in the horny layer[3], despite the large average vesicle size[138]. Moreover, the surfactant accumulates at the high stress sites within the vesicles and forms a highly curved area of the vesicle. Thus elastic liposomes deposited on the skin surface will evaporate and partially dehydrate, follow the local hydration gradient and trespass the strongly hydrated and deeper skin layers spontaneously[3] and intact without permanent disintegration[138].

The inter-cluster pathway and the inter-corneocyte pathway (honeycomb-like system) contain structural irregularities within the intercellular lipid lamellae. These irregularities can act like virtual channels through which elastic liposomes do penetrate [23]. In non-invasive transdermal immunisation when elastic liposomes labeled with a radio-active marker was applied, radioactivity was observed in the liver indicating the presence of these particles in systemic circulation, which might suggest that elastic liposomes permeate across the skin[139, 140]. The important difference between elastic and conventional liposomes is the high and stress-dependent adaptability of the former. When conventional vesicles are applied on the skin non-occlusively they are unable to deform, confine themselves to the upper layers of the stratum corneum, where they dehydrate and fuse with skin lipids[112].

1.5.3.2.2. As penetration enhancer

van den Bergh *et al* showed that hairless mouse skin pretreated with elastic vesicles increased the diffusion of ^3H_2O compared to pre-treatment with a buffer control, indicating a possible penetration enhancing mechanism[141]. In this mechanism, phospholipid vesicles, after entering the stratum corneum modify the intercellular lipid lamellae, facilitate penetration of free drug molecules into and across the stratum corneum[141]. Moreover elastic liposomes were reported to carry both entrapped and non-entrapped carboxyfluorescein into the stratum corneum and possibly to deeper layers of the skin[63], suggesting a possible penetration enhancing effect. In another study, elastic liposomes with ketotifen only outside the vesicles significantly improved drug permeation and deposition over elastic liposomes with

ketotifen only entrapped inside the vesicles, suggesting that the penetration enhancing effect may be of great importance in enhanced skin delivery of ketotifen by elastic liposomes[142]. However penetration enhancement is markedly influenced by the type, concentration and state of the surfactant used in these vesicles, as well as the nature of the permeant[133].

1.5.3.3. Elastic liposomes in dermal delivery

An extensive amount of research work has been conducted to study the *in vitro* skin delivery of various drugs from elastic liposomes. Some of the research findings of *in vitro* skin delivery of elastic liposomes are summarized in Table 1.1.

Table 1.1: Application of elastic liposomes in dermal delivery

Drug or marker utilized/therapeutic use	Composition of elastic liposome	Tissue utilized *in vitro*	ER in terms of permeation	Conditions used in permeation studies
Oestradiol[116]/hormone insufficiency	SPC: S. Chol (86:14%), SPC:S80 (86.7:13.3%), SPC:T80 (84.5:15.5%); hydrated with 7% v/v ethanol in water	Heat separated human epidermis	17 (2.1[B]) 17 (2.1[B]) 17 (2.1[B])	Application condition: non-occluded; Donor: 20µL; Receptor: aqueous sodium azide for first 12 h, 50% v/v ethanol in sodium azide for next 12 h
(+)-Catechin[143]/Chemo preventive and anticarcinogenic agent	EPC:CH: Deoxycholic acid (4:1:0.25); hydrated with 15% ethanol	Female nude mouse skin	4.75[A] (1.24[B])	Application condition: occluded; Donor:1mL; Receptor: citrate-phosphate buffer pH 7.4
5-FU[144]/skin cancer	SPC: S. Chol (84:16%); hydrated with 7% ethanol	Heat separated human epidermis	No improvement[A]	Application condition: non-occluded; Donor:20µL; Receptor: 50% aqueous ethanol
Diclofenac[145]/rheumatic diseases and actinic keratoses	SPC: S. Chol (44:15%); hydrated with 10% ethanol	Heat separated human epidermis	(2.0[B])	Application condition: not mentioned; Donor:1.5mL; Receptor: Phosphate buffer pH7.4
Cyclosporin A[146]/skin lesions and pruritus	SPC: S. Chol (1:0.28); hydrated with 0.9% NaCl solution	Kunming mouse abdominal skin	After 8 h of administration, elastic liposomes transported 1.16±0.26 µg/cm^2 of cyclosporin A and amounted to 1.88 ±0.06 µg 24 h later. Conventional liposomes failed to transfer cyclosporin A after 24 h.	Application condition: Non-occluded; Donor: 20µL; Receptor: 20% ethanol adjusted to isotonic by 0.9% NaCl

Retinol[147]/cosmetic product	EPC:T20 (6:1); hydrated with NTE buffer (pH 7.0, 145mM NaCl, 10mM TES (N-Tris(hydroxymethyl)-2-aminoethane sulfonic acid), 0.1mM EDTA)	Dermatomed human cadaver skin; epidermis skin model	(approx. 5.5[B])(approx.3.8[B]) Drug suspension showed no skin permeation	Application condition: non-occluded; Donor: 300µL; Receptor: Phosphate buffer solution (pH 7.0)
Dipotassium glycyrrhizinate[148]/acute or chronic dermatitis	PC:KG (2:1) PC:KG (4:1) PC:KG (8:1) HPC:KG (2:1) HPC:KG (4:1) HPC:KG (8:1)	Full-thickness pig ear skin	Flux was negligible, below detection limit	Application condition: non-occluded; Donor: 100µg; Receptor: 0.002% w/v aqueous sodium azide
Low-molecular-weight heparin[149]/anticoagulant	DOTAP:T 20 (86.9:13.1%) EPC:T 20 (86.9:13.1%) EPC:DCP:T 20 (82.9:4:13.1%)	Full thickness hairless mouse skin	400[A] 43[A] 30[A]	Application condition: non-occluded; Donor: 500µL; PBS containing 0.1% sodium azide
Hepatitis B surface antigen (HBsAg)/non-invasive topical delivery	SPC:S 80 (86:14% (w/w)) hydrated with phosphate buffer (pH 6.5)	Human cadaver skin	robust systemic and mucosal antibody response	Application condition: not mentioned; Donor: 10µg; Receptor: PBS pH 6.5
Methotrexate[119]/anti-neoplastic activity	PC:KG (2:1), PC:KG (4:1), HPC:KG (2:1), HPC:KG (4:1) Phosphate buffer (pH 7.4)	Full-thickness pig ear skin	5.2 (8.5[B]) 2.9 (4.8[B]) 5.9 (5.9[B]) 4.1 (4.1[B])	Application condition: non-occluded; Donor:200 µL; Receptor: Buffer pH 7.4

Melatonin [150]/ultraviolet induced erythema	SPC:SD. Chol (86:14%) 0.2M phosphate buffer (pH 6.5)	human cadaver skin	12.19 (4.70[B])	Application condition: not mentioned; Donor: 200 µL; Receptor: PBS pH 6.5
Ketotifen[142] /allergic rhinitis	SPC: T80 (84.5:15.5%) hydrated with 7% ethanol	Albino rabbit pinna skin	1.6 (1.7[B])	Application condition: non-occluded; Donor: 150 µL; Receptor: pH 7.4 isotonic phosphate buffer containing 0.11% (w/v) formaldehyde
Pergolide[114]/skin discoloration	L-595:PEG-8-L:sulfosuccinate (50:50:5), hydrated with pH 5.0 (0.05 M citrate buffer), pH 6.0 or pH 7.0 (0.1 M phosphate buffers) respectively	Non-occlusive co-treatment: 2 fold better permeation from elastic vesicles compared to buffer; non-occlusive pre-treatment with empty vesicles: no enhancement; Occlusion reduced the action of elastic vesicles, but could increase the drug transport since water is a good penetration enhancer for this particular drug. The highest pergolide skin permeation was obtained from an occluded saturated buffer solution, steady-state flux of 137.9 ng/h cm^{-2}. Volume of application had no effect on drug transport.		

CH: Cholesterol, DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane, EPC: egg phosphatidylcholine, ER: enhancement ratio, HPC: hydrogenated phosphatidylcholine, KG: Dipotassium glycyrrhizinate, SPC: soya-phosphatidylcholine, S. Chol: sodium cholate, S80: Span 80, SD. Chol: sodium deoxycholate, T80: Tween 80, T20: Tween 20

[A]: relative to drug solution or suspension [B]: Relative to conventional liposome

1.5.4. Preparation of liposomes

1.5.4.1. Phase transition temperature and bilayer fluidity

Lipids have a characteristic phase transition temperature (T_c) and they exist in different physical states above and below this temperature. The most consistently observed phase transition occurs above the T_c, in which the membrane passes from a tightly ordered "gel" or "solid" phase to a liquid-crystal "fluid" phase where the freedom of molecular movement is higher[151]. The most widely used method to determine T_c is microcalorimetry.

Phase transitions and fluidity of phospholipid membrane has been found to be very important both in the manufacture and exploitation of liposomes[60]. If liposomes are prepared below or above the T_c of the lipid, they will form gel state or liquid state vesicles respectively. It is very important to prepare liposomes above their T_c. Liquid state vesicles play predominant role in percutaneous penetration enhancement. They act not only in the superficial stratum corneum layers, but also induce lipid perturbations in the deeper layers of the stratum corneum where they can modify the intercellular lipid lamellae, whereas gel state vesicles only interact with the outermost layers of the stratum corneum. There they get fused which in turn might provide an additional permeation barrier[3]. The mechanism of action of fluid-state vesicles for skin interactions lies in the fact that they are more effective in enhancing drug transport into and across the skin[114]. However the membrane fluidity can be influenced by hydrocarbon chain length and unsaturation of different phospholipids[151]. On the whole, liposomal phase behaviour which affects bilayer fluidity determines liposomes' permeability, fusion, aggregation, and protein binding, which again markedly affect the stability of liposomes and their behaviour in vivo[60].

Ethanol, a key component of elastic vesicles was reported to exert a biphasic effect on the main T_c of fully hydrated PC[152]. In the absence of ethanol, the T_c value of the fully hydrated dipalmitoyl-PC bilayer is 41.6°C which decreased linearly with increasing ethanol concentration. At ethanol concentration of 50 mg/mL, maximum decrease in T_c

was about 2°C. Above this threshold limit the T_c increases with increasing ethanol content. This effect of ethanol on T_c could be due to the transformation of the partially interdigitated gel phase into the fully interdigitated gel phase of DPPC bilayer[153]. Recently it has been shown that above the threshold concentrations of ethanol, short-chain primary and secondary alcohols up to 1-heptanol can induce chain interdigitation in bilayers of dipalmitoyl-PC and distearoyl-PC[154, 155].

1.5.4.2. Method of preparation of liposomes

Numerous methods for preparation of both conventional and elastic liposomes are described in the literature[81]. Among them, the thin film hydration technique is most commonly used and was chosen in this project to prepare all vesicles. Therefore it will be discussed in detail; the other preparation methods will be discussed in brief.

1.5.4.2.1. Thin film hydration method

Lipid film hydration method also called mechanical dispersion is the most commonly followed technique to prepare both conventional and elastic liposomes originated by Bangham *et al*[58]. In brief, phospholipid with or without cholesterol (in case of conventional liposome) and surfactant (in case of elastic liposome) and lipophilic drugs are dissolved in organic solvent(s) such as chloroform, methylene chloride, methanol, ethanol, ether alone or mixtures of these solvents[60] in a 250 mL round bottom flask. This is then dried to form a lipid film onto the side of the flask by slowly evaporating the organic solvent using a rotary evaporator. Complete removal of organic solvent is assured by keeping the flask under vacuum overnight[76].

Next, the lipid film is hydrated by addition of an aqueous medium followed by rotation using rotary evaporator until the film has been completely dispersed. For hydrophilic drug loading, aqueous drug solution is added as hydration medium. In case of elastic liposome preparation, 3-10% ethanol can be added along with hydration medium. Upon hydration lipid bilayers are formed. It is believed that even before exposure to the hydration medium, the lipids in the film are oriented in such a manner as to separate

hydrophilic and hydrophobic regions from each other which resembles their conformation in the finished membrane preparation[76]. On hydration the lipids swell and peel off the support in sheets, generally to form MLVs[60]. The final milky white suspension is left to stand for 2 h at room temperature for complete swelling.

By this method, liposome preparation is easy and quick and vesicles are relatively stable on storage. This method has many drawbacks. For example, the volume enclosed within liposome membrane is usually only about 5-10% of total volume used, therefore rendering the method very wasteful for hydrophilic compounds[81]. Furthermore solute distribution may be uneven in liposomes, which should be considered carefully when preparing liposomes for commercial use. Size distribution is heterogeneous, liposomal particle size as large as 30µ and as small as 0.05µ can exist[156]. Finally this method is difficult for industrial scale up. For instance, film thickness is determined by the size of the round bottomed flask. The thickness will affect the efficiency of the hydration process. For a large batch size, it is not feasible to build a large flask to make the film with a proper thickness[60].

The other commonly used methods of liposome preparation include pro-liposomes, reverse phase evaporation (REV) and solvent injection method. Pro-liposomes (approx. 0.1µm in diameter) are dry, free-flowing product, which on addition of water, disperses to form an isotonic MLVs[76]. The process of pro-liposome involves casting a solution of lipid and material to be entrapped onto sorbitol which is readily soluble in water and poorly soluble in organic solvent[60]. REV method was first developed by Szoka and Papahadjopoulos in 1978[77] where lipid components are dried down onto a round bottom flask and the film is re-dissolved in diethyl ether[157]. Next, water soluble drug aqueous solution is added to it and the system is maintained under inert gas continuously[158]. Bath sonication of this system produces inverted micelles, which is placed in a rotary evaporator to remove the solvent under reduced pressure at room temperature with ~200 rpm. This causes the phospholipid-coated droplets of water to coalesce and produces a sticky viscous gel[159] which at a certain time collapses and forms an aqueous suspension (LUVs) within 5-10 min. This method has gained widespread application in producing

high encapsulation (up to 65%[159]) of water soluble drugs[77, 160]. However in this method, the compound to be entrapped is exposed to organic solvents. Enzymes, protein pharmaceuticals, RNA may undergo conformational changes; protein denaturation and breakage of DNA strands is possible due to the utilization of harsh solvents[81, 158]. Solvent injection method can be sub-divided into ether and ethanol injection method. The former was developed by Deamer and Bangham[161] where a lipid solution dissolved in diethyl ether is slowly injected into warm aqueous solution of the compound to be entrapped at 55-65ºC or under reduced pressure. Ether vaporization leads to the formation of single layer vesicles[81] (diameter ranging from 50-200 nm). This method is simple, easy to be scaled up and applicable for a wide variety of lipid mixtures and aqueous solution. However organic solvent used may be harmful for few compounds, rendering it not a useful method for protein incorporation[158]. Ethanol injection was first described by Batzri and Korn[162]. Briefly, lipid solution in ethanol is rapidly injected into an excess of buffer which leads to instantaneous formation of SUVs. This is a simple and rapid method which avoids sonication and exposure to high pressure, thus process is gentle. But removal of residual ethanol from the preparation may be problematic as the partial vapor pressure of ethanol at low concentration is smaller compared to that of water[158]. Moreover ethanol forms azeotrope with water and is difficult to remove under vacuum or distillation process[81].

1.5.4.3. Particle size reduction

MLVs are usually processed to reduce their size using a method which imparts high energy to lipid suspension. This can be achieved by sonication, high pressure homogenization or extrusion.

1.5.4.3.1. Sonication

Sonication is an effective method for reducing particle size for small batches. On the basis of sonicator type, there are two types of sonication techniques: probe and bath sonication. However difficulty in uniformly sonicating large batches of preparation,

generation of personal hazard and production of the limited size vesicles are shortcomings of the sonication method[76].

Probe sonication is conducted by immersing a metal probe below the liquid surface. Owing to high energy input of this method, the particle size of the MLV can be reduced rapidly and reproducibly[163]. Moreover, small and homogenously distributed SUV liposomes can be produced by this method. However there is a risk of lipid degradation by probe sonication due to high input of energy generation if sonicated for long time. Thus the sonicator vessel must be cooled efficiently during sonication[158]. However for large batch size, this method is of little value.

Bath sonication has the advantage of avoiding direct contact of the formulation with the probe, thus it is much mild and has less risk of lipid degradation[158]. Large volume can be sonicated and reproducible sizing is possible when the flask is placed in the same position each time. But this method may not reduce size to a large extent and is time consuming[76].

1.5.4.3.2. Extrusion

This method is very gentle in reducing size of MLVs by passing them through a series of membrane filter of defined pore size with much lower pressure (<100 p.s.i). Breakage and re-sealing of membrane occurs during the process. After repeated extrusion through 100 nm filter; liposome suspension gets progressively unilamellar with a size distribution around the pore size of the membrane[164]. Therefore size can be controlled by selecting the pore size of membrane[76].

The rate at which suspension will pass through the membrane depends on the original size and lipid concentration of liposomes since liposomes with less fluid membrane are difficult to break down[76]. This process can be applied to all liquid crystalline bilayer lipids. Size distribution is homogenous and the method is relatively gentle, fast and reproducible. But relatively low lipid concentration (<50mg/mL) is employed and long extrusion time is often required[158].

1.5.5. Characterization of conventional and elastic liposomes

Liposomes produced by different techniques have different physicochemical characteristics which have an impact on their behaviour in-vitro and performance in-vivo (disposition)[165]. The characterization of liposomes is important not only for formulation design but also for evaluation and quality control purposes. Characterization parameters for the purpose of evaluation of liposomes in skin delivery system can be classified into two broad categories, physical and chemical characterization.

Physical characterization evaluates various parameters, including size, shape, surface potential, entrapment efficiency (EE), lamellarity, phase behaviour and *in vitro* drug release profile[165, 166]. Liposome size has possibly the largest influence on physical properties and appearance of the formulation. Various techniques are available to measure particle size, most commonly used is the Dynamic Light Scattering technique.

The number of lamellae in liposomes is an important parameter to be monitored because it influences the entrapment of lipophilic drugs into liposomal bilayers. The average size and number of lamellae can give theoretical estimation of the total volume of enclosed aqueous space within the liposomal core[60]. Though knowledge of size distribution and preparation method of liposomes may give some idea on the presence of number of lamellae, it is preferable to follow an established technique which gives direct determination of the number. The most regularly applied technique is the electron-scanning microscopy.

To determine the EE of liposomes, it is required to separate the free drug from liposomes by applying methods mentioned in Table 1.2. Determination can be performed by analyzing the free drug and liposomal pellets; for the latter, lipid solubilizers (Triton X-100) are used to destroy the liposomes. Among the routinely used techniques, dialysis and ultrafiltration offers membranes with a molecular weight cutoff between those of drugs and lowest expected liposome fraction. However in ultracentrifugation, liposomes are pelleted to separate the free drug. Drawback of this method includes the risk of vesicle rupture and presence of small liposomes in the

supernatant. However the entrapment of hydrophilic drug is dependent on the volume of aqueous core. For the incorporation of an ionizable drug, entrapment will depend on the partition coefficient of the drug between the aqueous phase and bilayers, more specifically on the distribution coefficient.

The only difference in the characterization of conventional and elastic liposomes is the determination of bilayer elasticity for the latter. Measurement of vesicle membrane elasticity is accomplished by an extrusion method through small pore sized filters applying a fixed pressure. The elasticity value is expressed as deformability index.

The purity and potency of various liposomal constituents is studied under chemical characterization[167]. Table 1.2 shows various techniques to characterize physical and chemical parameters of liposomes.

Table 1.2: Liposome parameters and common techniques for their physical and chemical characterization[60, 168]

Physical Characterization	
Parameter	**Technique**
Mean vesicle size and size distribution	Dynamic Light Scattering, Size exclusion/molecular sieve chromatography, Electron microscopy, Ultracentrifugation, Ultrafiltration
Vesicle shape and surface morphology	Transmission electron microscopy, Freeze-fracture electron microscopy
Number of lamellae	NMR Spectroscopy, Small-angle X ray scattering, Freeze-fracture electron microscopy[169]
Surface charge	Micro-electrophoresis, Zeta potential
Encapsulation volume	Encapsulation of water soluble markers
Entrapment efficiency	Size exclusion/molecular sieve chromatography, Ultrafiltration, Ultracentrifugation/Dialysis
Bilayer Fluidity	Fluorescent probes, Spin label EPR, NMR probes, Calorimetry
Phase behavior	Freeze-fracture electron microscopy, Differential scanning colorimetery
Drug release	Diffusion cell/ dialysis

Elasticity measurement	Extrusion
Chemical Characterization	
Phospholipid concentration	Barlett assay, stewart assay, HPLC
Cholesterol concentration	Cholesterol oxidase assay and HPLC
Phopholipid peroxidation	UV absorbance, Iodometric and GLC
Phospholipid hydrolysis, Cholesterol auto-oxidation.	HPLC and TLC
Osmolarity	Osmometer

Physical and chemical characterizations are very important for meaningful comparison of different liposomes for different batches. One should ensure that no major changes occur on liposome storage so that well characterized product is supplied with optimal and reproducible clinical effects.

1.5.6. Stability of liposome

Storage instability is the most common problem in liposomes. On storage the physical characteristics of liposomes change, which should be regularly monitored by using appropriate techniques. However the chemical stability of the phospholipid and other components incorporated into liposomes is also an important parameter[60]. Chemical degradation of lipid affects the physical stability of liposomes. Lipid degradation is most commonly attributed to hydrolysis and oxidation; the lat ter may be prevented by adding anti-oxidants in the liposomal formulation. However hydrolysis is the most dangerous cause of lipid degradation, which significantly alters the physical stability leading to liposome aggregation, fusion and breakage.

The vesicle size distribution is the most important parameter to be monitored on a time basis because it affects liposomal efficacy and biodistribution. Generally liposomes consisting of only PC are prone to aggregate upon storage. Therefore small amount of

charged lipids are added to formulation[170]. Moreover change in particle size also alters the number of lamellae present in liposomes and the EE.

1.6. Compounds of interest

1.6.1. Ibuprofen

A number of products (including creams, gels and more complex transdermal systems) have been designed to deliver non steroidal anti inflammatory drugs (NSAID) to the skin surface for local activity[171]. Ibuprofen [2-(4-isobutylphenyl)-propionic acid] (IBU); chemical formula: $(CH_3)_2CHCH_2C_6H_4CH(CH_3)COOH$) is a NSAID belonging to the group of arylpropionic acid derivatives and shares its pharmacodynamic properties with the rest of the NSAIDs.

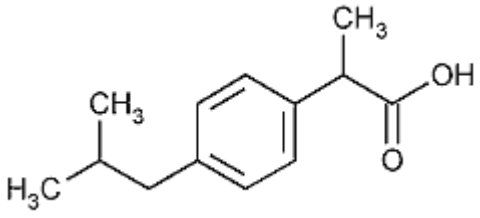


Figure 1.9: Chemical structure of Ibuprofen (IBU)

IBU is a small molecule (MW 206.29) with a log (octanol–water partition coefficient (*P*)) of around 3.72, and p*K*a 4.45[172] .

Table 1.3: The solubility of IBU as a function of pH (data adapted from Watkinson *et al*., [173])

pH	2.2	2.3	4.0	5.0	6.0	7.0	9.0	9.2
Solubility (mg/mL)	0.024	0.027	0.029	0.096	0.52	3.70	7.83	14.8

IBU is used in the management of mild to moderate pain, fever, and inflammation which are promoted by the release of prostaglandins in the body. IBU blocks the enzyme that produces prostaglandins (cyclo-oxygenase) which results in lower levels of

prostaglandins. As a result, inflammation, pain and fever are reduced. Therefore IBU is used topically to treat local inflammation (i.e. acne) and it works clearly beneath the transport-limiting barriers of the skin[174]. With pronounced analgesic properties, IBU is used in the long-term treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis[175, 176]. The systemic treatment of such diseases with NSAID has proven to be efficient; however like other drugs of this class, IBU has dose-dependent side effects, especially GI irritation, bleeding, ulceration or perforation after oral administration. Considering the fact that IBU is often used for a long-term period, it is therefore considered a good candidate for skin delivery which will minimize the adverse effects arising from oral administration[177, 178] by dispensing sub-cutaneous therapeutic doses through tissue penetration[179], at the same time will provide relatively consistent drug levels at the application site for prolonged periods[180, 181].

IBU delivery via passive diffusion is dose-dependent; better therapeutic effect will be achieved when the drug will permeate the skin better[174]. An effective permeation of IBU, however, is difficult to achieve due to its extremely poor skin permeability, even though IBU is relatively good compared to other NSAIDs in common use[182]. The maximum predicted flux value of unionized IBU is 0.61µg/cm^2/h[171]. In order to enhance permeation of IBU supersaturated solutions, eutectic systems, mucoadhesive patches, and vehicle containing non-ionic surfactants or fatty acid have been explored[183-187]. Elastic liposomes were found to show enhanced skin penetration for a range of drugs loaded (Table 1.1). Therefore development and evaluation of elastic liposomes with IBU could be a promising delivery vehicle of this drug. Thus IBU was chosen as a model drug in this study. However liposomal incorporation of poorly water soluble compounds is not only dependent on the physicochemical properties of the drug, factors including bilayer composition and their method of preparation have also been shown to be contributing factors[188].

1.6.2. Sodium fluorescein

Sodium fluorescein (NaFl) is an orange–red fine powder that is hygroscopic and is freely soluble in water (≥100 mg/mL), soluble in alcohol but practically insoluble in hexane and methylene chloride. The chemical formula and molecular weight of NaFl are $C_{20}H_{10}Na_2O_5$ and 376.3 respectively. In water it has an absorption maximum at 494 nm (blue) and emission maximum of 513 nm (green–yellow). NaFl is sensitive to pH, fluorescing maximally in the range of 8–9[189]. A solution of it is strongly fluorescent, even in extreme dilution. The fluorescence disappears when the solution is made acidic, and reappears when the solution is again made alkaline because in acidic condition, fluorescence is in equilibrium with spironolactone form.

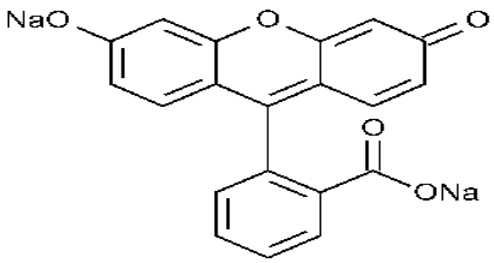


Figure 1.10: Chemical structure of sodium fluorescein (NaFl)

NaFl is the only fluorophore routinely used in experimental dermatology for in vivo study of skin. Increasingly wider application has been found in epidermal labeling, intradermal injection to investigate skin conditions in vivo when using non-invasive devices such as confocal scanning laser microscopy[189]. NaFl can be used to visualize and selectively stain subtle structures in the skin at the subcellular levels such as nuclei and lipids[190]. Moreover it has been used as an intravenous bolus injection for decades for the examination of the vasculature of the ocular fundus (fluorescein angiography) and as eye drops for the diagnosis of corneal erosions[189]. In this study, NaFl was chosen as a model molecule because of its water solubility, ease of analysis, availability and low cost. Also it has been incorporated into vesicular carriers as a marker for fluorescence microscopy to study the distribution of liposomes after skin penetration.

1.7. Objective of current study

The primary objective of current study was to design and evaluate elastic liposomes, in comparison to free drug solution and conventional liposomes, in topical delivery of IBU across human epidermis. The scope of the project included liposome development, investigation of influence of formulation variable on the physical characteristics (particle size, zeta potential, EE and bilayer elasticity) of liposomes and optimization based on their characteristics as well as determination of IBU penetration from conventional and elastic liposomes through human epidermis to establish the relationship between the formulation variable and skin penetration of IBU loaded liposomes.

METHODOLOGY

2.1. Materials

1. L-α-Phosphatidylcholine (PC) (Soybean, purity-95%); Physical state: Granules; MW: 758.07; Lot: SPC95-166; Avanti Polar Lipids, USA
2. Ibuprofen (IBU), 98% ; MW: 206.29; Lot: 026H1368; Sigma-Aldrich, USA
3. Cholesterol; MW: 386.66; Batch No.: C318; Allwest Scientific, Perth, WA
4. Fluorescein sodium salt (NaFl); MW: 376.27; Batch No.: 84325; Labchem, Azax Chemicals, Australia
5. Tween® 80; MW: 604.81; Batch No.: 064K0063; Sigma-Aldrich, USA
6. Sodium cholate hydrate, 99%; MW: 430.6; Lot: 104K0090; Sigma-Aldrich, USA
7. Sodium azide, not less than 99%; MW: 65.01; Batch No.:1001010; analytical grade; BDH Chemicals Ltd., England
8. Sodium sulphide; MW: 240.18; Batch No.: 312802; Ramprie Laboratories, WA
9. 0.2% w/w sulphuric acid, 98%; Batch No.: 04102038; Labscan Asia Co Ltd, Patumwan, Bangkok, Thailand
10. 2N hydrochloric acid, 32%; Batch No.: 0806328; Labserv, Biolab (Aust) Ltd, Clayton, Victoria, Australia
11. 30% Hydrogen peroxide; analytical grade; MW: 34.01; Batch No.: AF412330; Ajax Finechem, Australia
12. Sodium hydroxide; analytical grade; MW: 40.0; Batch No.: 06110161; Lab-Scan Analytical Sciences, Thailand.
13. Acetonitrile; HPLC grade; MW: 41.05; Lot: 096370; Fisher Scientific, Australia
14. Boric acid 99.5%; analytical grade; MW: 61.83; Batch No.: F1L048; Univar, Asia Pacific Specialty Chemicals, Australia
15. Potassium chloride; analytical grade; MW: 74.55; Batch No: 243524; Biolab (Aust) Ltd, Clayton, VIC
16. Orthophosphoric acid, 85%; analytical grade; MW 98.00; Code: 10173; BDH, Germany

17. Methanol, 99.9%; analytical grade; MW: 32.04; Lot No.: H43830; Mallinckrodt Chemicals, USA
18. Chloroform, 99.97%; MW: 119.38; Lot No.: 38243; EM Science, USA
19. Absolute alcohol; Batch No.: 201206; EX CSR Distilleries, Yarraville, VIC.
20. Water (deionised): passed through a milli 'Q' apparatus (Millipore Corporation, Bedford, MASS, USA) with an internal specific conductivity of 18 MΩ cm at 25°C

2.2. Equipment and Instrumentation

1. High Performance Liquid Chromatography (HPLC)

   An HPLC system (Shimadzu, USA) consisted of a pump (LC-20AT), auto sampler (SIL-20A), on line degasser (DGU-20A_5), equipped with a UV-Vis dual wavelength spectrophotometric detector (SPD-20A). Separation was achieved on a stainless steel C_{18} column (Apollo C_{18} 5 μm), 150 x 4.6 mm; Lot No.: 0604000194; Grace Davison Discovery Sciences, NSW
2. Zetasizer 3000HS; Malvern Instrument, UK
3. Vacuum rotary evaporator; Buchi Rotavapor R-200, Buchi Labortechnik AG, Switzerland
4. Fluorescent Spectrophotometer; Cary Eclipse, Varian Instruments; Walnut Creek, USA
5. Franz-type diffusion cells, customer-made, University of Queensland
6. Dermalab, Cortex Technology, Hadsund, Denmark.
7. Centrifuge; Eppendorf minispin AG 22331; Eppendorf, Germany
8. Nanosep® 300K Omega; Centrifugal devices, Lot: 09551484, PALL Life Sciences, USA
9. Dialysis tubing, high retention seamless cellulose tubing; MW 12000 cut off, average flat width 32 mm; Batch No.: 3110; Sigma-Aldrich, USA
10. pH meter; Microprocessor pH/mV/°C Meter, Model-8417N; Hanna Instruments, Singapore
11. Magnetic stirrer RW 20.n Ika labortechnik; Postfach, Staufen, Germany

12. An in-house purpose device for elasticity measurement; a sample holder made of 10 mL plastic syringe attached with a pressure gauge of 2.5 bar
13. Bath sonicator; Bransonic Ultrasonic cleaner 2510E-DTH; Branson Ultrasonic Corporation, USA
14. Probe sonicator, Microson™, ultrasonic cell disrupter, equipped with P-3 microprobe (3/32″, 2.4 mm diameter), output power: 40W, output frequency: 0.5 cycle, Farmingdale, New York, USA
15. Vortex mixer; Laboro Vor-mix, Scientific Equipment Manufacturers Pty. Ltd, Australia
16. Transmission Electron Microscope (TEM); JEOL 2100, equipped with an 11 Mpix digital camera capable of recording both images and diffraction data, operated at 120 kV; LaB6 filament; images saved using Gatan Digital micrograph software.
17. Field Emission Scanning Electron Microscope (FESEM); Leo Supra 55 Variable Pressure FESEM, Germany
18. Leica DC 100; Leica Microscopy Systems Ltd., Switzerland.
19. Oven (Memmert, Germany)
20. 0.1 µm Supor® Membrane, 25 mm, sterile, non-pyrogenic; Lot: 375147; Acrodisc® Syringe Filters; Pall Corporation, USA
21. 0.2 µm Supor® Membrane, 25 mm, sterile, non-pyrogenic; Lot: 375371; Acrodisc® Syringe Filters; Pall Corporation, USA
22. Polycarbonate 0.05 µm, 25 mm membrane filters; Batch No.: 256274, GE Water & Process Technologies
23. 0.02 µm, 10 mm, sterile, membrane filters; Batch No.: 09036A, Whatman International Ltd., England

2.3. Method

2.3.1. HPLC assay validation of Ibuprofen

2.3.1.1. Chromatographic conditions

IBU HPLC analysis method was adopted from a reported method with slight modification[191]. The drug elution was performed at ambient temperature at a flow rate of 1.2 mL/min and detected at the UV wavelength of 222 nm. The mobile phase used was 60% Acetonitrile and 40% phosphoric acid (50 mM, pH 3.0), which gave a retention time of 4.8 min for IBU and was run in an isocratic mode. The mobile phase was filtered through a 0.45 µm membrane filter prior to use. All samples were analyzed by HPLC using injection volumes of 20 µL. The HPLC assay of IBU was validated for selectivity, linearity, precision, recovery (accuracy), and sensitivity.

2.3.1.2. Preparing standard solutions

A stock solution of IBU was prepared by dissolving a known amount of IBU in methanol. Different concentrations of standard solution were prepared by proper dilutions of the stock with mobile phase (60% Acetonitrile, 40% phosphoric acid (50 mM, pH 3.0).

2.3.1.3. Selectivity

A forced degradation (stress testing) study described below[192] was performed to generate degradation products that were used to demonstrate specificity and selectivity of the method.

2.3.1.3.1. Preparation of stressed samples

Degradation products were generated by subjecting a standard solution of IBU (1 mg/mL) to four different stress conditions such as light (laboratory light, approximate 5660 flux), temperature (35±2ºC), acid hydrolysis (hydrochloric acid; 2M) and oxidation (hydrogen peroxide; 30%) . A stock solution (1 mg/mL IBU) was prepared in methanol. For acid and oxidation stresses, 20 µg/mL IBU was prepared by diluting stock solution with 2M hydrochloric acid, and 30% hydrogen peroxide respectively. For

temperature and light stress, dilutions were done with mobile phase. Samples were analyzed immediately after applying each stress to establish initial (t=0) level. Stressed samples were stored up to 3 weeks and were analyzed on suitable time interval. A standard solution (20µg/mL) of IBU and a blank were injected.

2.3.1.4. Linearity

The assay linearity was determined by using IBU standard solutions prepared from the mobile phase. Eight different standard concentrations of 0.5, 1, 2, 2.5, 5, 10, 15 and 20 µg/mL covering the entire range of expected entrapment and release study of IBU were prepared by diluting the IBU standard stock solution (1 mg/mL) with mobile phase and were injected directly into HPLC under conditions described in section 2.3.1.1. The calibration curve was constructed by plotting peak areas against different concentration of IBU standard solutions.

2.3.1.4.1. Linearity of Ibuprofen in release medium

The effect of release medium (Phosphate buffer solution; PBS 50 mM, pH 7.4 containing 0.05% sodium azide) on IBU detection in HPLC was performed by preparing a stock solution of IBU in release medium. Different standard concentrations were achieved by proper dilution of stock solution with mobile phase and release medium separately.

2.3.1.5. Precision

System precision was determined by analyzing six replicate injections of three different concentrations (0.5, 2.5 and 5.0 µg/mL) from the same standard solution.

Method precision, which measures the closeness of analytical results following a particular method, was determined by analysis of five independent standard solutions of three different concentrations (0.5, 2.5 and 5 µg/mL).

The variations in the results of both precisions were calculated in terms of percentage of relative standard deviation (% R.S.D.) of the determinations.

2.3.1.6. Intra- and inter-day repeatability

Intra-and inter-day repeatability were assessed at three concentrations (5, 2.5 and 0.5 µg/mL) covering specified range. All injections were carried out in triplicate. Intra-day repeatability was obtained by analyzing three sets of freshly prepared standards six times at different times in a day. The chromatographic peak areas were compared and the % R.S.D between measurements was estimated as a measure of intra-day repeatability.

Inter-day repeatability was measured as % R.S.D between analysis of the same three standards six times (stated above) on three consecutive days. The intra- and interday repeatabilities were assessed by the level of % of R.S.D obtained.

2.3.1.7. Recovery (Accuracy)

For determination of assay recovery, eight concentrations ranging between 0.5 and 20 µg/mL were analyzed by spiking an aliquot of empty liposome with a known amount of IBU. Spiked vesicles were disrupted with ethanol and all dilutions were done with mobile phase prior to HPLC analysis against a standard solution. The percentages of measured concentrations over added concentrations were calculated to determine the interference of liposome matrix on the IBU assay.

2.3.1.8. Sensitivity

Sensitivity of assay was determined by estimation of limit of detection (LOD) and limit of quantification (LOQ). A blank (methanol diluted with mobile phase) was injected 6 times on HPLC. The noise level was determined. LOD and LOQ were calculated based on 3.3 times and 10 times of noise level respectively using the calibration curve of peak height against IBU concentration.

2.3.2. Sodium fluorescein assay

2.3.2.1. Linearity

NaFl was analyzed by using the fluorescent spectrophotometer with an excitation wavelength of 494 nm and an emission wavelength of 513 nm. Different NaFl standard solutions were prepared from a stock solution of NaFl in water (at 1mg/mL) and diluting 5000 times with borate buffer pH 9. Linearity was performed by analysis of diluted standard solutions of NaFl. The calibration curve was constructed by plotting fluorescence intensity against concentration (ranging from 0.05 µg/mL to 0.30 µg/mL) of NaFl standard solutions.

2.3.2.2. Effect of light on sodium fluorescein fluorescence intensity

To determine the effect of light on NaFl fluorescence intensity, six different standard concentrations were left for a period of 24 h on bench top and fluorescence intensities were determined. The purpose of this experiment was to determine the extent of effect of light on sodium fluorescein fluorescence intensity under normal experimental conditions with various sample concentrations. The 24 hrs period covers the length of time used in release studies.

2.3.3. Solubility study of Ibuprofen

2.3.3.1 Determination of Ibuprofen solubility in water at room temperature

10 mg of IBU was dissolved in 0.5 mL of ethanol and was diluted with water up to 10 mL so that the final concentration of IBU in water is 1 mg/mL. As soon as water was added, IBU precipitated out and a milky white suspension formed, which was left for continuous stirring at room temperature overnight. This allowed most of the ethanol to evaporate from the system. After approximately 24 h, 1 mL of dispersion was taken out and was filtered through 0.45 µm filter and was diluted with mobile phase. This was injected into HPLC to determine the solubility of IBU in water.

2.3.3.2. Determination of Ibuprofen solubility in 50mM PBS pH 7.4 at room temperature

IBU is not readily soluble in PBS. Stirring and temperature affects its solubility. Again the solubility depends on the molarity of PBS. The purpose of the mentioned work was to determine the solubility of IBU in PBS 50 mM pH 7.4 at room temperature at various time intervals. An excess amount of IBU was added to 10 mL of 50 mM PBS pH 7.4 until the buffer was saturated. After shaking this IBU-PBS system manually for 5 min, 1 mL of the dispersion was withdrawn and filtered through 0.45 µ filter. This was diluted with mobile phase prior to injection into HPLC. The IBU-PBS dispersion was left for 2 days at room temperature with continuous stirring to favor further solubility and 1 mL was withdrawn after 0.1, 8, 24, and 48 h to determine the solubility of IBU in PBS pH 7.4. The IBU-PBS dispersion was used as the system was saturated with excess amount of IBU to permit the solubility study.

2.3.4. Stability of Ibuprofen and sodium fluorescein in release medium

Stability of IBU and NaFl in release medium (50 mM PBS pH 7.4, containing 0.05% sodium azide) was determined by keeping a standard solution of IBU and NaFl (1 mg/mL) in release medium for a period of 7 days at 35±2ºC. Triplicate samples of 10 and 5µg/mL of IBU and 0.2 and 0.1µg/mL of NaFl were prepared by dilution with mobile phase and borate buffer pH 9 respectively and were analyzed immediately after preparation as well as on day 0, 3, and 7. Analysis was performed according to conditions described under section 2.3.1.1 and 2.3.2.1. IBU potency was calculated as a percentage of IBU peak area over the total area of IBU and its related peaks.

2.3.5. Liposome preparation

2.3.5.1. Preparation of conventional liposomes

2.3.5.1.1. Film formation

Conventional liposomes were prepared by thin film hydration technique described by Bangham *et al*.[58]. In a typical procedure, the lipid phase consisting of PC (70-100 mg)

and cholesterol (0-30 mg) were dissolved in 10 mL of organic solvent (chloroform: methanol; 3:1v/v) and the mixture was introduced into a 250 mL of clean, dry, round bottom flask. The flask was then attached to a rotary evaporator and immersed in a temperature controlled water bath. The solvent was slowly removed with reduced pressure at above T_c of phospholipid (around 42ºC for 95% PC and 50-55ºC for cholesterol containing vesicles) with 100 rpm stirring speed. The drying process was continued for 10 min or more until the dry lipid film first appeared. The flask was then removed from the rotary evaporator and placed under vacuum in a vacuum oven overnight at ambient temperature to remove the final traces of organic solvent.

2.3.5.1.2. Film hydration & drug loading

Deposited lipid film was hydrated with 10 mL of aqueous hydration medium above T_c (around 42ºC for 95% PC and 50-55ºC for cholesterol containing vesicles) for 1 h with 100 rpm stirring speed using the rotary evaporator. Vesicles produced by thin film hydration method are multilamellar as shown in the literature[58]. IBU or NaFl was loaded into the vesicles via two different hydration media: IBU was dissolved in PBS pH 7.4, while NaFl was dissolved in water and both were added to the lipid film as hydration medium. In all liposomal formulations, the final total concentration of lipid system (PC/cholesterol/surfactants) was 10 mg/mL and IBU or NaFl concentration was 1mg/mL. All liposomes contained a total of 100 mg lipid or lipid phase (PC/cholesterol/surfactants; depending on the type of formulation) and 10 mg IBU or NaFl in 10 mL hydration medium. The resulting vesicles were swollen for 2 h at room temperature to get large MLVs, which were then sonicated using a bath sonicator for 30 min below room temperature followed by probe sonication for 1 min at 4±2°C, with energy output 40W, and 0.4 cycles to produce smaller vesicles. Finally, obtained liposomes were extruded ten times through 200 and 100 nm Supor® membrane filters (each). All samples were immediately flushed with nitrogen in a 10 mL glass vial and then capped with a plastic closure and stored in darkness at a temperature of 4±2°C.

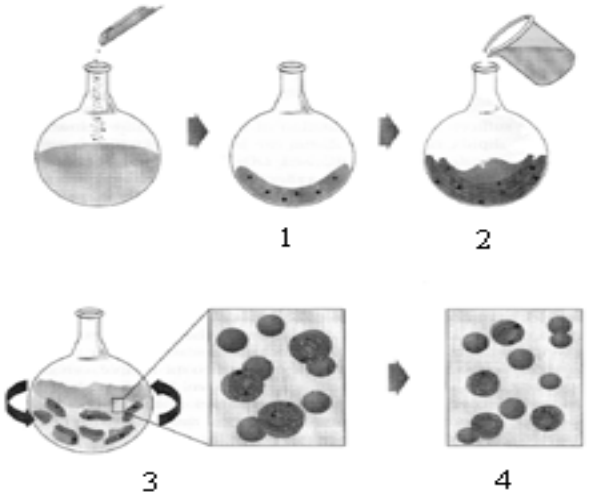


Figure 2.1: A representative diagram of liposome formation by Thin film hydration technique: 1: Formation of lipid film; 2: Addition of drug/probe solution as hydration medium; 3: Rotation using rotary evaporator; 4 MLVs which were downsized (Figure adapted from Lasic[193])

2.3.5.2. Preparation of elastic liposomes

2.3.5.2.1. Film formation

Elastic liposomes were prepared by thin film hydration technique described by Cevc *et al.*[194]. In brief, the lipid phase composed of PC (75-95 mg) and surfactant (5-25 mg) were dissolved in 10 mL of an organic solvent (chloroform: methanol; 3:1 v/v) in a 250 mL of clean, dry, round bottom flask. The solvent was then removed by rotary evaporator at above T_c of lipid (43°C) with 100 rpm stirring speed until a thin lipid film formed at the flask. Complete removal of residual solvent from the resulting film was performed by removing the flask from the rotary evaporator and keeping under vacuum in a vacuum oven overnight at ambient temperature.

2.3.5.2.2. Film hydration & drug loading

Resultant lipid film was hydrated with 9.3 mL of IBU or NaFl solution in PBS or water respectively at above T_c (around 42ºC) for 1 h with 100 rpm stirring speed. The flask was then left to cool down to room temperature. 0.7 mL of absolute alcohol was added

separately and dropwise to the liposomal dispersion with continuous stirring and manual shaking for approximately 5 min. Thus a total 10 mL of hydration medium was added. The ultimate hydration medium for elastic liposomes was a hydroalcoholic mixture containing 7% ethanol. IBU or NaFl was loaded into the vesicles in two different ways: IBU was dissolved in PBS pH 7.4 (1.075 mg/mL), while NaFl was dissolved in water (1.075 mg/mL). The volume of 9.3 mL of each of these solutions was used as hydration medium. For complete swelling, vesicles were left at room temperature for 2 h and were then sonicated with a bath sonicator for 30 min and probe sonicated for 1 min at 4°C, 40W, and 0.4 Hz. Liposomes were then finally extruded ten times through 200 nm Supor® membrane filters followed by ten times extrusion through 100 nm Supor® membrane filters.

2.3.5.3. Preparation of empty vesicles

Empty conventional and elastic liposomes were prepared and processed using the same method described above with hydration medium of water or PBS pH 7.4. All empty liposomes contained 10 mg/mL of lipid.

2.3.6. Physicochemical characterization of conventional and elastic liposomes

2.3.6.1. Determination of liposome size and size distribution

Liposome size was measured by photon correlation spectroscopy using a Zetasizer 3000HS. Briefly, liposomal dispersion was suitably diluted with hydration medium at 25±2°C, Samples were placed in quartz cells and were analyzed at 25±0.1°C and at a detection angle of 90°. Size of liposomes was expressed as average diameter (z-average) that was obtained by cumulative analysis performed by the Zetasizer 3000HS Software. The experiments were performed in triplicate and each sample was analyzed ten times to obtain mean diameter and polydispersity index[195]. For each formulation, the mean ±SD of three replicate determinations were used to express the result.

2.3.6.2. Determination of liposome zeta potential

To determine the type of surface charge on liposomal particles, zeta potential of liposomes was measured using Zetasizer with a zeta mode based on the Laser Doppler Anemometry principle. Samples were diluted with hydration medium and measurements were performed in triplicate. For each sample, the mean± SD of three determinations was repeated.

2.3.6.3. Liposome morphology

Liposomes were visualized by using a Jeol 2100 TEM with an accelerating voltage of 120 kV. Prior to TEM, a fine drop of liposome suspension was placed onto a carbon-coated copper grid to form a thin film. The grid was left at room temperature to allow the evaporation of moisture. The grids were then placed in the sample inlet chamber of TEM and observed. Crude and empty liposomes were also visualized. A series of micrographs were taken without the overlap of areas.

Morphological evaluation of liposomes was also performed using FESEM and optical microscope. For FESEM, one drop of liposomes was mounted on a carbon coated stub. Excess sample was removed carefully. A Cressington sputter-coater (208HR) was used to coat the samples with chromium. Samples were then examined under FESEM at an accelerating voltage of 1 kV. For examination under optical microscope, 2/3 drops of liposomes were spread on a glass slide and was covered with a cover slip before observed under the microscope (Leica DC 100).

2.3.6.4. Determination of entrapment efficiency

The EE of liposome was measured indirectly by determining the amount of non-entrapped IBU or NaFl in liposome dispersion by using centrifugation technique[196]. Briefly, an aliquot of liposomal dispersion, stored at 4±2°C, was placed in the upper chamber of a Nanosep® (300K Omega) which was then centrifuged at 4000 rpm for 40 min to separate the liquid (supernatant, which contains the un-entrapped IBU or NaFl). The liquid containing free NaFl or IBU was collected in the receptor chamber of the Nanosep® and their concentration was then determined by using Fluorescent

Spectrophotometer and HPLC respectively. EE was calculated from the equation below:

$$EE\ (\%) = \frac{W_{initial}-W_{free}}{W_{initial}} \times 100 \quad \text{.....(Equation1)}$$

Where, $W_{initial}$ is the total amount of NaFl or IBU used in the formulation

W_{free} is the amount of free NaFl or IBU found in the supernatant

2.3.6.5. Determination of bilayer elasticity

The elasticity of different formulations was measured by an in-house purpose built device, based on the description given in reports [136,197]. A schematic diagram of this device is presented in Figure 2.2 and a picture of device set-up is presented in Appendix 1. All elastic formulations were placed in a 10 mL sample holder which was attached to a stainless steel case containing a 0.05µm membrane filter with 25 mm diameter. The filtration was performed with the application of an external pressure of 2.0 bar. The volume of vesicle suspension which was extruded within 5 min was measured and vesicle size was monitored before and after extrusion. The experiment was performed in triplicate for each sample and duplicate formulations were analyzed.

According to van den Bergh *et al*, the elasticity of vesicles was expressed in terms of deformability index by the following formula[198]:

$$D = J^* (r_v/r_p)^2 \quad \text{.....(Equation 2)}$$

Where,

D= Deformability index

J= volume of suspension (mL) which was extruded within 5 min

r_v= Size of vesicles (nm) (after extrusion)

r_p= pore size (nm) of the filter

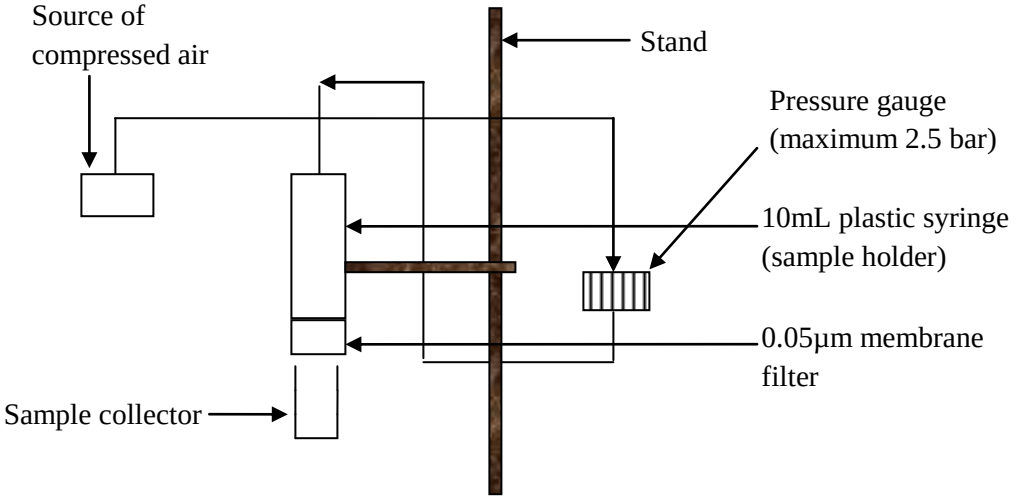


Figure 2.2: A typical diagram of the set up of extrusion device

2.3.7. Stability of liposomes under different storage conditions

The effect of storage condition on the size of NaFl loaded conventional liposomes was studied by keeping liposomes at three different temperature conditions i.e., 4±2°C (refrigerator), 25±2°C (room temperature), 35±2°C (hot room) for a period of 4 weeks. All liposomal suspensions were kept in sealed 10 mL vials after flushing with nitrogen. Samples were withdrawn periodically and the sizes were analyzed by Zetasizer.

2.3.8. In vitro release study of liposomes

Release study of liposomes was carried out using a dialysis method[199]. The details are described below.

2.3.8.1. Treatment of dialysis membrane

The dialysis membrane was first washed with running water for 3-4 h to remove the glycerin. This was then immersed in 0.3% w/v of sodium sulphide at 80°C for 1 minute to remove the sulphur. Sodium sulphide was then removed by washing the membranes with water at 60°C for 2 min followed by acidification with 0.2% v/v sulphuric acid.

The membranes were finally rinsed with hot water at 60°C to remove the acid and were stored in Milli 'Q' water at 4°C until used. The water was replaced with fresh water daily to minimize the risk of microbial growth.

2.3.8.2. Separation of non-entrapped sodium fluorescein or Ibuprofen from liposomes

In vitro release studies of NaFl and IBU liposomes were conducted by dialysis technique. In the present study, the dialysis tube with average flat width of 32 mm and molecule weight cut-off of 12,000 Da was used. Three optimized formulations containing NaFl and IBU along with their respective controls were chosen for this study. The effect of cholesterol on IBU release from conventional liposomes was also investigated. Free drug in each formulation was removed prior to release study by an initial 80 min dialysis. To do this, 8 mL of liposome was placed in the dialysis bag, which was fully immersed in 1 L of PBS pH 7.4 and was dialyzed for 80 min at room temperature to remove non-entrapped drug. Samples were taken from the receiver compartment (50 mM PBS pH 7.4) at predetermined time intervals and were analyzed for their NaFl or IBU content. A control drug solution (1 mg/mL) was also dialyzed in the same manner to ensure that complete removal of free drug was at around 80 min. After 80 min, purified liposomes were taken out for release study.

2.3.8.3. Determination of release rate of sodium fluorescein or Ibuprofen

As soon as free NaFl or IBU was separated, purified liposome dispersions containing approximately 1 mg NaFl or IBU were then transferred to a separate dialysis bag and immersed in a 50 mL release medium (PBS pH 7.4 with 0.05% sodium azide) in a 100 mL closed jar and was immediately transferred to 35±2ºC for release study as per set up in Figure 2.3. Sodium azide was added in the release medium to minimize the growth of microbes. Both ends of the dialysis membrane were tightly bound with threads. Control bags were prepared and tested along with the liposomal dispersions. Each control bag contained 1 mg/mL NaFl aqueous solution or 1 mg/mL IBU in PBS pH 7.4. The jar was kept on a magnetic stirrer with constant stirring speed. A solution of 1 mL was collected at 0.5, 1, 2, 3, 5, 7, 10, 12, 15, 20 and 24 h and replaced with fresh 1 mL release medium

to maintain sink condition. The release study was conducted in triplicate for each optimized formulation and controls. NaFl release was assayed spectrophotometrically at an excitation wavelength of 494 nm and an emission wavelength of 513 nm. IBU release was analyzed using HPLC assay method described in section 2.3.1.1.

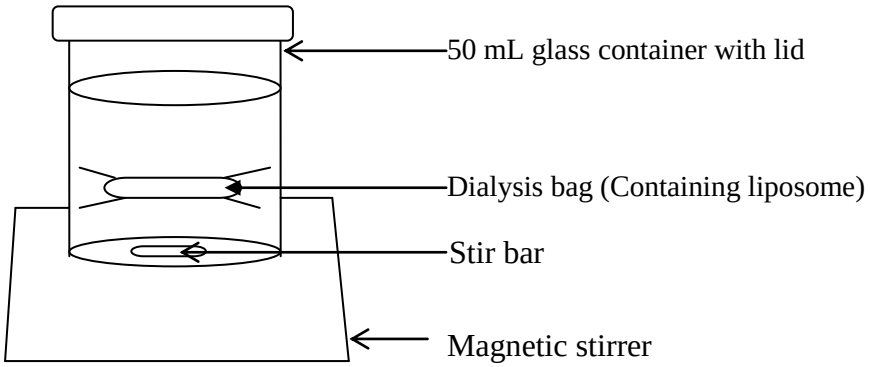


Figure 2.3: A typical diagram showing the set up of in vitro release study by dialysis method

2.3.9. In vitro permeation of Ibuprofen through human epidermis

2.3.9.1. Treatment of skin

Excised human skin from patients of Perth hospitals who had undergone abdominal plastic surgery was used under Curtin approved ethics protocol (No.HR 129/2008). Freshly obtained skin was wrapped in aluminum foil and stored in polyethylene bags at −20°C until use. This condition is sufficient for the skin to be stable for 3 months with regard to the penetration of drugs, as well as maintaining the thickness of the SC over a period of 6 months[200, 201, 202]. The skin was carefully checked through a magnifying glass to ensure that samples were free from any surface irregularities such as tiny holes or cervices.

2.3.9.2. Preparation of epidermis

The human epidermis was prepared by a heat separation technique[203, 204]. Prior to penetration study, whole skin was thawed and cut into suitable pieces. Each skin piece was soaked in water at 60°C for 1 min followed by careful separation of the epidermis from dermis using a scalpel. The obtained epidermal pieces of tissues were wrapped individually in parafilm in an aluminum foil and stored at a temperature of -20ºC until used. For penetration experiments, thawed epidermis was cut into small disks, cleaned, and then transferred onto the Franz diffusion cells.

2.3.9.3. Franz diffusion cell

The Figure 2.4 describes the setup of the Franz diffusion cell. The epidermal membrane was clamped between the donor and receptor compartments of the cell with nominal diffusion area of 1.13 cm^2 and a receptor compartment of a 3 mL capacity. The receptor was filled with PBS (50 mM, pH 7.4) which was constantly stirred by magnetic stirrer at 100 rpm. The epidermal side of the skin was exposed to ambient conditions while the dermal side was bathed by receiver. The receptor fluid was maintained at 35±1°C using a re-circulating water bath in order to ensure skin temperature. All bubbles were carefully removed between the underside of the skin and solution in the receiver compartment. To achieve higher reproducibility, the skin was pre-hydrated with PBS for 45 min before it was used for penetration study. A minimum of four diffusion cells were used for control and each optimized formulation.

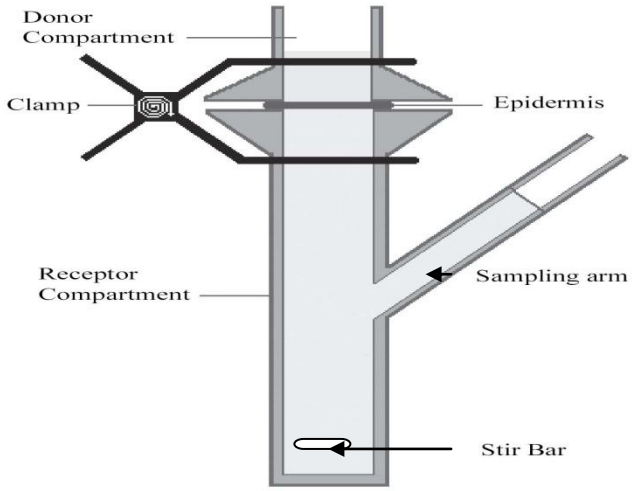


Figure 2.4: A typical representation of vertical Franz type diffusion cell with the skin harvested[205]

2.3.9.4. Liposome dosage regime and sampling time

A volume of 0.5 mL (at 1 mg/mL of IBU) of different liposomes (conventional, elastic liposomes) and plain drug solution containing IBU were applied non-occlusively to the epidermal surface of skin mounted in Franz type diffusion cells. Samples were withdrawn through the sampling port of the cell at predetermined time intervals over a period of 12 h and were analyzed. Receptor phase was immediately replenished with equal volume of fresh PBS. After 12 h, the experimental set up was dismantled and samples were removed from receptor compartments and analyzed by HPLC. The amount of IBU retained in the skin was determined by extraction of skin at the end of the experiment.

2.3.9.5. Extraction of Ibuprofen from epidermis

At the end of permeation experiment, the epidermal pieces were carefully removed. Each epidermal piece was washed three times with PBS pH 7.4 to remove any surface bound drug. 0.5 mL of methanol was added to each piece, which was vigorously vortexed for 2 min every 10 min over a period of 30 min. The epidermis was removed and another 0.5 mL of methanol was added to it. The same process was repeated. After

1 h, drug content in both methanol extractions were analyzed separately by suitable dilution with the mobile phase. However a 3rd extraction was applied by adding 0.5 mL of mobile phase to each epidermal piece followed by vortexing for 2 min every 30 min over a period of 2 h to ensure complete extraction of IBU. The total drug content in epidermis was calculated from the three extractions.

2.3.9.6. Calculation of permeation parameters

The cumulative amount of drug permeated per unit area was plotted as a function of time. The steady-state permeation rate or the flux ($J_{ss,}$ µg/cm^2/h) was calculated using the slope of the straight line portion of the curve i.e. cumulative amounts of the drug that permeated the membrane vs. time period of the experiment. Permeability Coefficient (*K*) and enhancement ratio (ER) were calculated from the following equations respectively.

$$\text{Permeability Coefficient } (K) = \frac{\text{Flux of individual Franz cell}}{\text{Concentration of drug in donor}} \ldots\ldots\ldots \text{Equation 3}$$

$$\text{ER} = \frac{\text{Transdermal flux from vesicular formulation}}{\text{Transdermal flux from plain drug}} \ldots\ldots\ldots \text{Equation 4}$$

2.3.10. Data analysis

Statistical analysis was carried out employing the Student's t-test using the software PRISM (Graph Pad). The mean of at least 3 experiments was taken together with the standard deviation (SD) for all data representation unless otherwise stated. In order to compare the skin penetration data in different samples, a linear mixed regression model was fitted. All data presented in skin penetration study were a mean± standard error (S.E) of 4-8 measurements. A value of $p<0.005$ was considered statistically significant.

RESULTS AND DISCUSSION

3.1. Validation of analytical method

3.1.1. HPLC assay validation of Ibuprofen

IBU assay was performed from a stock solution of IBU in methanol and appropriate dilution with mobile phase to prepare a range of standard concentrations (0.5-20 µg/mL) of IBU.

3.1.1.1. Selectivity

The purpose of doing specificity and selectivity of analytical assay is to ascertain that the analyte can be analyzed unequivocally in the presence of other expected components. Analytical method validation should be specific and selective, therefore be able to detect quantitative changes in drug substance over time. To achieve this, a forced degradation study was conducted to allow that IBU degraded under acid and oxidation stress. Typical HPLC chromatograms of IBU and its acid and oxidation degradation products are illustrated in Figure 3.1 showing a complete separation of IBU from its degradation products. The retention time of IBU, and its acid and oxidation degradation products were approximately 4.8 (Figure 3.1 (a)), 12.36 (Figure 3.1 (c)) and 5.73 min (Figure (d)) respectively. No degradation products were found for light and temperature stressed samples within the specified time period. Typical chromatograms of temperature and light stressed samples are shown in Appendix 2A and 2B respectively. For comparison purpose, a blank of empty liposome (PC: cholesterol 70:30) (Figure 3.1 (b)), 30% hydrogen peroxide in mobile phase (Appendix 2C) and 2M HCl in mobile phase (Appendix 2D) were injected separately into HPLC, where no detectable peak was found at the retention time of IBU. Additionally a known amount of IBU was spiked with empty liposome to determine the selectivity of the method. A typical chromatogram is also attached in Appendix 2E.

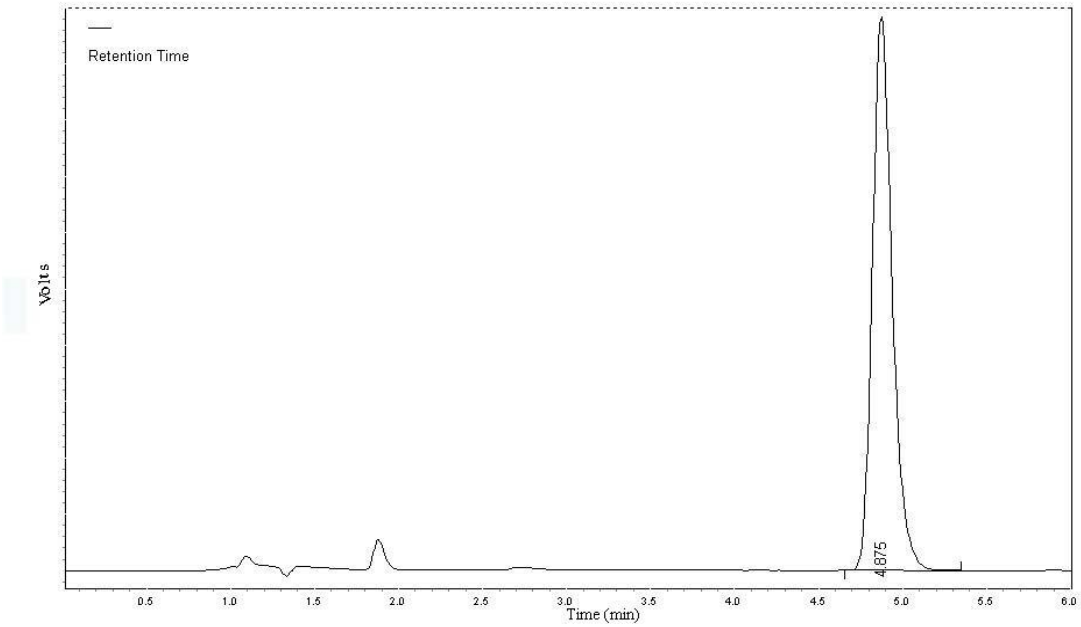


Figure 3.1(a): A typical HPLC chromatogram of 10 µg/mL of IBU in methanol as a standard

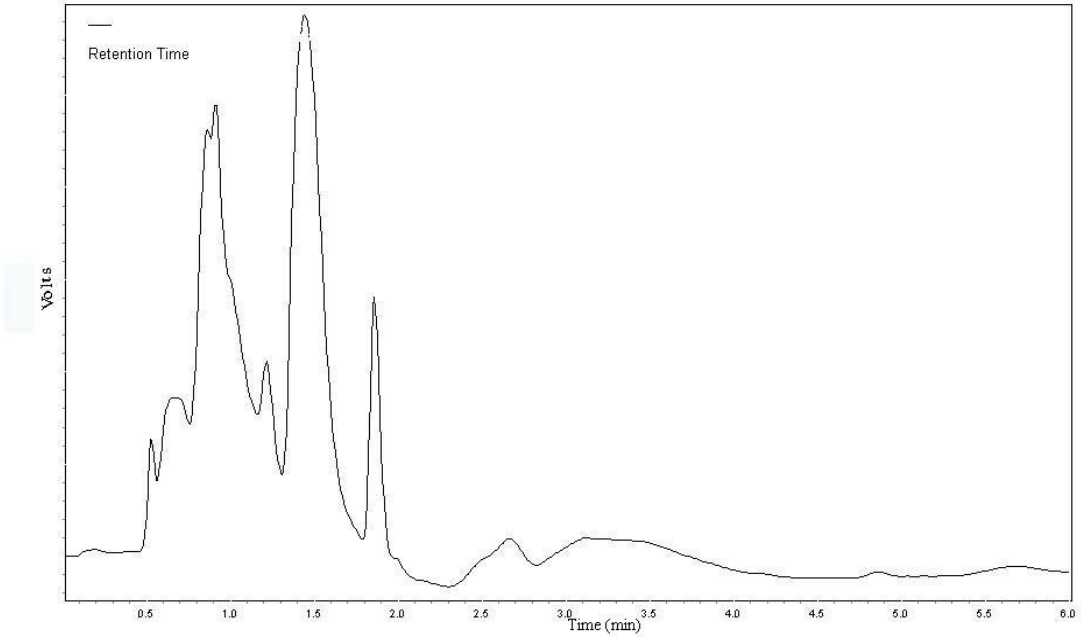


Figure 3.1 (b): A typical HPLC chromatogram of empty liposome (PC: cholesterol 70:30) as a blank

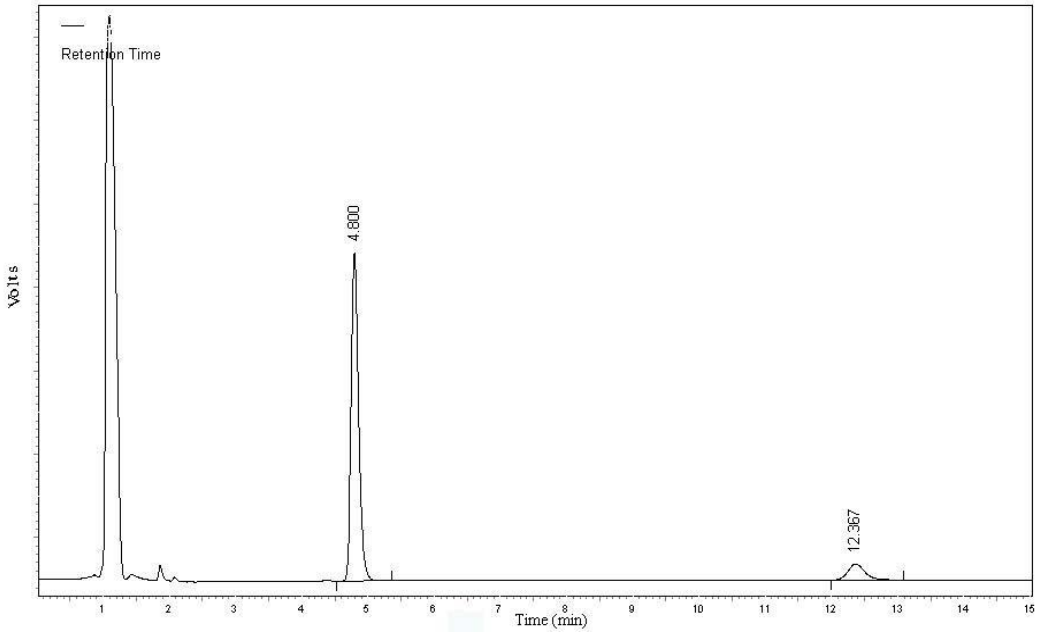


Figure 3.1(c): A typical HPLC chromatogram of IBU (retention time 4.8 min) and its acid stressed degradation product (retention time 12.4 min)

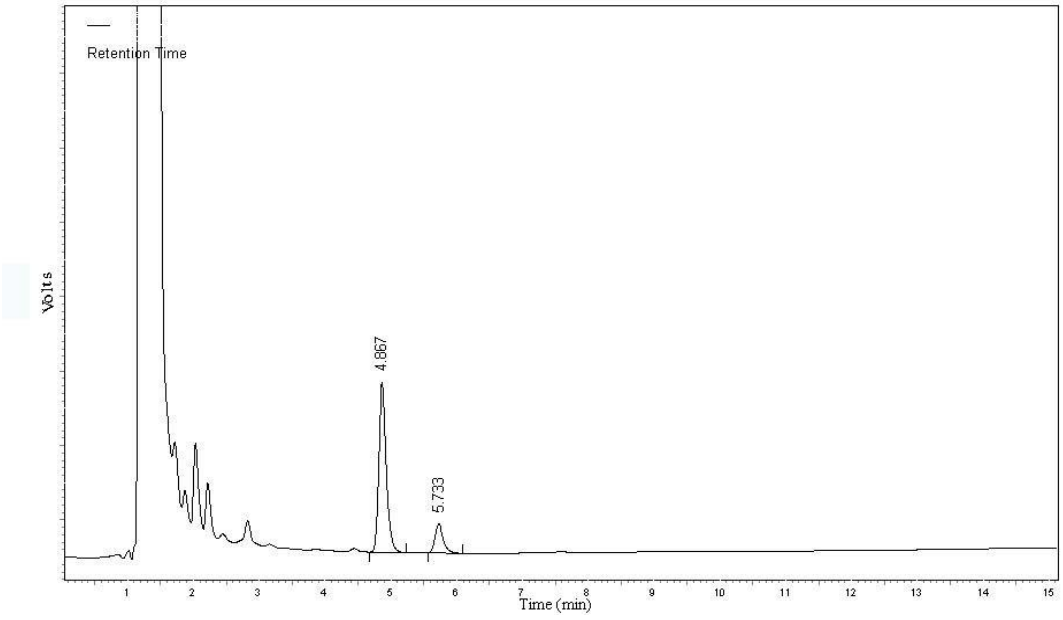


Figure 3.1(d): A typical HPLC chromatogram of IBU (retention time 4.9 min) and its oxidation stressed degradation product (retention time 5.7 min)

Thus it can be concluded that the assay method for IBU detection was selective and suitable for analysis of IBU in liposomes.

3.1.1.2. Linearity

Linearity study establishes the concentration range of analyte where the detector response was linearly proportional to analyte concentration. For 20 µl injection of IBU, the regression plot showed a good linear relationship over the concentration range of 0.5-20 µg/mL of IBU in mobile phase which covered the concentrations encountered in the analysis of IBU loading and release study. The correlation coefficient was 0.9999. The typical standard curve is shown in the Figure 3.2. Linearity (quoted as R^2) was evaluated by linear regression analysis, which was calculated by the least square linear regression method.

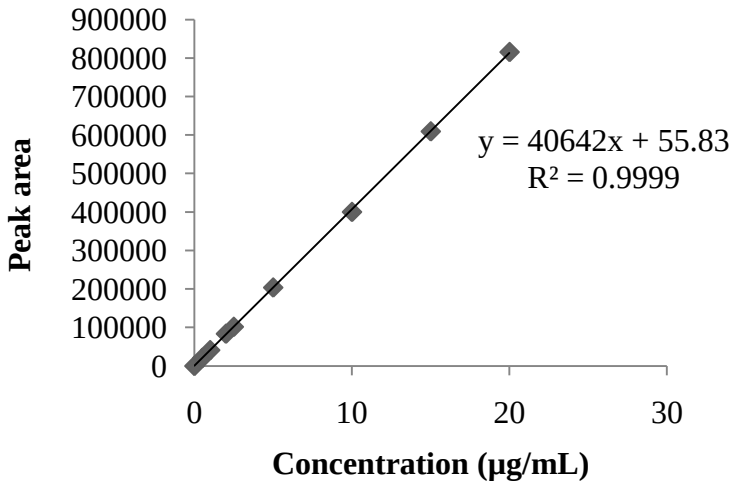


Figure 3.2: Standard curve for IBU in mobile phase, 20µL injection volume

3.1.1.2.1. Linearity of Ibuprofen in release medium

To investigate the retention time and area of IBU in release medium (50 mM PBS pH 7.4 containing 0.05% sodium azide) a stock solution of IBU was prepared in release medium. When different standard concentrations of IBU in PBS were injected into HPLC, data showed no significant difference in both peak area and retention time compared to those prepared in methanol and diluted with mobile phase. Therefore no separate calibration curve was prepared in release medium.

3.1.1.3. Precision

"The precision of an analytical method expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same sample under the prescribed conditions."[206]. Precision validation was performed for both system and method precision. The results of system precisions of the assay method are shown in Table. 3.1. The R.S.D. values found for 6 injections of 5, 2.5 and 0.5 µg/mL of IBU in mobile phase are 1.09, 0.05 and 0.27% respectively. For method precision, the R.S.D. values obtained for 5 preparations of the same three concentrations were 1.77, 1.54 and 1.78% respectively (Table 3.1). In both cases, the R.S.D. values were below the nominal acceptable level of 2.0% which indicates that the HPLC analytical method for IBU is precise. Detailed data of system and method precision are given in Appendix 3A and 3B.

Table 3.1: System precision and method precision of the HPLC method for determination of IBU

Concentration (µg/mL)	System precision R.S.D.[A](%)	Method precision R.S.D.[B](%)
5	1.09	1.77
2.5	0.05	1.54
0.5	0.27	1.78

[A] six replicate injections of each sample, [B] five replicate injections of each sample

3.1.1.4. Intra- and inter-day repeatability

Intra-and inter-day repeatability is assessed as the degree of reproducibility of the method when samples are analyzed on the same day or over a period of time. The analyses of assay variation at intra-and inter-days are summarized in Table 3.2. The highest value of % of R.S.D. analysis was found to be 0.16 (0.5 µg/mL) for intra-day measurements, while it was found to be 1.14 (5 µg/mL) for inter-day measurements. Both the intra-day and inter-day R.S.D. of IBU were below 2.0%, within the nominal acceptable level which indicates a considerable degree of precision and reproducibility of the assay method.

Table 3.2: Intra- and inter-day variations of IBU assay

Concentration[a] (µg/mL)	Intra-day R.S.D.[b](%)	Inter-day R.S.D.[c](%)
5	0.13	1.14
2.5	0.13	0.57
0.5	0.16	0.28

[a] n=3, at each concentration, triplicate samples were prepared and analyzed by HPLC

[b] Analyses of samples were performed at 0, 3 and 6 h in the same day and all data were included in the calculation of R.S.D.

[c] Analyses of samples were performed at 0, 1, and 2 day and all data were collected in the calculation of R.S.D.

3.1.1.5. Recovery (Accuracy)

Recovery is a measure of the extent of analyte recovered from the test matrix so as to assess the errors or loss occurred in extraction or in analysis in the experiment[206]. In another words, it is the closeness between experimental and true value[207]. Recovery studies were performed by spiking the empty liposomes with a known amount of IBU and the extent recovered in analysis was translated to percent recovered. The data is shown in Table 3.3. The average recovery of known amount of IBU was 99.33% with a R.S.D. of 1.25% over the concentration range of 0.5-20 µg/mL. The 95% confidence interval (CI) of the slope ranged from -0.10257343 to 0.2215060. Results indicate that IBU can be considered fully recovered in the presence of vesicle components and the assay is unbiased.

Table 3.3: Recovery of IBU[a] in lipid matrix

Prepared concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (%)
0.50	0.48	96.40
1.00	0.99	98.95
2.00	2.00	99.85
2.50	2.51	100.40
5.01	5.01	100.00
10.02	9.97	99.50
15.03	15.02	99.93
20.05	19.98	99.65
Mean ±S.D.		99.33±1.25
Regression Analysis		
Test		Result
Slope		0.059
Intercept		98.91
Correlation coefficient		0.1185
95% CI Slope		-0.10257343 to 0.2215060
95% CI Y-intercept		97.3334 to 100.5023

[a] n=3

3.1.1.6. Sensitivity

The sensitivity of HPLC method is determined by LOD and LOQ. LOD is the lowest concentration of the analyte the method can detect and LOQ is the lowest concentration that can be quantified by the method with accuracy and precision. For IBU, the LOD and LOQ determined in mobile phase were 2.7ng/mL and 8.2 ng/mL respectively with injection volume of 20µL.

3.1.2. Linearity of sodium fluorescein

Fluorescence analysis of NaFl was performed in fluorometer with an excitation and emission wavelength of 494 nm and 513 nm respectively[208]. A stock solution of NaFl was prepared in water; however different NaFl standard concentrations were prepared by dilution with water and borate buffer pH 9 respectively. The fluorescence profile of NaFl in water was found not reproducible and markedly smaller than that of in buffer (Figure 3.3(a) and (b)). For example, the average fluorescence intensities of NaFl at 0.25µg/mL in water and borate buffer pH 9 were 155.58 and 834.96 respectively. This can be explained by the fact that the fluorescence of NaFl is highly pH dependent[209]; in aqueous solution NaFl can exist in 4 different monomeric forms over the pH range of 0-13. Each form has its own absorption values as the absorption spectrum of these aqueous solutions are pH dependent. The protonation of the molecule results in a reduction of efficacy of the mechanism by which absorbed energy is converted into fluorescence emission, i.e. a pH associated quenching. Therefore in an acidic pH, the fluorescence is expected to be lower[210]. Another research group, Zhu *et al* found that the higher the pH the higher was the fluorescence of NaFl and maximum fluorescence was observed when the pH was beyond 8.4[211]. They found that the fluorescence intensity of NaFl increased 1.25 times when the solution pH value increased from 6.9 to 8.4. Considering our data and supportive literature findings, in this study, a separate linearity was performed in borate buffer pH 9 (Figure 3.3(b)). A stock solution of NaFl (1mg/mL) was prepared in water and different standard concentrations were prepared by diluting with borate buffer. The fluorescence intensity of NaFl was linear over a narrow concentration range (0.05µg/mL-0.30µg/mL). Concentration above 0.3µg/mL was not in the detectable range.

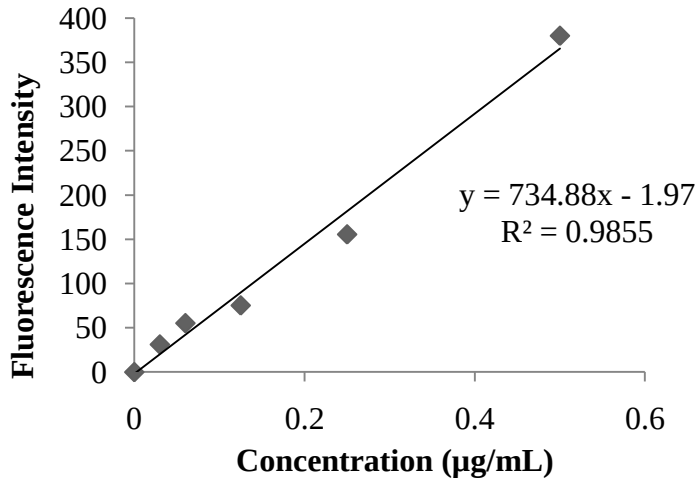


Figure 3.3(a): Fluorescence profile exhibited by NaFl standards (dilution with water; pH approx. 5.4), data represents as mean ±SD, n=3

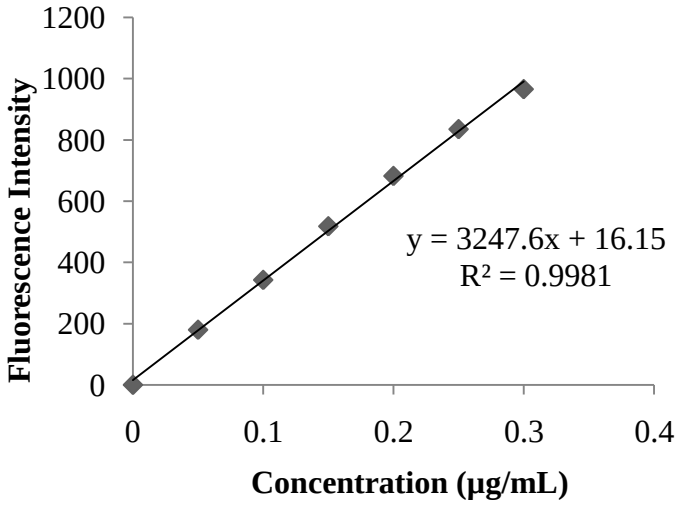


Figure 3.3(b): Linear fluorescence profile exhibited by NaFl standards (dilution with borate buffer pH 9), data represents as mean ±SD, n=3

3.1.2.1. Effect of light on sodium fluorescein fluorescence intensity

NaFl is not stable under light and may undergo photoquenching. A clear reduction in fluorescence intensity was observed when different standard solutions of NaFl were stored on bench top for a period of 24 h. Table 3.4 shows the % of decrease in fluorescence intensity of NaFl solutions stored on bench top without protection from light.

Table 3.4: % of decrease in fluorescence intensity of NaFl on bench top without light protection over 24 h at ambient temperature

Concentration (µg/mL)	Fluorescence intensity: Day 0	Fluorescence intensity: Day 1	% Decrease in Fluorescence intensity
0.3	965.66	823.91	14.67
0.2	682.13	632.76	14.47
0.1	342.65	277.57	18.99

Therefore in this study, all NaFl samples were covered with foil to protect them from photoquenching.

3.2. Determination of solubility (saturation concentration) of Ibuprofen

3.2.1. Determination of solubility of Ibuprofen in water at room temperature

A saturated solution of IBU in water was prepared at room temperature described under section 2.3.3.1. After 24 h, the solubility was determined and found to be 56.0 µg/mL, which is in a close agreement with that (60µg/mL) obtained by AR Mohammed[212].

3.2.2. Determination of solubility of Ibuprofen in 50 mM PBS pH 7.4 at room temperature

The saturated solution of IBU was prepared at 25°C by dissolving an excess amount of drug in 50 mM PBS pH 7.4. This solution was kept at continuous stirring for a period of 48 h and an aliquot was withdrawn at 0, 8, 24 and 48 h, filtered and analyzed for drug content by HPLC. The solubility of IBU in PBS pH 7.4 at 0.1, 8, 24 and 48 h were found to be 2.58, 4.37, 4.41 and 4.45 mg/mL respectively. Al-Saidan reported the solubility of 10.4±0.9 mg/mL for IBU in disodium hydrogen phosphate solution (100 mM) under the similar condition. He also showed that with the increase in molarity of disodium hydrogen phosphate solution, the solubility increased; maximum solubility (37.5±1.1 mg/mL) was found with 400 mM buffer[213].

Therefore it can be concluded that the solubility of IBU (4.45mg/mL) determined in current study with 50mM PBS pH 7.4 was reasonable and expected.

Solubility of IBU in both water and PBS were determined to know its solubility profile in respective hydration medium and also to maintain a sink condition in the in-vitro drug release study.

3.3. Stability of Ibuprofen and sodium fluorescein in release medium

IBU stability in the release medium was evaluated at 35±2ºC using HPLC method described under section 2.3.1.1. Neither any degradation product nor any significant change in area was found for two different IBU standard concentrations (10 and 5µg/mL) after 7 days storage at 35±2ºC.

Different standard solutions of NaFl (0.2 and 0.1µg/mL) in release medium stored at 35±2ºC for a period of 7 days did not show any change or decrease in fluorescence intensity (Table 3.5) provided that they were stored in amber glass containers.

Table 3.5: Stability of IBU and NaFl in release medium

Day	Potency (%)			
	IBU Concentration (µg/mL)		NaFl Concentration(µg/mL)	
	10	5	0.2	0.1
0	100.0	100.0	100.0	100.0
3	100.0	99.80	99.81	99.79
7	99.89	99.30	99.85	99.69

These data suggest that both IBU and NaFl should maintain their stability over the period of our release and skin penetration study. Because of the light sensitivity issue with NaFl, all samples containing NaFl were handled either in the dark or with amber glass container to prevent the photoquenching.

3.4. Preparation and characterization of liposomes

The purpose of this study was to develop and optimize different conventional and elastic liposomes on the basis of vesicle shape, size, zeta potential, EE and elasticity for dermal delivery using a water soluble dye NaFl and a model lipophilic drug, IBU. Formulation variables such as PC, cholesterol and type and concentration of surfactant; Tween 80 and sodium cholate were studied to identify and assess their influence on the size, charge, EE, bilayer elasticity, and in-vitro drug release of various liposomes. All other formulation and process variables such as lipid concentration, hydration time, speed of rotation and amount of drug used were kept invariant throughout the study to examine the influence of formulation variable on characterization, *in vitro* drug release and skin penetration study of liposomes.

3.4.1. Selection of process variable

3.4.1.1. Preparation technique, method of drug loading and type of lipid

Several methods are available for liposome preparation; among them the thin film hydration technique is the simplest and commonly used in the laboratory scale preparation. Thus this method was chosen in this project to prepare both conventional and elastic liposomes.

Efficient liposomal encapsulation of therapeutic agents can be achieved using passive and active loading procedures, depending on the nature of the drug[214]. In passive loading technique, which was selected in current project for liposome preparation, the therapeutic moiety is added during vesicle formation, while in active loading, the drug can be added to preformed liposomes by applying pH gradient technique. The mechanism of passive loading of hydrophilic compounds is based on the capture of the aqueous solution in the liposomal core, which is related to drug solubility in the aqueous solution, drug's ability to cross the lipid bilayer, the type of additive present in the formulation as well as to the molecular weight of the therapeutic compound[215]. Amphipathic and hydrophobic drugs can be associated with the lipid bilayer components and therefore their passive incorporation can be performed during the film formation.

The purity of lipid is very important in developing a quality liposomal formulation. Impure lipids for example, oxidized/hydrolyzed or lipid suspended in oil or triglycerides tend to destabilize the liposome[84]. Therefore PC, with purity 95% was chosen in the current project as the common ingredient for the development of all liposomes.

3.4.1.2. Selection of a solvent system

The selection of organic solvent to dissolve lipid and lipophilic components in liposomes is crucial in the formation of a thin, homogenous and clear film which again affects the uniform distribution of lipids in bilayer and particle size of vesicles[81]. It is known that high drug entrapment can be obtained by hydrating lipid film which is thin[216]. A thin film eventually produces vesicles with relatively homogenous size distribution.

Chloroform: methanol is a suitable solvent system for developing a fine and homogeneous film. They are mostly used by investigators. However because of the toxicity of chloroform, it should be avoidable. Therefore liposomes were developed initially using ethanol, ether, dichloromethane, methanol, and isopropyl alcohol alone. Among them utilization of dichloromethane left a badly solidified lipid at the bottom of the flask, therefore it was found not suitable for liposome preparation. However ethanol, methanol and isopropyl alcohol respectively formed film which was not homogenous and was thick (Table 3.6). Ethanol could be used to improve the safety, but it is not easy to remove it from the final product. In the current study though ethanol was used in hydration medium to prepare elastic liposomes, the development of traditional liposomes must be devoid of ethanol. Therefore after doing the above mentioned trials with various organic solvents (Table 3.6), an appropriate ratio (3:1) of chloroform: methanol was found to be the solvent mixture that can consistently produce satisfactory film, and was therefore chosen to prepare all liposome formulations.

Solvent residue must be kept well below the toxic level because the contaminant in chloroform is carbon tetrachloride, which is a known carcinogenic product. However there are no set maximum acceptable limits of solvents like chloroform[81]. To avoid

the concentration of impurities during evaporation high purity chloroform was used. Solvent evaporation under vacuum for overnight or at least 12 h was performed in all formulations to ensure maximum removal of organic solvents. Residual solvent traces in formulations were not determined in the present study due to shortage of time. However the use of a smaller batch size (10 mL in current study) is expected to produce less chloroform residue in phospholipid film in each batch.

Table 3.6: Effect of solvent system on the formation of lipid film

Type of Solvent	Observation
Ethanol	Thick and non-homogeneous film formed
Dichloromethane	No film formation, lipid badly solidified
Methanol	Thick and non-homogeneous film formed
Isopropyl alcohol (IPA)	Slow solubilization of the lipid in IPA, and formation of non-homogeneous film
Chloroform: methanol (3:1)	Clear, thin and homogeneous film formed

3.4.1.3. Effect of hydration medium

To study the effect of hydration medium on vesicular size distribution and zeta potential, empty pure PC liposomes were prepared and only bath sonicated before using for characterization (Table 3.7).

Table 3.7: Effect of hydration media on particle size distribution, zeta potential and physical appearance of empty pure PC liposomes

Hydration Medium	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)	Physical Appearance
Water	309.9±16.1	0.54±0.01	-5.9±0.6	Stable suspension
PBS pH 7.4	344.1±13.0	0.50±0.01	8.4±1.7	Stable suspension
Phosphate buffer saline pH 7.4 (200 mM NaCl)[A]	873.2±11.2	0.55±0.21	2.9±0.5	Complete phase separation
Phosphate buffer saline pH 7.4 (100 mM NaCl)[B]	850.4±8.1	0.67±0.41	4.8±0.7	Complete phase separation

PBS: Phosphate buffer solution. Data represented as mean± SD (n=3) except [A] and [B] which were done twice

Liposomal stability is dependent upon physical characteristics of both the suspended liposomes and the suspension medium. Empty PC liposomes hydrated with water and PBS were quite stable upon refrigeration and also produced smaller particles compared to those of phosphate buffer saline hydrated liposomes. However phosphate buffer saline was found not suitable for formation of a stable liposome product because of rapid settlement of particles. On refrigeration, a clear phase separation was observed.

Liposomes hydrated with water produced particles of lowest size (309.9±16.1 nm) whereas PBS yielded slightly bigger particles, which however was not statistically significant (P>0.1). The bigger particles formed from phosphate buffer saline could be due to the ionic interactions that might have increased the bilayer thickess, and consequently caused an expansion within liposome particles. Polydispersity index is a measure of the size distribution of particles in a tested sample. The polydispersity index of all empty PC liposomes were more than 0.3 which means particle size distribution was not homogenous. This may be because vesicles were only bath sonicated which could be affected by the position of the sample inside the flask[76]. However the zeta potential found in PC liposomes hydrated with water was negative whereas in vesicles hydrated with PBS and phosphate buffer saline were positive. Mohammed *et al* also reported a similar zeta potential (-5.9±0.6) of empty PC

liposomes (PC= 12.16 mg/mL) hydrated with water and prepared by film hydration technique[212]. However zeta potential of PBS hydrated liposomes was more positive than that of phosphate buffer saline hydrated liposomes which potentially could contribute to the better stability of liposomes.

The zeta potential of particles is the overall charge that the particles acquire in a particular medium. It is not an actual measurement of the individual particle surface charge; rather, it is a measurement of the electric double layer produced by the surrounding ions in solution including adsorbed counter ions[217]. These counter ions play a major role in the zeta potential measurement. According to the electric double layer theory, zero electrophoretic mobility was expected for neutral liposomes. However it has been reported that liposomes composed of neutral phospholipids (i.e. PC) acquire a negative surface charge in water. Makino *et al* found that neutral liposomes exhibited a negative zeta potential in solution of low ionic strength and higher ionic strength caused the zeta potential of neutral liposomes to reverse sign[218]. At low ionic strength, the phosphatidyl groups (negatively charged) are located at the outer portion of the head group. Makino *et al* proposed that this reversal of zeta potential is triggered by changes in the direction of the choline group (positive) which at higher ionic strength approaches the outer region of the bilayer while the phosphatidyl group hides behind the PC bilayer[218]. As water has no ionic strength, and PBS and phosphate buffer saline have higher ionic strength, a negative zeta potential in water hydrated liposome and positive zeta potential in PBS and phosphate buffer saline hydrated liposomes could be explained by Makino's theory. However zeta potential in phosphate buffer saline hydrated liposomes were less positive compared to that of PBS hydrated liposomes (Table 3.7). This could be due to the presence of anion (Cl^-) in the saline which might have adsorbed on PC molecule resulting in a less positive charge of liposomes.

The aggregation and particle settlement is expected in case of liposomes hydrated with phosphate buffer saline. In theory, agglomeration state varies significantly, depending upon a variety of factors. One possible factor is the influence of ionic strength due to the addition of electrolytes such as Na^+ and Cl^- ions. Increasing the ionic strength of the hydration medium may lead to an agglomeration state likely

through charge shielding and condensation of the charge at the electric double layer[219, 220]. Therefore liposomes hydrated with PBS containing NaCl showed phase separation on refrigeration. This can be further supported by another study where Sager *et al* reported that a buffered saline hydration medium influences the particle agglomeration state[221].

Particle size is one of the most important factors in percutaneous penetration. Smaller vesicles were reported to show better transdermal flux than that of larger ones[63, 222]. Both water and PBS hydrated liposomes produced stable liposome suspension and size difference in between the two was not significant. Liposome charge has been reported to exert an effect on skin penetration. Ogiso *et al* found that *in vitro* penetration of Melatonin entrapped in negatively charged liposomes was higher than that of positively charged ones ($P<0.05$)[223]. This was further supported by Yu and Liao who reported a significant increase in in-vitro permeability of triamcinolone acetonide through rat skin by negatively charged SUVs[108]. Therefore based on charge, PBS was not considered as preferred hydration medium. After considering all the above mentioned factors on particle size and zeta potential, water was finally chosen as the hydration medium to prepare all liposomes.

3.4.1.4. Effect of method of size reduction

Producing MLVs by thin film hydration technique usually yields particles with heterogeneous size distribution which then need to be controlled by downsizing. Size reduction can be done by sonication, extrusion and homogenization.

In the present study, both bath and probe sonication were applied to reduce particle size. It was found that only bath sonication was not enough to reduce size considerably; additional one minute probe sonication effectively reduced particle size. Finally additional ten times extrusion through 200 and 100 nm polycarbonate filters (each) were performed which was found to be efficient in producing well-defined and reproducible vesicles. Extrusion is rapid and amenable to scale up procedures[224].

Although in theory the application of high frequency in probe sonication can lead to the production of smaller SUVs, in practice the probe sonicator, which was used can only generate a maximum 1 Hz frequency and its prolonged application resulted in

the destabilization of liposomes. Extrusion process is important for producing homogenous size of liposomes although its sole in downsizing liposomes is limited. To avoid the contact of liposomes with probe sonicator for long time/ with high frequency, extrusion was chosen, which improved polydispersity index. Extrusion is not expected to have negative effect on the amount of SUV obtained especially when elastic liposomes are extruded. The latter can squeeze and pass through filters irrespective of their size.

Table 3.8 shows the effect of combined downsizing techniques on particle size and polydispersity index of empty pure PC liposomes. It is clear from the table that vesicle size after downsizing reduced considerably compared to that of crude (1559.0± 29.2 nm) liposomes and those that were bath sonicated (309.9±16.1 nm). One minute probe sonication and ten times extrusion through a series of filters reduced particle size most efficiently. Effect of sonication and extrusion on polydispersity index of liposomes was quite clear. As expected, vesicle size distribution was heterogeneous (with very high polydispersity index) when they were characterized before downsizing. Sonication reduced particle size, creating relatively more homogenous particle distribution. Repeated extrusion through a fixed pore size membranes produced highly reproducible and most homogenous size distribution.

Table 3.8: Effect of downsizing technique on particle size distribution of empty pure PC liposome

Downsizing	Particle size (nm)	Polydispersity Index
No downsizing (Crude)	1559.0±29.2	1.0±0.00
Bath sonication (30 min)	309.9±16.1	0.540±0.01
Probe sonication (one min at 40 W, 0.5 cycle)	188.6±7.8	0.44±0.02
Extrusion (10 times through 200 and 100 nm filters)	153.0±2.9	0.351±0.00

Each downsizing technique was used in combination with the previous one. Data represented as mean± SD (n=3)

Particle size has been reported to play a crucial role in dermal penetration. Large vesicles with a size >600 nm are not able to deliver their contents into the deeper layers of the skin and these liposomes stay in/on the top of the stratum corneum. Liposomes with size <300 nm delivered their contents to some extent into deeper layers of the skin[222]. Verma *et al* showed enhanced penetration of carboxyfluorescein containing liposomes with diameter of 120 nm compared to that of larger ones (greater than 300 nm) in human abdominal skin model concluding that smaller particles better penetrated the skin[222]. In order to achieve efficient downsizing of a reasonable size batch, all three techniques of size reduction were combined to produce small and relatively homogeneous vesicles.

3.4.1.5. Hydration time and speed of rotation

Hydration time and speed of rotation are also important factors to be considered in designing liposome formulation. The time allowed for the hydration of the lipid layer with the aqueous phase/drug solution determines the amount of drug entrapment which again influences the size of MLVs. Drug entrapment with similar lipid composition was enhanced by slower rate of hydration[164]. The hydration time of 1 h and rotation speed of 100 rpm was chosen because these are the conditions mostly chosen by researchers to develop a successful liposome formulation[193].

3.4.2. Characterization of empty conventional and elastic liposomes hydrated with water

Unloaded or empty liposomes were prepared with water as hydration medium according to the method described under section 2.3.5.3. Formulations with different composition were prepared with a view to characterize them based on particle size and zeta potential and eventually to be used for comparing with that of NaFl loaded liposomes (Table 3.9). Here empty pure PC liposome was used as a bench mark for comparison purpose.

Table 3.9: Characterization of empty conventional and elastic liposomes hydrated with water

Formulation ratio (% w/w)	Size (nm)	Zeta potential (mV)
Pure PC	153.1±2.9	-5.9±0.6
PC:CH 70:30	131.3±3.7	-27.2±0.7
PC:T80 85:15	90.9±5.8	-6.7±0.4
PC:S. Chol 85:15	69.3±0.8	-15.2±3.3

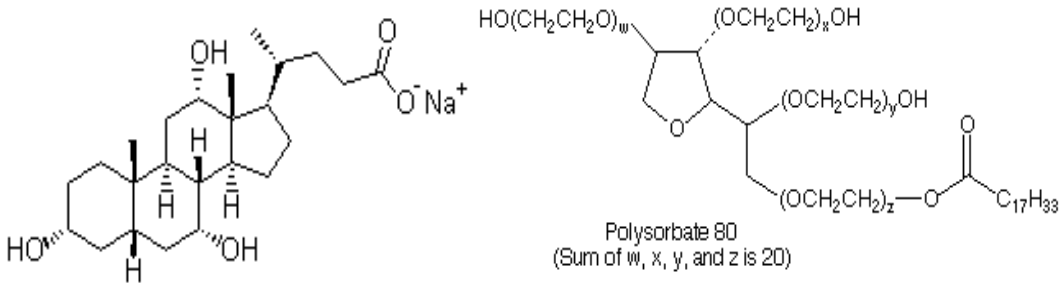
CH: Cholesterol; PC: Phosphatidylcholine; S. Chol: sodium cholate; T80: Tween 80. Characterization of liposomes was done after ten times extrusion through 200 and 100 nm Supor® membrane filters. Data represented as mean± SD (n=3)

Table 3.9 shows that empty pure PC liposome had the biggest particle size compared to that of other formulations. Cholesterol incorporation in PC bilayer reduced size to 131.3±3.7 nm. However a significant drop in vesicle size was observed when surfactants were included to PC liposome. Addition of Tween 80 and sodium cholate produced elastic liposomes with an average size of 90.9±5.8 nm and 69.3±0.8 nm respectively. The effect of cholesterol and surfactant on particle size of liposomes is discussed in detail in section 3.4.3.1 and 3.4.4.1 respectively.

The effect of formulation variables on zeta potential of empty liposomes was prominent. Pure PC liposomes showed a negative zeta potential in water whereas addition of cholesterol in vesicle bilayer increased the negative charge on PC molecule. When 15% w/w of Tween 80 and sodium cholate were added to PC liposomes, the zeta potential obtained were negative; more negative than pure PC liposome and less negative than cholesterol containing liposomes. Neutral PC is reported to produce a net negative charge in presence of low ionic strength (such as water) medium[212]. The increase in negative charge of cholesterol containing liposome is probably because the polar head of cholesterol contains hydroxyl groups, which may easily combine with choline in the polar region of PC to produce a type of dipole tropism that increases the liposomal surface negative charges[225].

However the reason for the more negative zeta potential of elastic liposomes compared with that of empty pure PC liposomes could be due to the partial

hydrolysis of Tween 80[226]. Another study reported that Tween 80 may cause more negative zeta potential due to the shift in the shear plane of the particle[227]. The inclusion of sodium cholate in liposomes induced a negative surface charge due to the adsorption of cholate anion on PC molecule. Essa *et al* found that estradiol loaded ultradeformable liposomes had higher negative zeta potential (−29 ±2.4 mV) due to the adsorption of cholate anion, compared to the conventional estradiol liposomes[228]. However the zeta potential in Tween 80 containing elastic liposomes was less negative than that of sodium cholate due to the fact that sodium cholate is anionic (Figure 3.4 (a) and 3.4 (b)). The latter might have adsorbed on PC to increase the negative charge of liposomes.



(a) (b)

Figure 3.4: Structure of sodium cholate (a) and Tween 80 (b)

3.4.3. Characterization of conventional liposomes loaded with sodium fluorescein

Conventional liposomes can be prepared from phospholipid with or without cholesterol. The beneficial role of cholesterol within liposomal drug carriers is well recognized. Because of its flat and rigid fused ring structure[229] cholesterol generates more rigid vesicles. Thus in current study conventional liposomes were prepared and characterized with a view to show the effect of cholesterol content on liposome size, zeta potential and EE of NaFl. Because the analysis of NaFl is simple and less time consuming, it was chosen to prepare various traditional and elastic liposomes. Moreover NaFl is a small molecular weight compound and freely soluble in water, therefore it can be used as a model hydrophilic compound. According to passive loading theory, it is entrapped in the aqueous core of liposomes. Four different

conventional liposomes loaded with 1mg/mL of NaFl were prepared with a range of PC (70-100 mg) and cholesterol (0-30 mg). They were coded as NaFl CON 0 (NaFl loaded liposome containing no cholesterol i.e. pure PC), NaFl CON 10, 20 and 30 denote NaFl loaded conventional liposome prepared from PC: cholesterol ratio of 90:10, 80:20, and 70:30 respectively.

Methods of characterization of liposomes immediately after preparation and upon storage are required for adequate quality control of the product. The methods have to be reproducible, precise and rapid in the context of their use in industrial and even in laboratory setting. Therefore all liposomes were characterized within a day of their preparation.

3.4.3.1. Vesicle size distribution

As soon as NaFl loaded SUVs were formed, they were characterized for particle size distribution, zeta potential and EE. However vesicle sizes were determined both before and after extrusion (ten times through a series of 200 and 100 nm Supor® membrane filters). The mean diameters of all different vesicles are shown in Table 3.10. Sonicated vesicles which contained no cholesterol, i.e. pure PC vesicles had relatively bigger particle size (401.1±10.1 nm) compared to other cholesterol containing vesicles. Compared to NaFl CON 0, addition of 10% w/w cholesterol in the formulation, particle size reduced significantly (187.4±6.4 nm, $P<0.005$). Further addition of cholesterol reduced particle size more, with the lowest size achieved upon addition of 30% w/w cholesterol (153.1±6.6, $P<0.005$). All extruded vesicles reduced in size after extrusion; however NaFl CON 0 showed the maximum size reduction upon extrusion (60.01%). Extruded conventional liposomes also showed a similar pattern of size reduction upon addition of cholesterol. NaFl CON 30 showed the smallest size (133.46±7.01 nm, $P<0.005$) after extrusion compared to that of pure PC (160.7±3.4 nm) (Table 3.10).

Liposomal size reduction induced by cholesterol can be explained by the condensation effect of cholesterol on the bilayer. Surface pressure measurements on monolayers of nonionic surfactant/ cholesterol mixtures demonstrated a condensing effect of cholesterol showing a decrease in the effective area per molecule as the

cholesterol content of the monolayer increased[230]. Similar effect was also observed by Semple *et al* when they found cholesterol inclusion within liposomal bilayers resulted in an increased packing densities of phospholipids molecules[231] which is thought to result from the accommodation of cholesterol in the molecular cavities formed by surfactant monomers assembled into vesicles[232]. This condensation effect clearly contributed to the effect of cholesterol on size reduction of bilayer vesicles.

The polydispersity index of less than 0.1 indicates a narrow size distribution and greater than 0.3 indicates wider size distribution[233]. However polydispersity indices of various NaFl loaded conventional vesicles were found to be $\leq$ 0.3, which is reasonably narrow size distribution. After extrusion polydispersity index of all liposomes reduced slightly. Ideally mechanical dispersion method yields MLVs with a wider size distribution[1]. Sonication and extrusion steps should result in the formation of vesicles with a narrow size range, although standardizing sonication power is not easy[160].

3.4.3.2. Zeta potential

Knowledge of zeta potential of a liposome preparation can help to understand dispersion and aggregation processes[234] and to predict the fate of the liposomes in vivo. Zeta potential has often been used for characterizing colloidal drug delivery systems[235] to determine the type of interaction between the active substance and the carrier; i.e. whether the drug is encapsulated within the body of the particle or simply adsorbed on the surface. In this study zeta potential was found to be highly negative in all NaFl loaded conventional liposomes compared to that of empty pure PC (Table 3.9) and cholesterol containing liposomes (Table 3.10). Drug loading showed an effect on the zeta potential of liposomes which suggest that there was a drug-liposome interaction. The highly negative zeta potential of NaFl loaded conventional liposomes could be a result of the carboxylate (from NaFl) anion adsorption on surface of liposomes. Carboxylic acid functional groups of NaFl are almost fully ionized in aqueous solutions above pH 9. pKa of fluorescein is 6.4. The pH of all NaFl loaded liposomes was found to be 7.3 to 7.4; at this pH NaFl is expected to be highly ionized (>50%). Because of the ionization it is likely that the ionized

carboxylate functional group of NaFl was absorbed at the surface of PC molecules creating a highly negative zeta potential.

3.4.3.3. Entrapment efficiency

The EE of liposomes is governed by the ability of the formulation to retain drug molecules in the aqueous core or in the bilayer membrane of the vesicles. Purification process such as ultracentrifugation has been reported to be responsible for losing a large quantity of entrapped molecule probably due to the increased leakage of lipid membranes because of the high speed used in such method[196]. In this study a moderate speed centrifugation method in conjunction with the use of an ultrafiltration device (Nanosep®) was applied for a relatively short time to separate unentrapped NaFl molecule from the trapped one. Nanosep®s having a filter membrane can easily separate the liquid even at a low speed leaving the liposomal pellet on top of the filter.

Table 3.10 shows the EE of NaFl loaded various conventional liposomes, which was found to be dependent on the amount of cholesterol present in their structure. Vesicles with no cholesterol had an EE of 13.8±0.2%. A similar EE was found by Coderch *et al*. who reported 15.9% EE from pure PC liposomes containing 10 mg/mL lipid and 1 mg/mL of NaFl from REV vesicles[208]. However EE was found to increase with each inclusion of cholesterol in the formulation. 30% w/w inclusion of cholesterol increased EE significantly (24.8±0.5%, $P<0.002$) compared to that of pure PC.

Table 3.10: Characterization of NaFl loaded conventional liposomes

Code	PC: CH (w/w)	Size-1 (nm)	Size-2 (nm)	P.I.-1	P.I.-2	Zeta Potential (mV)	EE (%)
NaFl CON 0	100:0	401.9±10.0	160.7±3.9	0.26±0.09	0.25±0.11	-32.7±1.0	13.8±0.2
NaFl CON 10	90:10	187.4±6.4	140.0±1.9	0.30±0.06	0.29±0.06	-39.3±0.1	20.7±1.0
NaFl CON 20	80:20	151.8±10.2	141.7±4.3	0.29±0.04	0.28±0.04	-42.2±2.5	22.3±2.0
NaFl CON 30	70:30	153.1±6.6	133.4±7.0	0.25±0.06	0.23±0.05	-46.6±1.4	24.8±0.5
NaFl CON 40	60:40	Characterization was not done because liposomes were not formed					

CH: cholesterol; PC: Phosphatidylcholine; NaFl CON 0: sodium fluorescein (NaFl) loaded conventional liposome with no cholesterol, NaFl CON 10, 20 and 30 denote NaFl loaded conventional liposome prepared from PC: CH ratio of 90:10, 80:20, and 70:30 respectively.

Size-1, P.I.-1 = Size and polydispersity index of vesicles determined after sonication but before extrusion

Size-2, P.I.-2 = Size and polydispersity index of vesicles determined after extrusion through 200 and 100 nm Supor® membrane filters

EE= Entrapment efficiency

Data represented as mean± SD (n=3)

The increase in EE of hydrophilic NaFl with inclusion of cholesterol may be due to the increased stability of the liposomal membrane due to increased membrane rigidity. Gregoriadis and Davis demonstrated that inclusion of 50% mol/mol cholesterol within a liposome formulation increased stability and reduced permeability of liposomal bilayers[236]. It was thought that cholesterol took place in the cavities formed by lipid molecules and filled up the empty spaces. This space filling action combined with the interaction of cholesterol with phospholipid significantly reduced the permeability of small hydrophilic solutes and ions[237,238]. Moreover above the T_c (50-55ºC) cholesterol makes the membrane more ordered and inhibits the gel from transition into the liquid phase, hence it effectively prevented leakage of drug[239, 240]. Water soluble NaFl is initially effectively entrapped inside the aqueous core of liposomes. Addition of cholesterol reduced the leakage of entrapped NaFl and as a result increased EE.

When 40% w/w cholesterol was added to PC liposomes, a hard film developed at the bottom of the flask which was very difficult to hydrate. At the end of 1 h hydration, most of the film left on the flask and some formed into hard lipid flakes. No satisfactory vesicles were formed. Such phenomena may be due to the crystallization of cholesterol at high concentration. It has been reported that above a certain mole fraction, cholesterol shows crystallinity, even in the presence of bilayer membranes[229]. The conditions required for this behaviour is strongly dependent on the type of phospholipid with which cholesterol is bound[94].

3.4.3.4. Bilayer elasticity

Vesicular elasticity, expressed as deformability index, is a term which is used to measure the elasticity of elastic liposomes to differentiate them from conventional liposomes. The former can squeeze and penetrate through the very tiny pores of SC whereas the latter cannot because of their rigid structure. Among the various NaFl loaded conventional liposomes, CON 0 and CON 30 were the most different in terms of their characteristics and composition. Therefore they were selected for elasticity measurement. NaFl CON 30 showed poor elasticity of about 3.61 which was less deformable than that of NaFl CON 0 (6.72±0.7) (Table 3.11). Conventional liposomes lack the propensity for highly curved structures, therefore generating greater resistance against particle deformation. Inclusion of cholesterol in liposome

bilayer is known to further restrict the flexibility of the lipid hydrocarbon chains, making it more rigid[241]. Thus pure PC liposome, showed better deformability than cholesterol containing liposome. More discussion on elastic liposome and their elasticity measurement will be done later in this chapter.

Table 3.11: Elasticity measurement of NaFl loaded conventional liposomes

Code	PC: CH (w/w)	Size (nm)[a]	Polydispersity[a] Index	*J* (mL)	*D*
NaFl CON 0	100:0	101.9±2.3	0.21±0.10	1.6±0.1	6.72±0.7
NaFl CON 30	70:30	98.3±2.8	0.23±0.03	0.9±0.1	3.61±0.7

CH: cholesterol, PC: phosphatidylcholine, NaFl CON 0 and 30 denote sodium fluorescein (NaFl) loaded conventional liposome with no CH and 30% w/w of CH.

[a]size and polydispersity index were determined after extrusion through 50 nm polycarbonate membrane filters

J= Volume of suspension passed through filter within 5 min

D= Degree of deformability (Deformability index)

Data represented as mean± SD (n=3)

Optimization of liposome composition is the first step in developing a liposomal formulation with desired properties for the delivery of bioactive agents. The primary properties that should be considered for evaluation of liposomes include size, zeta potential, EE and elasticity, all of which are affected by the amount and structure of the membrane constituents used[160]. Therefore, a proper understanding of the physicochemical properties of liposomes as a function of the liposomal membrane composition will guide us to understand how to modulate liposome properties to improve their in vivo performance. Though NaFl CON 30 showed less deformation than that of NaFl CON 0, applying the criteria of small particle size and high EE, NaFl CON 30 was considered to be the best conventional liposome here in this context. Smaller particles are expected to lead to deliver an increased amount of drug into the deeper skin strata[63, 222]. Consequently vesicles with high EE would carry more drug into the skin.

3.4.4. Characterization of elastic liposomes loaded with sodium fluorescein

NaFl loaded elastic vesicles were prepared by the procedure described in section 2.3.5.2. The basic composition of elastic liposomes comprised of PC (the stabilizing agent), surfactant (the destabilizing agent) and 7% ethanol in order to impart elasticity to vesicle membrane. In the preparation steps, ethanol was added separately to vesicles after 1 h hydration. This is because at around 40ºC, ethanol might evaporate or even cause breakage of liposomes.

In the current study two surfactants were used: sodium cholate and Tween 80 with varying ratios for optimization of formulation. Tween 80 and sodium cholate are good excipients for skin drug delivery because of their low toxicity and low irritation. Tween 80 is pharmaceutically acceptable and biocompatible. Characteristics of various elastic liposomes prepared from Tween 80 and sodium cholate are presented in Table 3.12 and 3.13. To simplify the names, vesicles were coded by the name of the compound entrapped and the type and weight percentage of surfactant. For example, NaFl T80 5 and NaFl S. Chol 5 denoted NaFl loaded elastic liposome where 5% w/w Tween 80 and 5% w/w sodium cholate were used in conjunction with PC respectively.

3.4.4.1. Vesicle size distribution

The particle sizes of both types of elastic liposomes loaded with NaFl were measured both before and after extrusion. Before extrusion, there was a marked decrease in size with increase in level of surfactant (Table 3.12 and 3.13). For instance, NaFl T80 5 had an average vesicle size of 158.3±4.3 nm. When 15% w/w Tween 80 was used, size reduced significantly (132.9±3.3 nm, $P<0.02$). Similarly NaFl S. Chol 5 had an average diameter of 129.7±2.5 nm whereas inclusion of 15% w/w sodium cholate reduced size considerably (115.7±1.5 nm, $P<0.03$). The effect of Tween 80 on liposome size may be considered due to a steric repulsion occurring between the Tween molecules, which are exposed from the outer and inner leaflet of the liposomal bilayer[225]. The Tween surfactants exposed from the outer leaflet would tend to increase the liposome particle curvature, whereas the Tween surfactants

exposed to the inner leaflet may do the opposite. Hence, it is thought that addition of Tween surfactants reduced the initial liposomal size since there were more Tween surfactants in the outer leaflet than that in the inner leaflet of the bilayer[225].

After extrusion, however a further marginal size reduction was noticed with both Tween 80 and sodium cholate containing elastic liposomal formulations when surfactant increased from 5% w/w to 15% w/w (Table 3.12 and Table 3.13). These results are expected because of the elasticity phenomena of elastic vesicles which can be pushed through fixed pore size filters. Upon extrusion elastic vesicles eventually produce similar sized particles, which is close to the pore size of the filter membrane. However a very significant drop of vesicle size was observed for both types of elastic liposomes when surfactant concentrations were increased above 15% w/w. This may be due to the formation of micelles, which are relatively smaller in size. This can be suggested from the data reported for the lecithin/sodium cholate systems where vesicles and mixed micelles were found to coexist at 20% sodium cholate, only mixed micelles being found at 30% [94].

For all elastic liposomes, the polydispersity index reduced after extrusion which suggests that particles with narrow size distribution were produced. However when 25% w/w surfactant was used, although smaller particles were formed, a relatively large polydispersity index (0.63±0.04 in 25% w/w Tween 80 and 0.71±0.03 in 25% w/w sodium cholate) was observed (Table 3.12 and 3.13) with both elastic liposomes before extrusion. This phenomenon could be attributed to the formation of micelles.

Table 3.12: Characterization of NaFl loaded elastic liposomes prepared using PC and Tween 80

Code	PC:T80 (w/w)	Physicochemical Property						Elasticity measurement			
		Size-1 (nm)	Size-2 (nm)	P.I.-1	P.I.-2	Zeta Potential (mV)	EE (%)	Size-3 (nm)	P.I.-3	*J* (mL)	*D*
NaFl T80 5	95:5	158.3±4.3	136.1±2.8	0.52±0.02	0.35±0.06	-12.0±1.4	15.6±1.1	118.1±2.4	0.22±0.01	3.5±0	19.7±1.0
NaFl T80 10	90:10	134.4±3.4	124.3±0.6	0.44±0.01	0.36±0.01	-12.8±0.1	13.3±1.0	112.9±2.4	0.21±0.04	4.3±0.1	22.2±1.7
NaFl T80 15	85:15	132.1±3.3	128.4±2.0	0.46±0.01	0.35±0.02	-14.7±0.5	14.4±1.5	109.8±2.6	0.22±0.05	7.6±0.1	35.8±0.6
NaFl T80 20	80:20	112.4±1.0	104.5±3.4	0.43±0.03	0.37±0.01	-13.6±1.1	6.4±0.5	88.0±2.3	0.17±0.03	3.3±0.2	10.4±1.2
NaFl T80 25	75:25	104.3±3.5	93.7±1.4	0.63±0.04	0.27±0.01	-11.6±0.0	6.7±1.0	86.4±3.1	0.23±0.02	2.7±0.1	8.0±0.2

PC: Phosphatidylcholine, T80: Tween 80, NaFl T80 5, 10, 15, 20 and 25 denote sodium fluorescein (NaFl) loaded elastic liposome containing 5, 10, 15, 20 and 25% w/w T80 respectively in conjunction with PC.

Size-1, P.I.-1 = Size and polydispersity index of vesicles determined after sonication but before extrusion

Size-2, P.I.-2 = Size and polydispersity index of vesicles determined after extrusion through 200 and 100 nm Supor® membrane filters

Size-3, P.I.-3 = Size and polydispersity index of vesicles determined after extrusion through 50 nm polycarbonate membrane filters

EE= Entrapment Efficiency

J= Vol of suspension extruded within 5 min, *D*= Degree of deformability (Deformability index)

Data represented as mean± SD (n=5) except the elasticity measurements which were done thrice

Table 3.13: Characterization of NaFl loaded elastic liposomes prepared using PC and sodium cholate

Code	PC:S. Chol (w/w)	Physicochemical Property						Elasticity measurement			
		Size-1 (nm)	Size-2 (nm)	P.I.-1	P.I.-2	Zeta Potential (mV)	EE (%)	Size-3 (nm)	P.I.-3	*J* (mL)	*D*
NaFl S. Chol 5	95:5	129.7±2.5	122.3±2.8	0.44±0.01	0.38±0.01	-18.6±1.1	20.0±0.6	110.7±1.7	0.22±0.03	2.9±0.1	14.2±0.2
NaFl S. Chol 10	90:10	118.6±2.6	113.4±4.3	0.43±0.01	0.40±0.06	-19.7±3.1	17.8±2.4	106.4±2.5	0.20±0.00	3.6±0.2	16.5±1.7
NaFl S. Chol 15	85:15	115.7±1.5	109.4±1.6	0.46±0.05	0.37±0.06	-20.8±0.5	17.7±2.9	104.0±1.1	0.21±0.04	7.0±0.1	30.3±0
NaFl S. Chol 20	80:20	106.4±1.9	98.6±7.5	0.48±0.07	0.39±0.04	-20.1±2.2	9.5±0.7	74.1±1.5	0.24±0.00	3.0±0.3	6.6±1.0
NaFl S. Chol 25	75:25	91.4±1.5	74.8±2.1	0.71±0.03	0.43±0.02	-15.8±1.3	6.0±0.9	70.6±0.8	0.16±0.03	2.9±0.2	5.8±0.4

PC: Phosphatidylcholine, S. Chol: sodium cholate, NaFl S. Chol 5, 10, 15, 20 and 25 denote sodium fluorescein (NaFl) loaded elastic liposome containing 5, 10, 15, 20 and 25% w/w S. Chol respectively in conjunction with PC.

Size-1, P.I.-1 = Size and polydispersity index of vesicles determined after sonication but before extrusion

Size-2, P.I.-2 = Size and polydispersity index of vesicles determined after extrusion through 200 and 100 nm Supor® membrane filters

Size-3, P.I.-3 = Size and polydispersity index of vesicles determined after extrusion through 50 nm polycarbonate membrane filters

EE= Entrapment Efficiency, *J*= Vol of suspension extruded during 5 min

D= Degree of deformability (Deformability index)

Data represents as mean ±SD of n=5 except the elasticity measurement which were done thrice

3.4.4.2. Zeta potential

The mean values of zeta potential of all NaFl loaded elastic liposomes were found to be negative (Table 3.12 and 3.13) and the addition of surfactant at various ratios mostly did not affect the zeta potential significantly which is consistent with previous literature reports[242, 243,244]. Compared with empty elastic liposomes (Table 3.9), the zeta potential found in NaFl loaded elastic liposomes were more negative. This is likely due to the adsorption of carboxylate ion from NaFl on PC molecule.

3.4.4.3. Entrapment efficiency

The effect of surfactant concentration on the EE of elastic liposomes containing NaFl (Table 3.12 and 3.13) clearly shows that EE decreased with an increase in concentration of surfactant. The maximum EE found in Tween 80 and sodium cholate elastic liposomes were around 15.6% and 20.1% respectively, which was at 5% w/w inclusion of surfactant. The possible reason of decrease in EE up to 15% w/w surfactant content could be due to the property of surfactants which make the vesicle bilayers soft and more leaky, subsequently increase the tendency of trapped molecule to be leaked through the bilayers. As expected above 15% w/w of surfactant, EE reduced significantly in both types of elastic liposomes. 20% w/w surfactant dropped EE to 6.4% (in Tween 80) and 9.5% (in sodium cholate). This is due to the possible coexistence of mixed micelles and vesicles at higher concentrations of surfactant leading to lower drug entrapment in elastic formulations[245,246]. Lasch *et al*[247] and Lopez O *et al*[248] studied the formation of mixed micelles due to the solubilisation of phospholipid in liposomes by non-ionic surfactants such as Triton X-100, octyl glycoside by a variety of techniques. They reported that transformation of liposomes to mixed micelles was a concentration-dependent process. Vesicle to micelles transformation was mainly governed by the progressive formation of mixed micelles within the bilayer[247, 248].

3.4.4.4. Bilayer elasticity

Bilayer elasticity is the critical feature of all elastic liposomes. It is the stress dependent adaptability of elastic vesicles which differentiates them from traditional liposomes and other types of lipid suspensions. The surfactant present in the formulations causes fluidization of bilayer that is responsible for increase in elasticity of vesicle membrane[233]. This in turn permits elastic liposomes to squeeze themselves through pores that are much smaller than their own diameter[112]. This is achieved by combining at least two amphiphilic components (phospholipid and surfactant) with sufficiently different packing characteristics into a single bilayer. The high ultradeformability permits elastic liposomes to penetrate the skin spontaneously and minimizes the risk of complete vesicle rupture in the skin.

Ethanol is not an essential component in the development of our elastic liposomes as its concentration was kept low. Surfactants were essential. In ethosomes and niosomes (containing 30% ethanol), ethanol, however may interact with the polar head group region of the phospholipid/surfactant molecules resulting in the reduction of melting point, thereby increasing the fluidity of the vesicles. In present study, 7% ethanol was used. In case of elastic liposomes, ethanol is expected to impart some bilayer elasticity on vesicle membrane, which, however may be small. It should be pointed out that the difference in elasticity of various elastic liposomes observed in this study was due to the presence of different amount of surfactants, as ethanol concentration was kept constant in all formulations. Thus the effect of surfactants on bilayer elasticity was explained.Comparative measurement of elasticity of the bilayer of different elastic liposomes was carried out by extrusion measurement using 50 nm membrane filters (Table 3.12 and 3.13). The results indicate that elasticity of vesicles depend on surfactant concentration. With an increase in Tween 80 concentration from 5% to 15% w/w membrane elasticity (*D*) increased from 19.7±0.7 to 35.8±0.6 whereas for sodium cholate vesicles deformability increased from 14.2 to 30.3.

A dramatic drop of elasticity however was observed when the surfactant content was increased more than 15% w/w with minimum elasticity obtained at the surfactant concentration of 25% w/w (from 35.8±0.6 to 8.0±0.2 for Tween 80 vesicles and from

30.3 to 5.8±0.4 for sodium cholate vesicles). This is because when the critical micelle concentration (CMC) of surfactant is reached lipid vesicles and mixed micelles coexist. The resultant mixtures are reported to be less deformable in nature with less skin permeability in comparison to elastic liposomes [22, 247, 248].

Elastic liposomes are reported to pass through narrow pores of skin irrespective of their size[22], which means they are able to deform. Therefore no vesicle leakage is expected. To support this phenomenon, NaFl loaded elastic liposomes were extruded through 50 nm membrane filters and were then centrifuged to separate the free dye by using Nanosep®. The concentration of NaFl in the supernatant was found to remain the same which indicates that elastic liposomes are able to hold their structural integrity when squeezing through the narrow pores. This further suggests that the entrapped drug (i.e. inside elastic elastic liposomes) will be able to penetrate through the small pores of the stratum corneum to reach the region in the skin where drug molecules normally cannot reach by themselves. To confirm that the extrusion process not interrupted by free dye adsorption on the filter, 1mg/mL of NaFl solution was extruded through 50 nm filter and no change in the dye concentration was found after extrusion.

In order to cross the intact stratum corneum, elastic liposomes should be capable of passing through pores of diameter less than 50 nm under influence of suitable transdermal gradient[194]. Only properly optimized and moderately loaded carriers can pass through pores smaller than their own diameter. Our study showed that increasing the concentration of surfactant beyond a certain level brings no advantages in terms of elasticity of liposomes which ultimately will not help in deep skin transportation efficiency. Only optimum ratio of lipid and surfactant can lead to maximum flexibility. This is why it is crucial in optimizing the elastic liposomal formulation of the bilayer membrane[245]. In our case the maximum elasticity was found with 15% w/w surfactant. Although 85:15 PC: surfactant did not show highest entrapment, considering that higher elasticity would lead to better penetration, elastic liposomes composed of 15% w/w surfactant were chosen for further studies.

3.4.4.5. Morphology of sodium fluorescein loaded liposomes

NaFl loaded conventional and elastic liposomes with different composition were examined under TEM. Their micrographs are shown in Figure 3.5(a)-3.5(d). Vesicles were appeared as homogenous, regular, and spherical shape in conventional and elastic liposomes (containing 15% w/w surfactant). Elastic liposomes prepared using 20 and 25% w/w surfactants were also visualized under TEM, where no spherical structures were found. A typical TEM micrograph of NaFl T8025 is shown in Appendix 4. The absence of spherical structure demonstrates that these liposomes formed structures other than vesicles; however from TEM micrograph it was difficult to define them as micelles. Such a phenomenon observed with elastic liposomes prepared from 20% and 25% w/w surfactant suggests structures other than vesicles because of the possible coexistence of mixed micelles and micelles. This was supported by the report on lecithin surfactant system where the investigators showed vesicles and mixed micelles formed at 20% and micelles formed at 30% w/w of sodium cholate[249]. A typical micrograph of extruded (through 50nm membrane filter) NaFl T80 15 is shown in Figure 3.5 (e). No change in vesicle shape was observed after extrusion which indicates vesicles can deform.

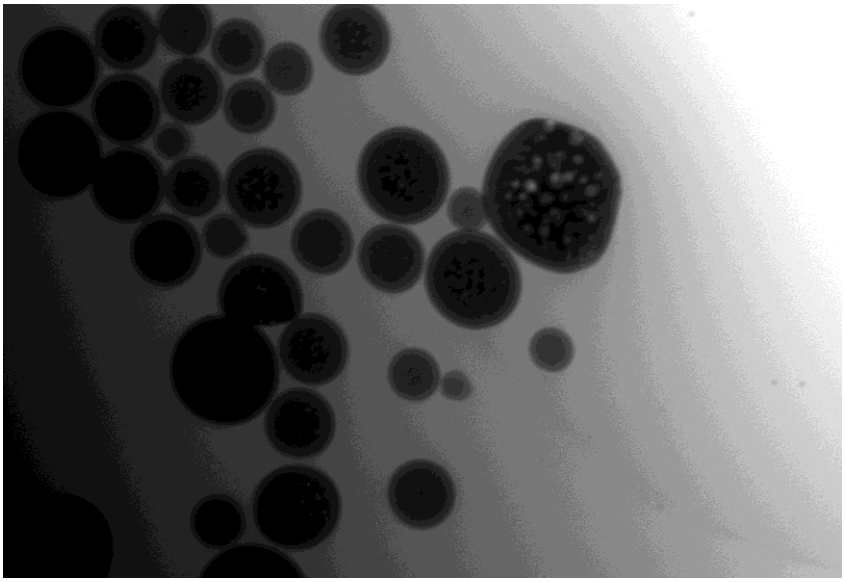


Figure 3.5 (a): TEM micrograph of sonicated NaFl T80 15 (NaFl loaded elastic liposome containing 15% w/w of Tween 80) (scale 0.5µm). The tiny white spots on the vesicle are because of incompletely dried material. TEM imaging using a strong beam led to vesicle disruption and damage.

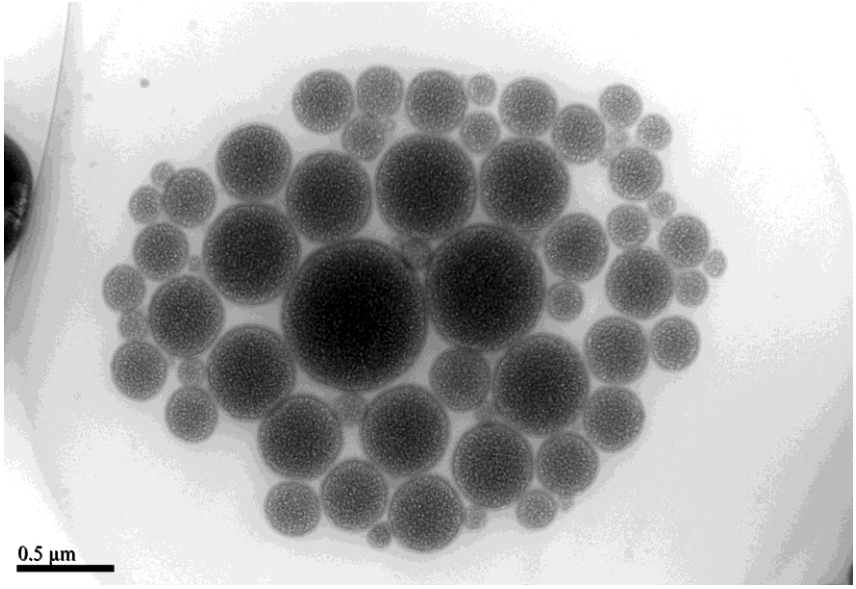


Figure 3.5 (b): TEM micrograph of sonicated NaFl S. Chol 15 (NaFl loaded elastic liposome containing 15% w/w of sodium cholate)

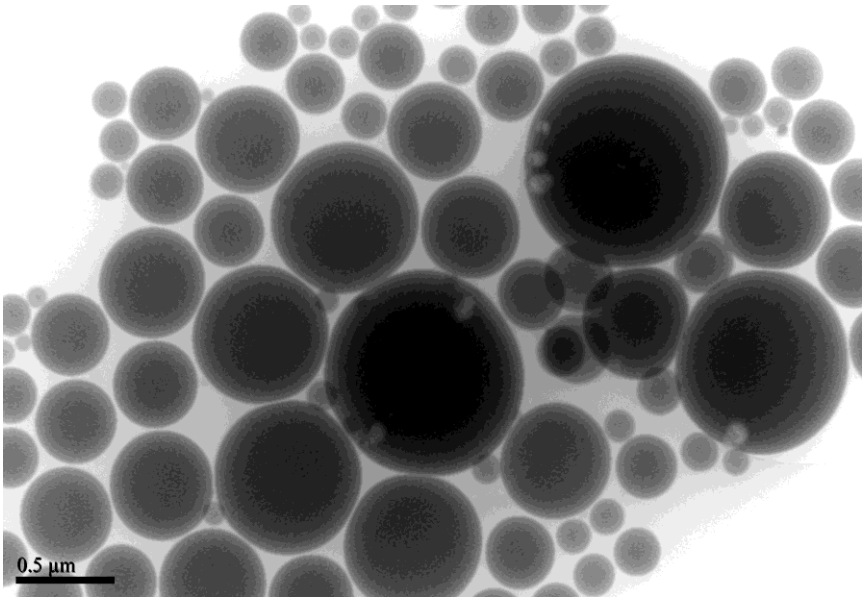


Figure 3.5 (c): TEM micrograph of sonicated NaFl CON 0 (NaFl loaded conventional liposome containing no cholesterol. i.e. Pure PC)

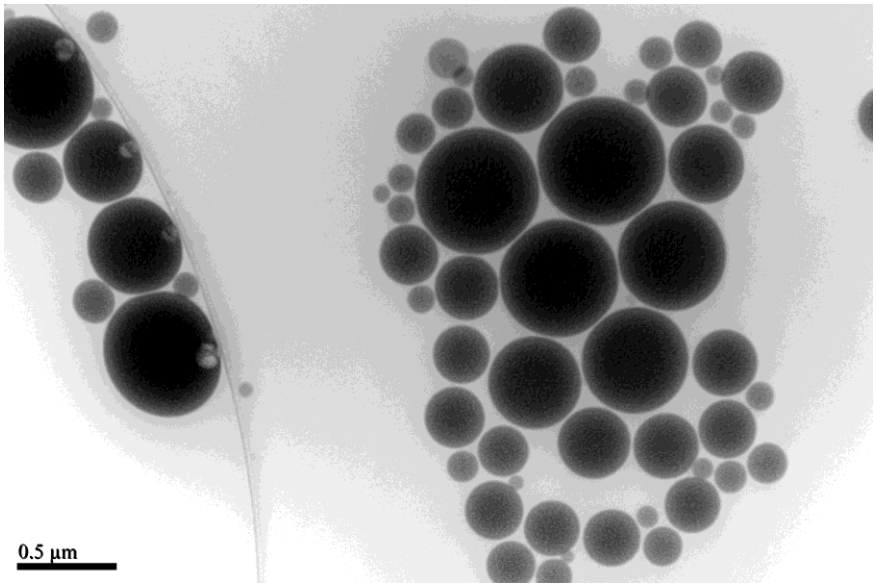


Figure 3.5 (d): TEM micrograph of sonicated NaFl CON30 (NaFl loaded conventional liposome containing 30% w/w of cholesterol). The tiny white marks on vesicles at the copper grid are due to incompletely dried material. TEM imaging using a strong beam led to vesicle disruption and damage.

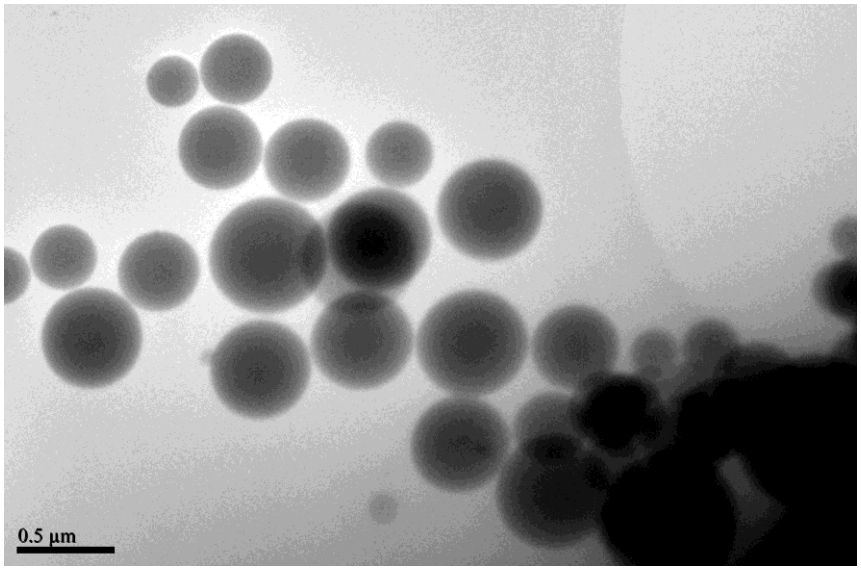


Figure 3.5 (e): TEM micrograph of NaFl T80 15 (NaFl loaded elastic liposome), extruded through 50 nm membrane filter

Micrograph shows no vesicle rupture after extrusion through 50 nm filter, which indicates that elastic liposomes are able to deform or change their shape while passing through small sized pores of the stratum corneum.

3.4.5. Effect of cholesterol on storage stability of sodium fluorescein loaded conventional liposomes

Average particle size of liposomes determined after their preparation changes upon storage. This is largely due to aggregation, fusion, or rupture of liposomes and occurs virtually in all liposome dispersions. Thus the ability of the system to maintain its original size distribution gives an indication of physical stability of liposome system. From a pharmaceutical point of view, it is important to formulate liposomes that can be stored for a long period of time. Degradation processes particularly oxidation and hydrolysis can lead to an increase in particle size of liposomes[250]. In this study, the stability of conventional liposomes loaded with NaFl was investigated by particle size analysis for a period of 4 weeks at 4±2°C, 25±2°C and 35±2°C. The results are presented in Table 3.14.

Effect of storage condition on the stability of liposomes showed that all vesicles had tendency to increase in size upon storage with an exception of that at 4±2ºC where no significant size increase was detected. At 35±2ºC, the maximum increase in size (42.9%) was found with NaFl CON 0, which had no cholesterol in the bilayer. It was observed that the presence of cholesterol in the lipid mixture reduced the tendency of liposome to grow in size. The stabilization effect of cholesterol on size was much more pronounced when liposomes were stored at 35±2ºC. 10, 20 and 30% w/w cholesterol in bilayer showed significant stability of liposomes compared to that of no cholesterol. Lowest increase (14.4%) in size was found with NaFl CON 30. At room temperature, vesicular instability was less pronounced than that of at 35±2ºC but more than that of at 4±2ºC (Table 3.14). Table 3.15 shows the % of increase in diameter of various NaFl conventional liposomes stored at different temperatures at the end of 4 weeks.

Table 3.14: Effect of storage condition on size of NaFl loaded conventional liposomes

Physical stability study at 4±2ºC; Particle size (nm)					
Code	**PC/CH (w/w)**	**T=0 week**	**T=1 week**	**T= 2 week**	**T= 4 week**
CON 0	100:0	160.7±3.9	161.0±3.0	161.7±5.4	165.7±6.2
CON 10	90:10	140.0±1.9	140.5±2.3	141.9±2.0	142.9±0.8
CON 20	80:20	141.7±4.3	143.2±3.7	144.3±4.1	145.3±4.5
CON 30	70:30	133.4±7.0	134.7±6.1	135.2±7.9	135.9±8.6
Physical stability study at 25±2ºC; Particle size (nm)					
CON 0	100:0	160.7±3.9	162.2±4.2	170.5±1.0	174.3±1.8
CON 10	90:10	140.0±1.9	141.5±1.5	144.1±1.3	145.8±2.7
CON 20	80:20	141.7±4.3	143.2±5.0	145.1±5.6	148.4±4.2
CON 30	70:30	133.4±7.0	136.0±7.5	138.9±6.4	142.7±2.8

Physical stability study at 35±2ºC; Particle size (nm)					
CON 0	100:0	160.7±3.9	174.9±6.8	196.8±4.7	229.7±0.6
CON 10	90:10	140.0±1.9	147.6±2.6	157.2±2.8	177.3±3.1
CON 20	80:20	141.7±4.3	149.0±3.8	153.8±2.4	167.4±3.2
CON 30	70:30	133.4±7.0	142.3±7.1	148.2±4.6	152.7±3.5

CH: cholesterol, PC: Phosphatidylcholine, CON0, 10, 20 and 30 denote sodium fluorescein (NaFl) loaded conventional liposome prepared from PC: CH ratio of 90:10, 80:20, and 70:30 respectively. To avoid repeatation, NaFl is removed from each formulation code. Data represented as mean± SD (n=3)

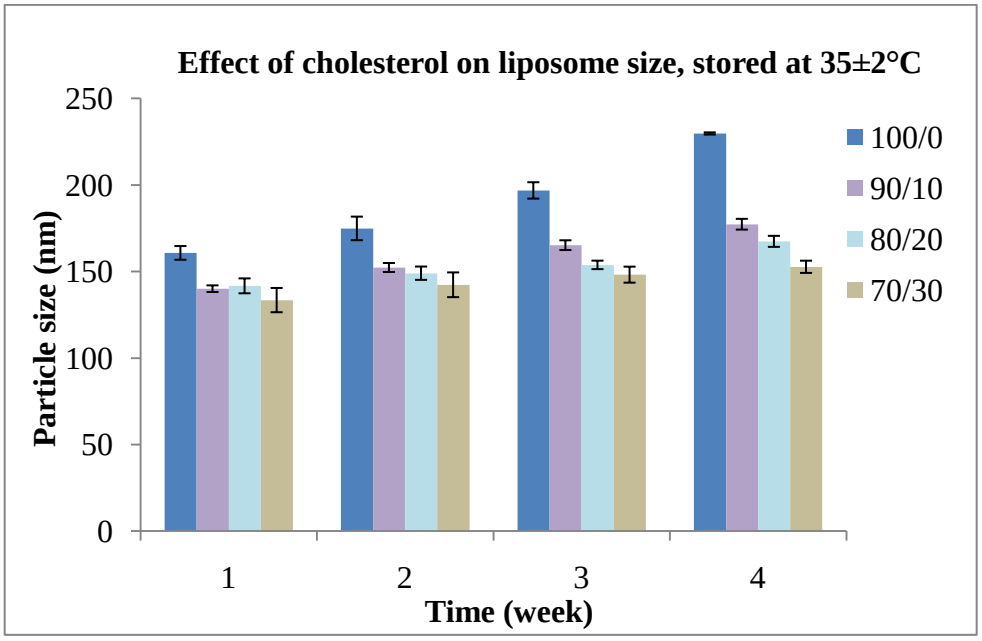


Figure 3.6: Effect of cholesterol on particle size of various NaFl loaded conventional liposomes stored at 35±2ºC over a period of 4 weeks

Table 3.15: % of increase in particle size of various NaFl loaded conventional liposomes stored at three different storage conditions at the end of 4 weeks

% of increase in particle size at the end of 4 weeks			
Code	**4±2ºC**	**25±2ºC**	**35±2ºC**
NaFl CON 0	3.09	8.47	42.94
NaFl CON 10	2.06	4.11	26.59
NaFl CON 20	2.54	5.92	18.13
NaFl CON 30	1.80	5.42	14.41

CON0, 10, 20 and 30 denote sodium fluorescein (NaFl) loaded conventional liposome with 0, 10, 20 and 30% w/w of cholesterol.

It is known that changes in average particle size of liposome dispersions are strongly affected by lipid composition. From Table 3.14 it is clear that cholesterol produced better stability in PC vesicles in all storage conditions. In presence of cholesterol in phospholipid bilayers, the amount of hydroperoxides produced by ionizing radiation is dramatically reduced. This decreased production of hydroperoxides can be explained by the protective effect by cholesterol, which is due to a steric hindrance to the radical chain propagation reaction through the unsaturated lipids. This effect can be explained by the property of cholesterol which modifies the physical state of bilayer from the solid-ordered phase to liquid-ordered phase[251]. Because of the presence of cholesterol between two adjacent unsaturated acyl chains of phospholipid, the order of bilayers in the liquid-crystalline phase is increased by promoting a tighter packing of the bilayer. This results in increased resistance to liposome oxidative degradation[252]. Water has been shown to play a major role in radiation-induced oxidative damage, with the aqueous phase being a source of reactive oxygen species [253-255]. Cholesterol reduces the content of water at the hydrophobic–hydrophilic interface of the bilayer, influences resistance of liposomes to oxidative damage and increases their stability[256]. Therefore cholesterol has been shown to have anti-oxidative properties in biological membranes and liposomes[257-259].

In an aqueous medium, liposomal phospholipids can hydrolyze to free fatty acids and lysophospholipids. Formation of free hydrolytic products might limit the shelf life of liposome. Elevated content of lysophospholipids tend to destabilize the bilayer and the membrane becomes more sensitive to vesicle fusion[250]. The stabilizing function of cholesterol was reported by Samuni *et al* [260] who measured the Laurdan's GP (Generalized Polarization) values for various egg PC- cholesterol liposomes (The increase in GP values reflects a decrease in bilayer hydration) and confirmed in their study that the presence of cholesterol in lipid bilayers decreased the hydration of the bilayer. This effect can again be explained by the phenomena that cholesterol increases bilayer packing in the head group and acyl chain regions, thereby decreasing the rate of diffusion of water into the bilayer[261-264]. Furthermore, decrease in water content and consequently protons and hydroxide ions results in a decrease of direct hydrolytic degradation caused to the membrane lipids. Altogether cholesterol reduces particle fusion and promotes better stability.

From Table 3.15, we can see that instability increased when the system temperature increased. Thus it is evident that the instability is mostly dependent on temperature. The increase in temperature is responsible for frequent collision between liposomes, which enhances thermal motion of liposomal bilayer, and accelerates the speed of liposome fusion[265]. Therefore NaFl CON 0 which showed marked particle aggregation at 35±2ºC, showed slight increase in size (3.09%) in refrigerated condition, whereas CON 30 that was most stable in all storage conditions showed lowest size increase (1.80%) at 4±2ºC. It is therefore highly recommended that all liposomes should be stored in refrigerated condition.

3.4.6. Development of Ibuprofen loaded liposome

IBU loaded conventional liposomes were prepared by following the thin film hydration technique as described under section 2.3.5.1. Initially all liposomes were prepared by dissolving IBU (1 mg/mL) with the lipid phase in the organic solvent followed by film hydration with 10 mL of water. In current study because water was used as a hydration medium for NaFl liposomes, for comparison purpose water was also chosen as the

hydration medium to prepare IBU liposomes initially. However we encountered the problem of IBU crystallization in water which resulted in an artificially high EE. This led to the decision made on choosing PBS pH 7.4 as hydration medium instead of water. Initially IBU loaded pure PC liposome prepared using water as hydration medium showed a very high EE (around 98%) which was unexpected for a compound like IBU and was very different to that reported by Mohammed *et al.* Mohammed *et al* reported an EE of 29.5±0.6% of IBU loaded pure PC liposome (PC=12.41 mg/mL PC and IBU=0.625 mg/mL) when liposomes were hydrated with water[212]. Therefore after taking into consideration that IBU has a very poor water solubility (60µg/mL[212]), the following investigation was conducted to reveal the true EE of IBU loaded liposomes.

A known amount of IBU was dissolved in approximately 0.5mL of ethanol. Sufficient amount of water was added to obtain a final drug concentration of 1 mg/mL. As soon as water was added the solution started to turn turbid due to the formation of IBU crystals. This dispersion (dispersion-1) was left on a stirrer overnight to allow the ethanol evaporates. Next day an aliquot of this dispersion was observed under light microscope to visualize the appearance of the drug crystals that originated from a solvent-water system. Another saturated solution of drug in pure water (dispersion-2) was prepared where drug crystals were visible by naked eye. This dispersion was prepared to compare with the crystals of dispersion-1.

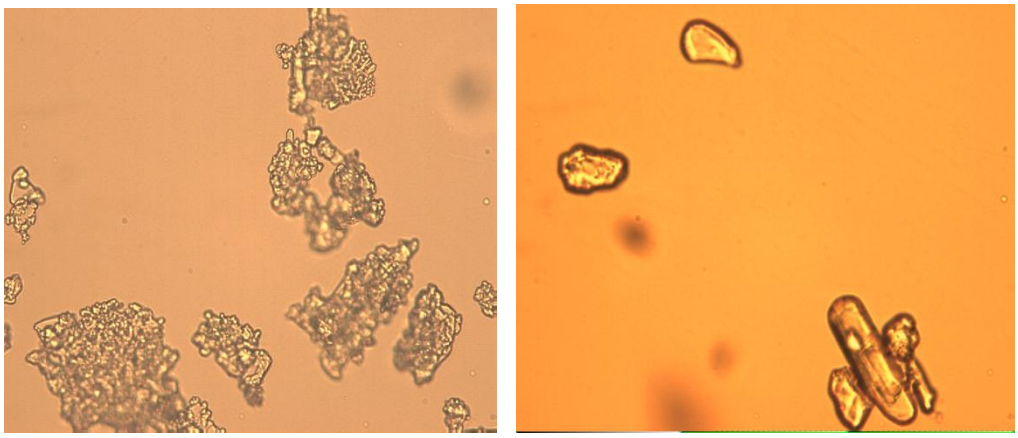


Figure 3.7: (a): Drug crystals found in dispersion-1 (at 40 X magnification) (b): Drug crystals found in dispersion-2 (at 40 X magnification)

The second investigation was to prepare IBU loaded pure PC liposome and to leave on bench top without downsizing for a period of 2 h. Large vesicles were supposed to settle down by this time along with the undissolved drug. An aliquot of the bottom layer of liposome suspension was sampled and observed under the light microscope.

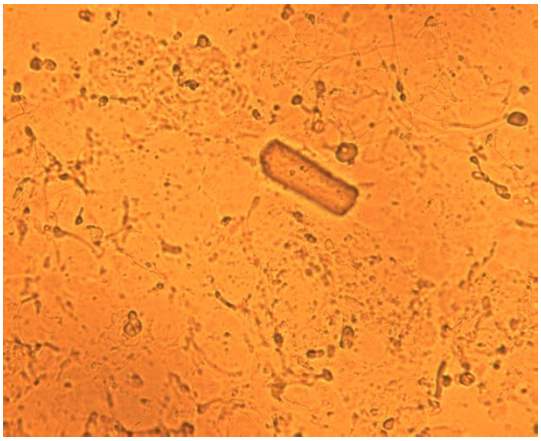


Figure 3.7(c): Bottom layer of Ibuprofen (IBU) loaded liposome showing drug crystal (at 40X magnification)

The presence of IBU crystal in liposomal suspension (Figure 3.7 (c)) confirmed our suspicion that the high entrapment of IBU was a result of poor solubility of IBU in water.

Based on the above finding, a washing protocol was developed to determine the true EE of IBU loaded pure PC liposome. Briefly liposomal vesicles were centrifuged using Nanosep®. The supernatant was removed completely from liposomal pellet and the pellet was washed twice with 50 mM PBS pH 7.4 by applying centrifugation speed of 4000 rpm for 20 min each. The two washes were collected and analyzed separately for drug content. A third wash of liposomal pellet using PBS was performed where no detectable drug was analyzed by HPLC. Drug contents in three supernatants (including two washes) were pooled to calculate the cumulative drug content. The EE was calculated from the equation stated in section 2.3.6.4, and was found to be around 25.3%. Therefore it was evident that the previous EE of 98% was incorrect. The artificially high EE was a result of a large amount of drug being insoluble in water, which upon centrifugation was unable to pass through the Nanosep®. Consequently drug

content “appeared” very high in liposomal system but in reality, only a very small quantity of drug was truly entrapped inside or associated with the liposomes.

Although IBU loaded liposomes could be prepared by hydration with water and the EE could be determined by using washing protocol, but an ideal liposome formulation should not contain undissolved drug in the system. Therefore a dialysis method was applied to separate the free IBU from liposomes. An exhaustive dialysis of 80 min (40 min in PBS and 40 min in water) was found enough to remove the free drug.

The second problem we encountered arose when cholesterol was added to the formulation. Vesicles composed of 10% w/w cholesterol formed with no difficulty but inclusion of 20% and 30% w/w cholesterol in IBU loaded PC liposomes resulted in the formation of very hard films which were not possible to be hydrated with water at 55ºC for 1 h. The hydration time was prolonged and beads were used to aid in hydration and swelling of films. Unfortunately this did not improve hydration. Even hydration with bath sonication above T_c[266] was attempted which produced hard small lipid flakes. No vesicles formed.

As water was found to be not suitable hydration medium for IBU conventional liposomes, PBS pH 7.4 was selected. IBU at pH 7.4 is highly ionized; therefore there was no solubility problem. Many potential penetrants are weak acids or weak bases and therefore ionization is possible. For such compounds it may be better to use buffer to prepare the liposome so that the solubility is maximized. On the other hand, drug in free acid or free base form (i.e. unionized) is better for skin permeation. At higher pH, IBU ionization is high, flux is high but permeability is low. In other words, high pH would ionize IBU and improve solubility[173] but skin permeability may be compromised as a result[171]. However the effect of drug ionization may not affect its skin permeability if it is trapped inside the liposomes because liposomes have its own mechanism of skin penetration. Ionisation effect would be only applicable while studying the penetration profile of ionized and unionized free drug solution. To ensure all IBU in soluble form and the maximum amount can be incorporated into liposomes, PBS pH 7.4 was eventually chosen as the hydration medium for IBU loaded liposome preparation.

3.4.7. Comparison of empty liposomes hydrated with PBS to that of hydrated with water

Empty liposomes were prepared in the same way as described in section 2.3.5.3 and were hydrated with PBS. Table 3.16 shows the size and zeta potential of various empty liposomes hydrated with PBS.

Table 3.16: Comparison of empty liposomes hydrated with PBS to that of hydrated with water

Formulation ratio (% w/w)	Size (nm)		Zeta potential (mV)	
	PBS	water	PBS	water
Pure PC	204.9±8.2	153.1±2.9	8.4±1.7	-5.9±0.6
PC/CH 70:30	138.7±3.0	131.3±3.7	-10.8±0.9	-27.2±0.7
85/15 PC/T80	123.6±6.6	90.9±5.8	3.6±0.3	-6.7±0.4
85/15 PC/S. Chol	127.7±3.2	69.3±0.8	-1.1±0.6	-15.2±3.3

CH: Cholesterol, PC: Phosphatidylcholine, S. Chol: sodium cholate, T80: Tween 80. Characterization of liposomes was done after ten times extrusion through 200 and 100 nm Supor® membrane filters. Data represented as mean± SD (n=3)

Hydration medium was found to show pronounced influence on size and zeta potential of various empty conventional and elastic liposomes. All empty liposomes hydrated with water produced smaller sized vesicles compared to that of hydrated with PBS. The zeta potential of different liposomes was also found to be dependent on hydration medium. All PBS hydrated empty liposomes had less negative zeta potential compared to that of water hydrated liposomes. The possible reason of bigger particle size in PBS hydrated liposomes could be due to the ionic interaction that might have expanded the bilayer thickness, resulting in an increase in particle size. It is reported that in presence of higher ionic strength medium (such as PBS pH 7.4), the positively charged choline head group of PC approaches to outer bilayer region, which causes to put a net positive charge on particles (section 3.4.1.3).

The size and zeta potential of various empty liposomes, however was found to be influenced by cholesterol and surfactants. Both cholesterol and surfactant containing liposomes produced smaller particle size compared to that of pure PC liposomes. Inclusion of cholesterol in liposomes caused a negative zeta potential on the particle. This could be due to the reason that the presence of cholesterol on bilayer increases the adsorption of negative ions on PC molecule from buffer. However compared to pure PC liposome, Tween 80 and sodium cholate containing elastic liposomes had more negative zeta potential which could be because of the hydrolysis of Tween 80 and adsorption of cholate ion (sodium cholate) on PC molecule.

3.4.8. Characterization of Ibuprofen loaded conventional liposome

The effect of cholesterol content on size distribution, zeta potential and EE of various IBU loaded conventional liposomes was investigated. Vesicles were prepared from a range of PC (70-100 mg) and cholesterol (0-30 mg). They were coded as IBU CON 0 (pure PC, containing no cholesterol), and IBU CON 10, 20 and 30 (containing 10, 20 and 30% w/w cholesterol respectively).

3.4.8.1. Vesicle size distribution

The effect of cholesterol content on IBU loaded liposome size, zeta potential and EE was investigated. Table 3.17 shows various characteristics of IBU conventional liposomes. Before extrusion all vesicles reduced in particle size with subsequent addition of cholesterol. The maximum vesicular diameter found was with IBU CON 0 (298.6±11.3 nm) whereas IBU CON 30 showed the lowest particle size of 182.7±6.7 nm. Before extrusion, all formulations showed large polydispersity index indicating wider size distribution. After extrusion, the size of all liposomes dropped considerably. The biggest size reduction was observed in pure PC liposome. However the particle size of IBU CON 10 was higher than that of CON 0, but reduced in liposomes containing 20% and 30% w/w cholesterol. The cholesterol-induced size reduction of various conventional IBU liposomes can be explained by the condensation effect of cholesterol

in bilayer, a decrease in effective area per molecule[230] due to an increased packing density, leading to decreased particle size.

The polydispersity index of all IBU liposomes reduced considerably when vesicles were extruded. Upon extrusion the particle size usually becomes smaller and produces more uniformly shaped particles due to extrusion through a fixed pore sized filters repeatedly.

3.4.8.2. Zeta potential

Almost all particles in contact with a liquid acquire an electric charge on their surface. Compared with the zeta potential of empty pure PC and 30% w/w cholesterol containing liposome, the zeta potential of IBU loaded liposomes were more negative. This could be due to the reason that IBU is highly ionized at pH 7.4 (pKa=4.6). Being a carboxylate anion its adsorption on liposome forms part of an electrical double layer on liposome particle, which in turn was detected as a weak net negative charge on the surface of liposomes[267].

Table 3.17 shows that the zeta potential of various IBU loaded conventional liposomes were cholesterol dependent which is supported by the study of Minghui *et al* in which they showed the zeta potential of salidroside nanoliposomes gradually increased in magnitude with the increasing cholesterol content[268]. In the current study, pure PC liposome had a positive charge (1.8±0.4). 10% w/w addition of cholesterol caused a weak negative charge (-0.8±0.3) on particle. However 20 and 30% w/w cholesterol produced more negative charge. Such elevation in the negative zeta potential was also in agreement with the data reported by Liu *et al* who found progressively higher negative zeta potential due to subsequent addition of cholesterol to egg PC liposomes. This could be explained by the phenomena that incorporation of cholesterol into the bilayer reduces the surface binding affinity of the cations from the buffer solution on the bilayer surface[269].

3.4.8.3. Entrapment efficiency

The influence of cholesterol inclusion within the lipid composition on IBU EE was determined by varying cholesterol content from 0 to 30% w/w. The result of drug EE of various IBU loaded conventional liposomes indicated that as the concentration of PC decreased, EE decreased. The highest EE found was with CON 0, which was around 33.8%. However EE reduced when 10 % w/w cholesterol was added to the system. The lowest EE found was with CON 30, around 21.0% (Table 3.17).

Table 3.17: Characterization of IBU loaded conventional liposomes

Code	PC:CH (w/w)	Size-1 (nm)	Size-2 (nm)	P.I-1	P.I-2	Zeta potential (mV)	EE (%)
IBU CON 0	100:0	298.5±11.3	143.0±3.7	0.85±0.11	0.33±0.02	1.8±0.4	33.8±2.0
IBU CON 10	90:10	252.6±3.3	157.8±3.6	0.72±0.10	0.34±0.03	-0.8±0.3	27.2±1.6
IBU CON 20	80:20	235.1±6.2	154.7±4.2	0.55±0.08	0.34±0.04	-4.8±1.0	22.7±2.2
IBU CON 30	70:30	182.7±6.7	147.5±3.8	0.52±0.05	0.37±0.02	-12.2±0.5	21.0±1.8

CH: Cholesterol; PC: Phosphatidylcholine; IBU CON 0: Ibuprofen (IBU) loaded conventional liposome with no cholesterol, IBU CON 10, 20 and 30 denote IBU loaded conventional liposome prepared from PC: CH ratio of 90:10, 80:20, and 70:30 respectively.

Size-1, P.I-1 = Size and polydispersity index of vesicles determined after sonication but before extrusion

Size-2, P.I-2 = Size and polydispersity index of vesicles determined after extrusion through 200 and 100 nm Supor® membrane filters

EE= Entrapment efficiency

Data represented as mean± SD (n=5)

At pH 7.4, ionization of IBU is more than 50%. Ionized species tend to be strongly associated with the liposomal lipid membrane by means of electrostatic interactions with the zwitterionic phospholipid of liposomes[270]. Drug-liposome membrane interaction can be expressed as δ_{mem} which is the logarithm of the ratio of P^{N}_{mem} to P^{I}_{mem} for the liposome water system where P^{N}_{mem}= Partition coefficient of neutral species in liposomal membrane; P^{I}_{mem}= Partition coefficient of ionized species in liposomal membrane[172]. The larger the δ_{mem} value, the smaller the affinity of ionized species for the liposome phase, relative to that of the neutral phase partitioning. The δ_{mem} value of IBU is shown in Table 3.18. However the partitioning of ionized drugs to lipid membrane is influenced by the acidity and basicity of drugs. The charged forms of amphiphilic (anionic) acids have less affinity for PC-based membranes than charged forms of amphiphilic (cationic) bases compared to uncharged species respectively[172]. Generally when ionized species move toward the aqueous exterior the first charge it experiences are those of the negatively charged phosphates. Further movement would let the ionized drug come closer to the vicinity of positively charged trimethylammonium groups (from choline). In case of ionized weak acids, for example IBU, this movement may lead to weakened membrane binding because of less affinity of ionized IBU for the liposomal membrane (Table 3.18).

Table 3.18: IBU-liposomal membrane water system[271]

Parameters for liposomal membrane water system	
$\log P^{N}_{mem}$	3.80±0.03
$\log P^{I}_{mem}$	1.81±0.05
δ_{mem}	1.99

In current study, EE of IBU loaded liposomes decreased with an increase in cholesterol content. Maximum entrapment was obtained from pure PC IBU liposome. This could be explained by the binding of ionized IBU with liposomal membrane which would allow anionic head group of IBU to be associated with the positively charged choline of PC and more of the hydrophobic portion of IBU would insert into the lipid membrane in a

configuration similar to that of cholesterol. This is further supported by Yang *et al*. IBU is an amphipathic molecule with an ionizable carboxylic acid at PBS pH 7.4. Yang *et al* from the proton NMR spectroscopy suggested that the negatively charged carboxyl group of IBU may interact with the positively charged trimethyl head group of PC, while the phenyl and isobutyl groups of the IBU may take part in the Vander Waals interaction with the acyl regions of the lipids [243]. Therefore it is assumed that binding of ionized IBU with the liposomal membrane would be due to drug partitioning and ionic interaction; while this binding may be comparatively smaller than its non-ionized form, yet considerably interfered with the EE of IBU loaded liposomes.

The decrease in entrapment of IBU in liposomes with each addition of cholesterol could be explained by the membrane binding affinity of IBU. Cholesterol prefers to align in the hydrophobic region of the membrane and provides a limited space available for the ionized IBU by lowering the partitioning of drug molecules to the bilayer membrane. Therefore the higher amounts of cholesterol might have competed with IBU for the packing space within the bilayer excluding the drug as the amphiphiles assemble into liposomes[212]. Moreover as cholesterol increases, the negative charge increased (Table 3.17); this would tend to repel negatively charged IBU from the bilayer further, resulting in reduced EE.

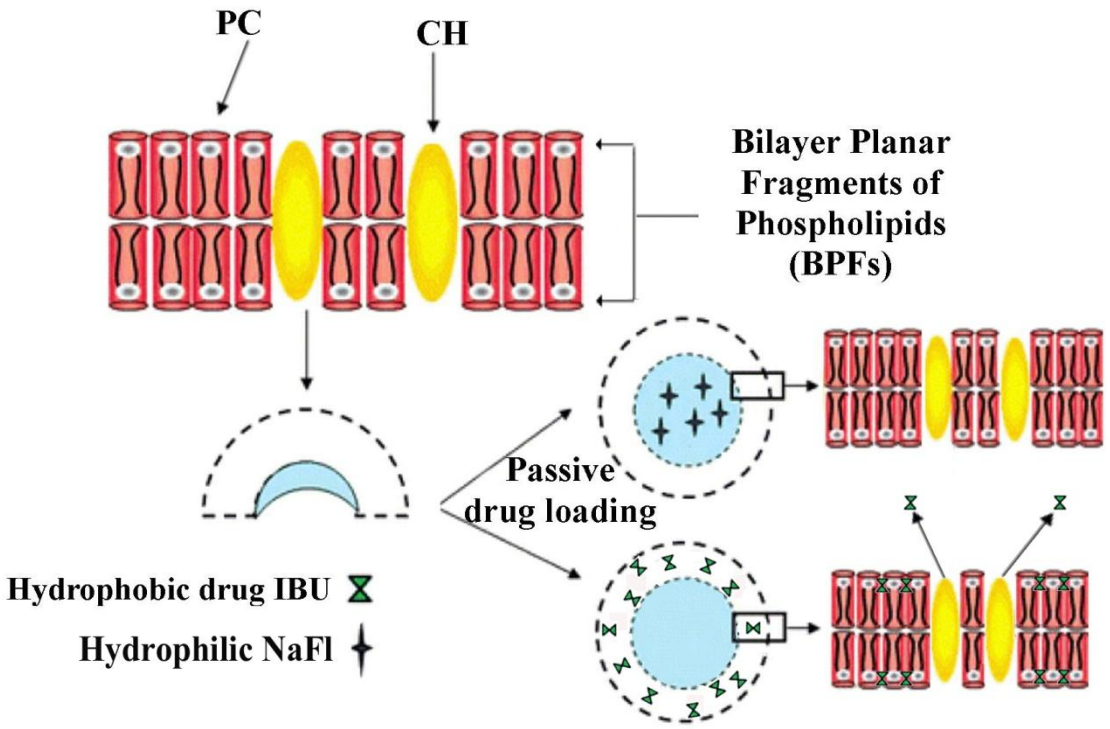


Figure 3.8: A typical diagram of passive drug loading: possible location of trapped molecule (figure adapted from Jaafar-Maalej *et al* [241] and modified)

3.4.8.4. Bilayer elasticity

To determine elasticity of IBU loaded conventional liposomes, two most different formulations were chosen; IBU CON 0 and IBU CON 30. Their deformability indexes were found to be 8.4±1.3 and 4.8±0.8 respectively. As expected conventional liposomes showed poor elasticity. However the higher elasticity found with pure PC liposome is due to the absence of cholesterol in the formulation. Cholesterol is reported to restrict the mobility of phospholipid tails, making it more rigid for particle deformation[248].

Table 3.19: Elasticity measurement of IBU conventional liposome

Code	PC:CH (w/w)	Size (nm)	Polydispersity Index	*J* (mL)	*D*
IBU CON 0	100:0	115.2±5.5	0.23±0.01	1.6±0.2	8.4±0.3
IBU CON 30	70:30	112.7±2.7	0.25±0.02	0.9±0.2	4.8±0.8

CH: Cholesterol; PC: Phosphatidylcholine; IBU CON 0 and IBU CON 30 denote Ibuprofen (IBU) loaded conventional liposomes with no CH and 30% w/w CH respectively.

Size and polydispersity index of vesicles determined after extrusion through 50 nm polycarbonate membrane filter

J= Vol of suspension extruded within 5 min

D= Degree of deformability

Data represented as mean± SD (n=2)

Optimization of IBU loaded conventional liposomes was based on particle size and EE. IBU CON 0 was chosen as the best formulation because of its smaller vesicular diameter and highest EE. Furthermore the elasticity of pure PC IBU liposome was better than that of IBU CON 30. As not much deformability is expected in conventional liposomes, the relatively better elasticity was considered as an additional selection criterion.

3.4.9. Characterization of elastic liposomes loaded with Ibuprofen

Tween 80 and sodium cholate were utilized to develop IBU loaded elastic liposomes. Table 3.20 and Table 3.21 show various characteristics of IBU elastic liposomes. To simplify the names, they were coded by the name of the drug incorporated and the type and weight of surfactant added to formulation. For example, IBU T80 5 and IBU S. Chol 5 represent IBU loaded elastic liposome which contained 5% w/w of Tween 80 and sodium cholate respectively.

3.4.9.1. Particle size distribution

All IBU loaded elastic liposomes were characterized for particle size both before and after extrusion (Table 3.20 and Table 3.21). The more surfactant was added to bilayer, the smaller was the particle size which is consistent with the finding obtained from NaFl loaded elastic liposomes in which all surfactant containing liposomes had sizes much smaller than those without surfactant (Table 3.16). Table 3.20 shows that the highest vesicular diameter was found with elastic liposome containing Tween 80 at 5% w/w (171.83±6.20 nm), which reduced to 146.8±2.69 nm when 15% w/w Tween 80 was added. Similar size reduction pattern was observed with sodium cholate, where IBU S. Chol 5 had the largest particle size of about 181.23 nm; 15% w/w sodium cholate reduced size to 148.2±3.05 nm (Table 3.21). This result resembled that of PEG-grafted liposomes; a finding by Yoshida *et al*[272] where decreased particle size was reported with increased surfactant. However a significant drop in size was observed when surfactant in the system above was above 15% w/w; 110.4±1.13 nm and 113.75±6.85 nm for 25% w/w Tween 80 and sodium cholate respectively. This could be due to the presence of micellar structure at higher concentration of surfactant. After extrusion, the particle size of various elastic liposomes did not reduce significantly, but polydispersity index became smaller indicating relatively narrower particle size distribution. In both types of elastic liposomes size reduction occured significantly in 25% surfactant containing elastic liposomes (P<0.05 and <0.03 for Tween 80 and sodium cholate respectively) compared to that of 5% surfactant.

3.4.9.2. Zeta potential

The zeta potential found in Tween 80 containing elastic liposomes were slightly less positive and that in sodium cholate containing elastic vesicles were slightly more negative compared to that of empty elastic liposomes (Table 3.20, 3.21 and 3.16). The possible reason could be due to the adsorption of anionic IBU on liposomal surface[267].

3.4.9.3. Entrapment efficiency

The EE in all elastic liposomes reduced when the surfactant content was increased (Table 3.20 and 3.21). Maximum drug encapsulation was found in vesicles with 5% w/w of surfactant (30.7±1.4% and 26.7±2.4% for Tween 80 and sodium cholate respectively). Up to 15% addition of surfactant, the reduction in EE was not significant (P>0.05). A significant drop in EE was observed only when more than 15% edge activator was added. This could be due to the presence of vesicles and mixed micelles at 20% surfactant and mixed micelles at 25% surfactant[247, 248].

Table 3.20: Characteristics of IBU loaded elastic liposomes prepared using PC and Tween 80

Code	PC:T80 (w/w)	Physicochemical Property						Elasticity measurement			
		Size-1 (nm)	Size-2 (nm)	P.I.-1	P.I.-2	Zeta Potential (mV)	EE (%)	Size-3 (nm)	P.I.-3	*J* (mL)	*D*
IBU T80 5	95:5	171.8±6.2	150.6±2.6	0.46±0.06	0.34±0.01	2.2±0.7	30.7±1.4	115.3±3.0	0.21±0.02	2.6±0.1	13.8±1.2
IBU T80 10	90:10	152.3±3.2	141.6±2.7	0.43±0.05	0.35±0.04	2.1±0.5	27.5±1.7	114.8±0.6	0.22±0.05	3.4±0.1	17.9±0.6
IBU T80 15	85:15	146.8±2.7	136.0±2.7	0.43±0.05	0.33±0.01	2.1±0.2	24.4±1.9	106.8±1.3	0.19±0.10	6.8±0.0	31.1±1.5
IBU T80 20	80:20	119.8±2.3	114.7±2.3	0.45±0.01	0.34±0.02	2.4±0.2	19.4±3.3	96.4±2.3	0.26±0.11	1.8±0.1	6.7±1.3
IBU T80 25	75:25	110.4±1.1	105.3±1.2	0.45±0.03	0.33±0.02	2.1±0.2	16.9±2.2	96.8±0.8	0.24±0.00	1.1±0.2	4.1±0.4

PC: Phosphatidylcholine; T80: Tween 80, IBU T80 5, 10, 15, 20 and 25% denote Ibuprofen (IBU) loaded elastic liposome containing 5, 10, 15, 20 and 25% w/w T80

Size-1, P.I-1 = Size and polydispersity index of vesicles determined after sonication but before extrusion

Size-2, P.I-2 = Size and polydispersity index of vesicles determined after extrusion through 200 and 100 nm Supor® membrane filters

Size-3, P.I-3 = Size and polydispersity index of vesicles determined after extrusion through 50 nm polycarbonate membrane filters

EE= Entrapment Efficiency

J= Vol of suspension extruded within 5 min

D= Degree of deformability

Data represents as mean ±SD (n=5) except the elasticity measurements which were done twice

Table 3.21: Characteristics of IBU loaded elastic liposomes prepared using PC and sodium cholate

Code	PC:S. Chol (w/w)	Physicochemical Property						Elasticity measurement			
		Size-1 (nm)	Size-2 (nm)	P.I.-1	P.I.-2	Zeta Potential (mV)	EE (%)	Size-3 (nm)	P.I.-3	*J* (mL)	*D*
IBU S. Chol 5	95:5	181.2±5.4	155.0±5.4	0.58±0.03	0.32±0.02	-2.9±0.1	26.7±2.4	118.6±3.0	0.24±0.03	2.1±0.1	11.8±1.5
IBU S. Chol 10	90:10	162.0±7.0	145.2±4.5	0.51±0.01	0.33±0.02	-2.2±0.4	25.2±0.7	115.8±1.1	0.21±0.10	2.9±0.2	15.5±0.4
IBU S. Chol 15	85:15	148.2±3.0	142.2±2.0	0.45±0.03	0.39±0.01	-2.5±1.1	23.0±0.7	111.4±2.6	0.22±0.12	5.8±0.2	28.8±2.1
IBU S. Chol 20	80:20	125.1±3.9	117.8±2.0	0.45±0.02	0.33±0.02	-2.8±0.1	17.8±1.1	104.4±4.6	0.21±0.10	1.2±0.1	5.2±1.3
IBU S. Chol 25	75:25	113.7±6.8	109.5±0.8	0.58±0.01	0.36±0.01	-3.5±0.1	14.9±0.7	92.3±2.1	0.25±0.3	1.0±0.1	3.4±1.0

PC: Phosphatidylcholine; S. Chol: sodium cholate, IBU S. Chol 5, 10, 15, 20 and 25% denote Ibuprofen (IBU) loaded elastic liposome containing 5, 10, 15, 20 and 25% w/w S. Chol.

Size-1, P.I-1 = Size and polydispersity index of vesicles determined after sonication

Size-2, P.I-2 = Size and polydispersity index of vesicles determined after extrusion through 200 and 100 nm Supor® membrane filters

Size-3, P.I-3 = Size and polydispersity index of vesicles determined after extrusion through 50 nm polycarbonate membrane filters

EE= Entrapment Efficiency

J= Vol of suspension extruded during 5 min

D= Degree of deformability

Data represents as mean ±SD (n=5) except the elasticity measurements which were done twice

3.4.9.4. Bilayer elasticity

The deformability of various IBU loaded elastic liposomes was found to be surfactant concentration dependent (Table 3.20 and 3.21). The relationship was non-linear, more in a bell shape. Interestingly maximum elasticity was obtained when both Tween 80 and sodium cholate were at 15% w/w surfactant. Above this, a significant drop of elasticity was noticed in both types of elastic vesicles (Table 3.20 and 3.21). It is known that only an optimum concentration of surfactant can provide maximum bilayer elasticity that results in better skin permeability[246]. Increasing this surfactant content above a certain level does not bring any benefit in skin penetration due to bilayer solubilization by surfactants[245] and formation of micelles whose aggregate tend to block the pores of membrane therefore producing apparently low degree of deformation.

3.4.9.5. Morphology of Ibuprofen loaded liposomes

IBU loaded various conventional and elastic liposomes were observed under TEM and SEM where they appeared as spherical structures (Figure 3.9 (a) to Figure 3.9 (d)). A typical SEM micrograph of IBU CON 0 is shown in Appendix 6 and a TEM micrograph of 1 month old IBU S. Chol 15 stored at 4±2ºC is shown in Appendix 7.

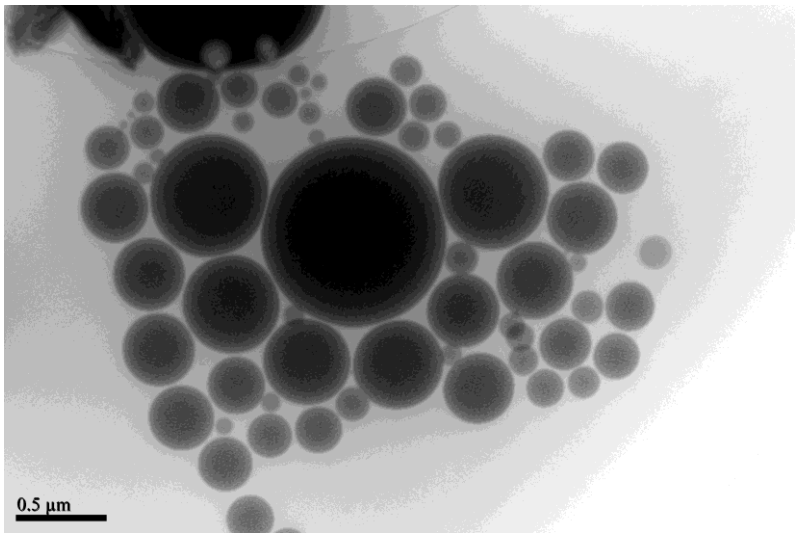


Figure 3.9 (a): TEM micrograph of crude IBU S. Chol 15 (Ibuprofen loaded elastic liposome containing 15% w/w of sodium cholate)

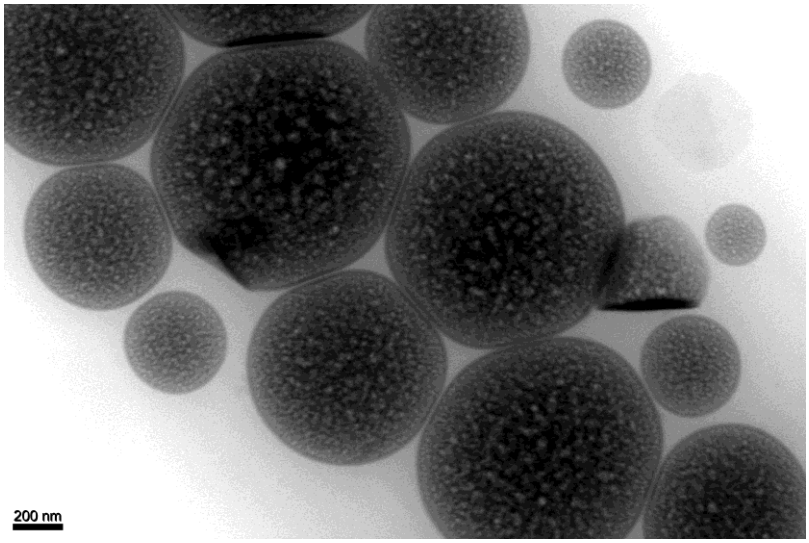


Figure 3.9 (b): TEM micrograph of sonicated IBU T80 15 (Ibuprofen loaded elastic liposome containing 15% w/w of Tween 80). The white features inside vesicles could be inner dried liposomal components which were visible on a narrow focus.

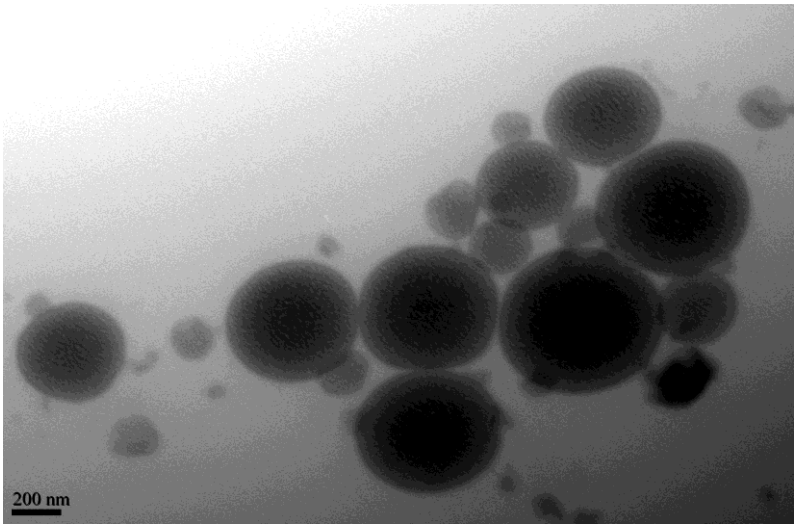


Figure 3.9 (c): TEM micrograph of sonicated (through 100nm filter) IBU CON 0 (Ibuprofen loaded conventional liposome containing no cholesterol)

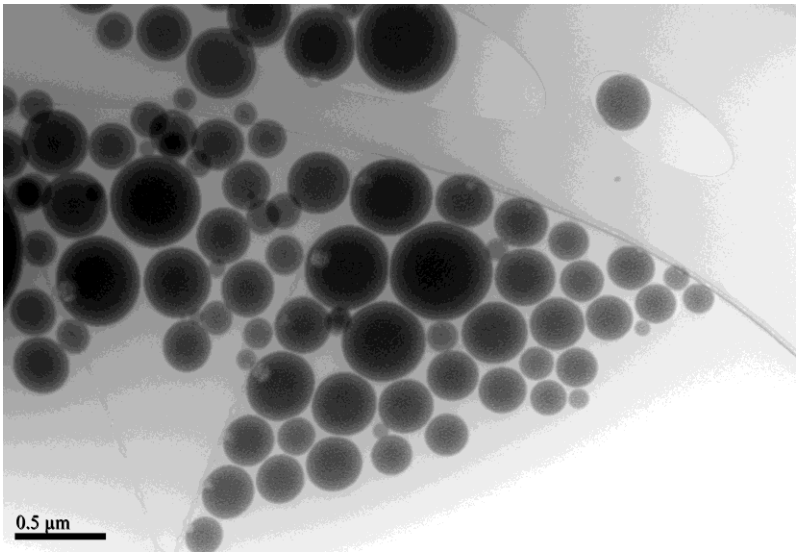


Figure 3.9 (d): TEM micrograph of extruded IBU CON 30 (Ibuprofen loaded conventional liposome containing 30% w/w cholesterol)

3.5. *In vitro* release study

The *in vitro* release profile of the entrapped drug may not predict exactly how a delivery system would behave in vivo but it will allow us to compare the release characteristics of different formulations. In the current study, a dialysis method, described under section 2.3.8.3 was used to investigate the release rate of NaFl and IBU from various liposomes using 50 mL PBS (50mM, pH 7.4 containing 0.05% sodium azide) as receptor. Drug release by dialysis has the advantage of separating free drug from liposomes without the application of centrifugal force which potentially can destroy liposomal structure. The release study was conducted using various optimized formulations and respective controls.

The drug release or diffusion from liposomes is governed by the transfer of the molecules from the liposome system to the external aqueous phase and diffusion of molecule through the dialysis membrane from the external phase to the receptor fluid[233]. A fixed surface area of dialysis membrane, fixed quantity of drug inside the dialysis bag and fixed volume of final formulation should be employed to eliminate variation within identical formulations. Any defects could lead to huge variations in the release profile of same liposomes and would generate untrue or unmeaningful data for formulations. Moreover if sufficient agitation is not employed, a stagnant

layer may form, which will represent an opposing force to drug release from the formulation through the dialysis membrane, leading to a faulty and unsatisfactory release profile. In this study all these factors were controlled to ensure the generation of meaningful data.

3.5.1. In vitro release of sodium fluorescein from liposomes

Evaluation of *in vitro* drug release from various NaFl encapsulated liposomes was conducted 35±2ºC by the dialysis method. The *in vitro* release behaviour of the free NaFl (i.e. Control), a conventional liposome (NaFl CON 30) and two elastic liposomes (NaFl T80 15 and NaFl S. Chol 15) is profiled by plotting cumulative percentage of NaFl released over time (h) and the results are presented in Figure 3.10. To maintain the sink condition drug concentration in the release medium should be kept below 10% of saturation[199]. As NaFl is freely soluble in water, maintaining a sink condition in buffer was not a issue.

To study the effect of liposome formulation on the release of NaFl, a control sample containing only the drug solution was used to generate data on drug diffusion throughout the dialysis membrane. Drug release from liposome dispersions was found to be a function of the formulation variables. Significant prolonged NaFl release was achieved with the liposome formulations in comparison with that of free drug solution (control; 96.46% after 90 min). NaFl release from 30% w/w cholesterol containing liposome was the slowest, 43.82±3.52% over 24 h while elastic liposomes provided relatively faster release (62.10±1.23% with Tween 80 and 57.84±1.45% with sodium cholate) over the same period of time. From the release profile it appears that drug release from liposomes was a biphasic process consisting of an initial faster release for the first 2 h, followed by a relatively slow release, lasting over the next 22 h. The initial burst effect of drug release varies with the liposome type and lipid composition. For example, in NaFl CON 30, during the first 2 h 28.15±4.24% of NaFl released whereas Tween 80 and sodium cholate containing elastic liposomes released 49.25±1.87% and 42.19±1.85% of NaFl.

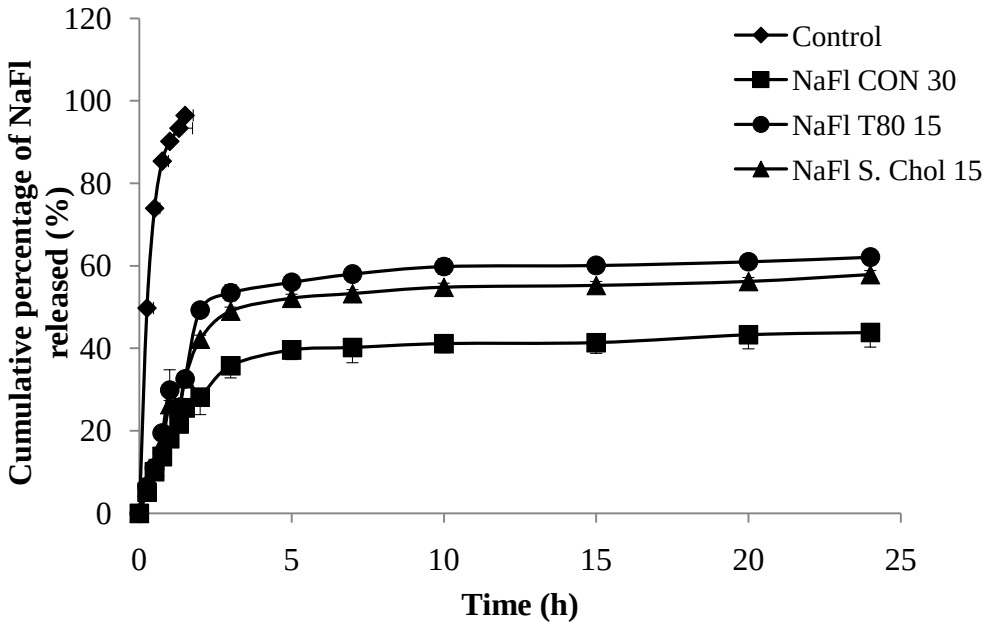


Figure 3.10: Cumulative percentage of sodium fluorescein (NaFl) release as a function of time across dialysis membrane from different liposomes and control in water over 24 h. Control represents 1mg/mL NaFl solution in water, NaFl CON 30 represents NaFl loaded conventional liposome composed of 70% w/w phosphatidylcholine (PC) and 30% w/w cholesterol, NaFl T80 15 and NaFl S. Chol 15 represent NaFl loaded elastic liposomes containing 15% w/w Tween 80 and sodium cholate in conjunction with PC respectively. Data represents as mean ±SD (n=3).

The slow release from cholesterol containing liposome can be explained by the stabilizing effect of cholesterol in the bilayers. At above the T_c (55ºC for cholesterol containing liposome), cholesterol modulates membrane fluidity by restricting the movement of the relatively mobile hydrocarbon chains, reducing bilayer permeability[273] and decreasing the efflux of the encapsulated drug, which results in prolonged drug retention[274]. On the contrary, the faster drug release from elastic liposomes compared to that of conventional liposome was due to the high fluidity provided by the incorporation of edge activator[233]. The significantly lower release of NaFl from both conventional and elastic liposomal formulations as compared to the diffusion of free NaFl indicated a sustained release from liposome formulations.

So far no published literature on the release profile of NaFl from liposome was found which employed the same conditions that were used in present study. However Henriksen et al[275] showed around 15% of carboxyfluorescein (100 mM carboxyfluorescein solution in 60 mM Tris Buffer pH 8) released from liposome containing 50 mg/mL PC after 5h following a fractional dialysis method. Compared with Henriksen's report, the release of NaFl observed from different liposomes in current study was faster in the same period of time, about 39.5% and 52-56% from conventional and elastic liposomes respectively. This is expected as the lipid concentration in current study is 10 mg/mL, much lower than that of Henriksen's study. In addition, NaFl is more water soluble than carboxyfluorescein and a relatively low dose of NaFl was used in our study (37.6 mg/mL carboxyfluorescein in Henriksen's, report whereas in current study, 1mg/mL). Higher lipid concentration eventually lead to slow release of entrapped drug and higher drug concentration will probably take time to diffuse through the dialysis membrane.

For an ideal topical delivery, improved skin permeation as well as sustained release of drug is desirable. Though drug release was slower from cholesterol containing liposomes than that of elastic liposomes, traditional liposomes are not able to penetrate deeper into the skin which may not help ultimately. Considering the permeability through skin, elastic liposomes are advantageous which allow for drug delivery into the skin.

3.5.2. In vitro release of Ibuprofen from liposomes

3.5.2.1. Effect of cholesterol

The same dialysis method was applied to investigate the effect of cholesterol on IBU release from various conventional liposomes. Results of the *in vitro* release study are shown in Figure 3.11. The release profile was found to be apparently biphasic in nature; initial rapid drug leakage was observed where about 39-48% of the entrapped drug was released from different IBU conventional liposomes in the first 2 h of incubation. However during the following 22 h, a slow release occurred in which a maximum of 26% of IBU was leached from different conventional liposomes. The initial burst release of IBU could be because highly ordered lipid particles cannot

accommodate large amounts of drug. A significant amount of IBU was associated with or adsorbed to the surface of liposomes, which released quickly after being exposed to large volume of buffer[276]. The slow release in next 22 h could be attributed to the release of IBU which was trapped deep inside bilayer of liposomes.

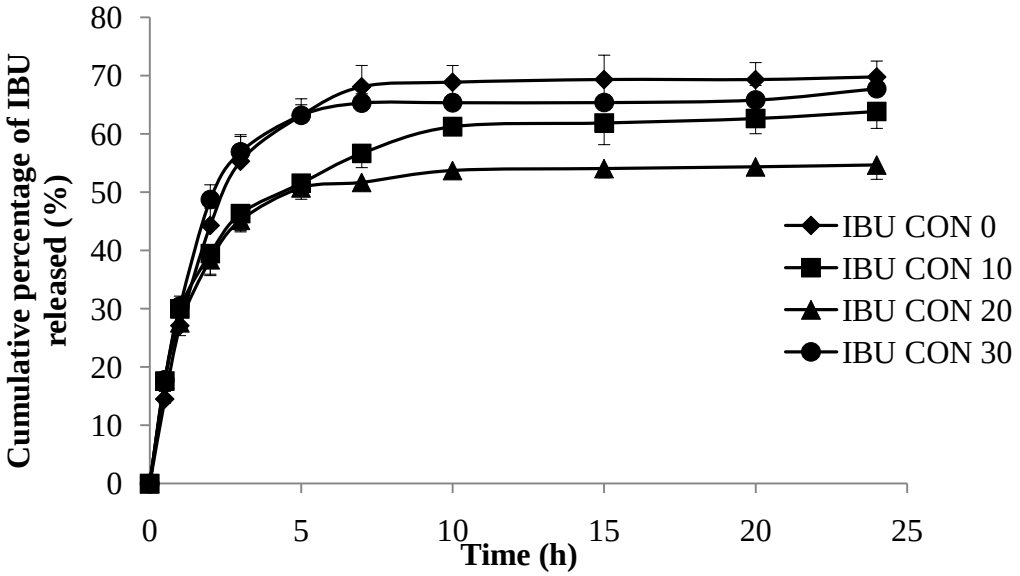


Figure 3.11: Cumulative percentage of Ibuprofen (IBU) release as a function of time across dialysis membrane from different conventional liposomes over 24 h. IBU CON 0, IBU CON 10, IBU CON 20, IBU CON 30 represent IBU loaded conventional liposomes containing Phosphatidylcholine (PC) with 0, 10, 20 and 30% w/w cholesterol. Data represents as mean ±SD (n=3).

From Figure 3.11, it is observed that cholesterol free IBU conventional liposome showed slightly higher release (approximately 69.8%) after 24 h compared to those containing 10% cholesterol (IBU CON 10) in the system where 63.87% of IBU released. Significant slower drug release was observed from IBU CON 20 releasing approximately 54.7% of drug over 24 h compared to that of pure PC liposomes (P<0.002). However further increase in cholesterol content (up to 30%) in the lipid bilayer showed a faster release (67.78%) compared with that of IBU CON 20. IBU release from IBU CON 30 not very significantly different from that of pure PC liposome (P=0.0426).

The faster release profile of IBU from pure PC liposomes could be explained by the absence cholesterol in the structure. It is generally accepted that cholesterol decreases the leakage and permeability of entrapped drug from liposomal bilayer. Cocera *et al* [277] reported that cholesterol produced an optimum hydrophobicity which decreased the formation of transient hydrophilic holes, by decreasing membrane fluidity, producing a more ordered structure, responsible for drug release through liposomal layers[158]. However it is reported that higher amount of cholesterol in liposome beyond a certain concentration may disrupt the regular linear structure of vesicular membrane and increase drug release consequently[76].

The release of IBU from various cholesterol containing conventional liposomes was investigated by Mohammed *et al*[212] who reported approximately 30% of IBU release from 16:4 µmol of PC: cholesterol liposome (equivalent to 12.16 mg/mL PC and 1.546 mg/mL cholesterol) over a period of 5 h. In our study, higher drug release from 10% w/w cholesterol containing liposome was obtained. This difference in release rate could be due to several reasons. First of all, Mohammed *et al* investigated drug release from MLVs whereas SUVs were prepared in current study. Bilayer lamellarity is an important parameter that affects the drug release. It is generally accepted that drug release from MLVs is slower than that of SUVs due to having more concentric lamellae in case of the former, which acts as a drug diffusion barrier. Moreover Mohammed *et al* formulated all IBU liposomes from water, whereas PBS was used as hydration medium in present study. The effect of solubility and ionization on drug release might have an influence here.

3.5.2.2. Effect of Surfactants

An *in vitro* release study was performed on IBU loaded optimized elastic liposomes (IBU T80 15 and IBU S. Chol 15) with free IBU as control. For comparison purpose, cumulative release rate of IBU CON 0 was also included in the plot. The release profile is shown in Figure 3.12. The release of IBU from formulations was found to be biphasic in nature. Initial rapid release was observed which lasted during the first 3 h when maximum 66% of drug was released. Then it was followed by a steady release during the next 21 h. Drug release from free drug was found to be highest than that of formulations; 95% IBU was released into the receptor from control

within 1 h, whereas the average release from liposomes was around 28.6%. Therefore all IBU loaded liposomes showed sustained release.

As expected drug release from elastic liposomes was higher than that of conventional liposome. At the end of 24 h, 69.8, 74.06 and 81.89% of IBU released from pure PC, Tween 80 and sodium cholate containing liposomes respectively. This is due to the presence of bilayer softening agent in elastic liposomes which tend to make the liposomes more leaky, thus provides faster drug release.

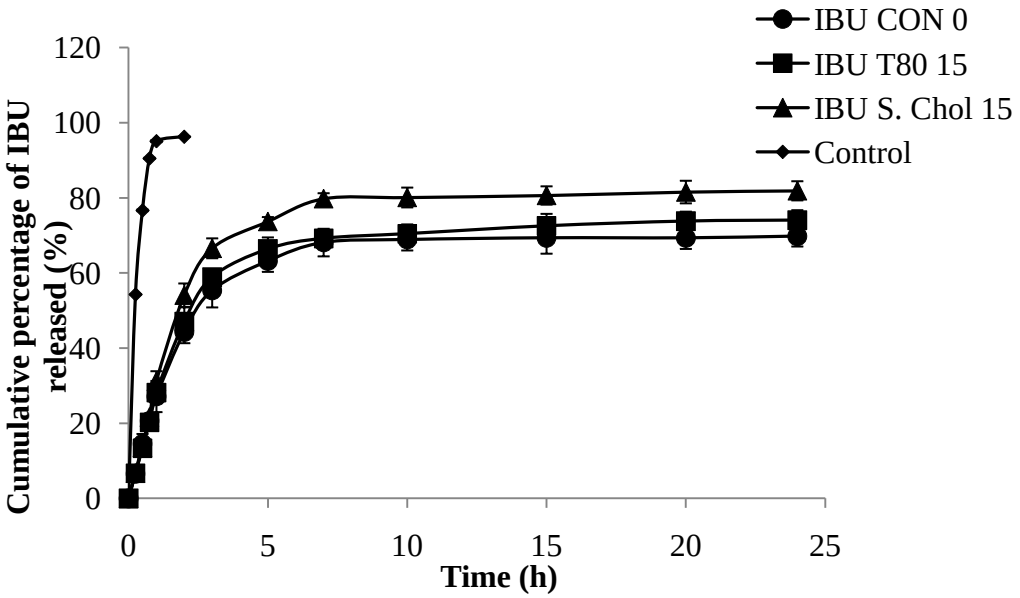


Figure 3.12: Cumulative percentage of Ibuprofen (IBU) release across dialysis membrane from different liposomes and control in PBS pH 7.4 over 24 h. IBU CON 0 represents IBU loaded conventional liposomes containing no cholesterol, IBU T80 15 and IBU S. Chol 15 represent IBU loaded elastic liposomes containing 15% w/w Tween 80 and sodium cholate respectively in conjunction with phosphatidylcholine (PC). Control: 1mg/mL of IBU solution in PBS pH 7.4. Data represents as mean ±SD (n=3).

Compared with the release rate of NaFl, IBU liposomes showed relatively faster drug release. This could be due to the relatively stronger electrostatic repulsion between negatively charged ionized carboxyl groups of IBU and phosphate groups in PC in pH 7.4 media[278].

3.6. *In vitro* permeation of Ibuprofen through human epidermis

The non-occlusive *in vitro* skin penetration study, using Franz type diffusion cells mounted with excised human abdominal epidermis, was conducted with two elastic and one conventional liposome formulations, and IBU solution as the control. For comparison purpose, the amount of IBU in all formulations and control was kept constant (0.5 mg). The results, plotted as cumulative amount of IBU permeated per unit area as a function of time are presented in Figure 3.13, cumulative percentage of IBU permeated per unit area as a function of time in Figure 3.14. Various transdermal permeation parameters were calculated and are summarized in Table 3.22.

In this penetration study, a pre-hydration time of 45 min was selected. A volume of 0.5 mL of formulations and control were applied as donor to cells. Samples were withdrawn from the receptor containing 50 mM PBS pH 7.4 at predetermined time intervals over 12 h. Fresh buffer was added each time. Withdrawn samples were analysed for their drug content at each time point. To confirm skin integrity, skin resistance test or conductivity test was carried out on the skin both before starting the experiment and at the end of the experiment. The conductivity values obtained are tabulated in Appendix 8. As the data suggest, the skin integrity was intact before and after the penetration experiment, therefore, the experimental data generated can be considered as the valid data for skin penetration study.

Results of *in vitro* skin penetration study (Figure 3.13) showed that IBU permeation from the control (free IBU solution in PBS pH 7.4) was lowest (8.5±0.9 $\mu g/cm^2$), whereas elastic liposomes showed maximum (45.4±12.7 $\mu g/cm^2$, 43.6±13.1 $\mu g/cm^2$), 5.34 folds of that of the control. In comparison, IBU permeation from conventional liposome (12.7±0.5 $\mu g/cm^2$) was not statistically significant different from that of the control ($P>0.3$). Furthermore, the cumulative amount of drug penetration from both Tween 80 and sodium cholate containing elastic liposomes were significantly different from that of the conventional liposome and control. However, no significant difference was found between the two elastic liposomes although elastic liposomes of Tween 80 appear to be slightly better than that of sodium cholate (Figure 3.13).

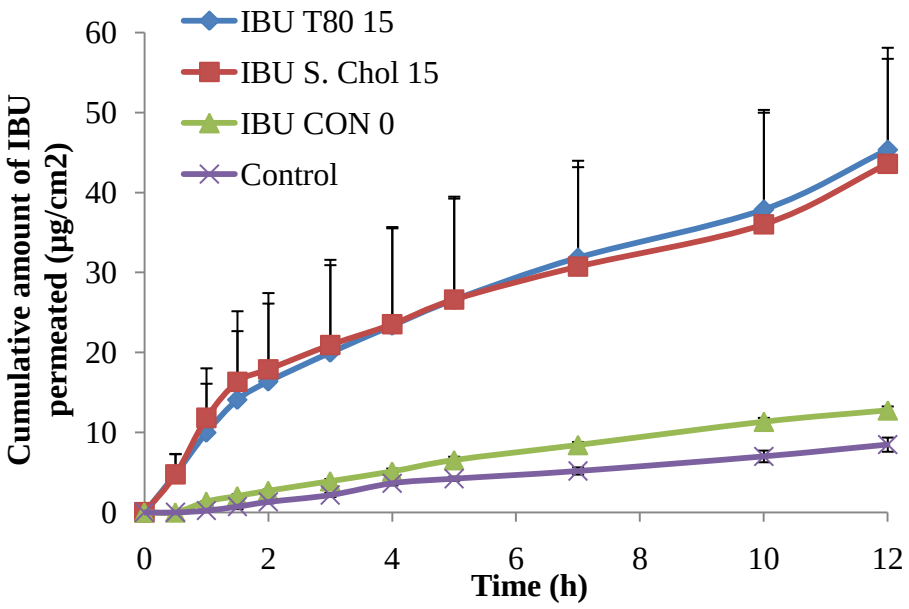


Figure 3.13: Cumulative amount of Ibuprofen (IBU) permeated across human epidermis from different liposomes and control in PBS pH 7.4 over 12 h. IBU T80 15 and IBU S. Chol 15 represent IBU loaded elastic liposomes containing 15% w/w of Tween 80 and sodium cholate in conjunction with Phosphatidylcholine (PC) respectively. IBU CON 0 represents IBU loaded conventional liposome with no cholesterol. Control: 1mg/mL of IBU solution in PBS pH 7.4. Data represents as mean ±S.E.M (n=4-8).

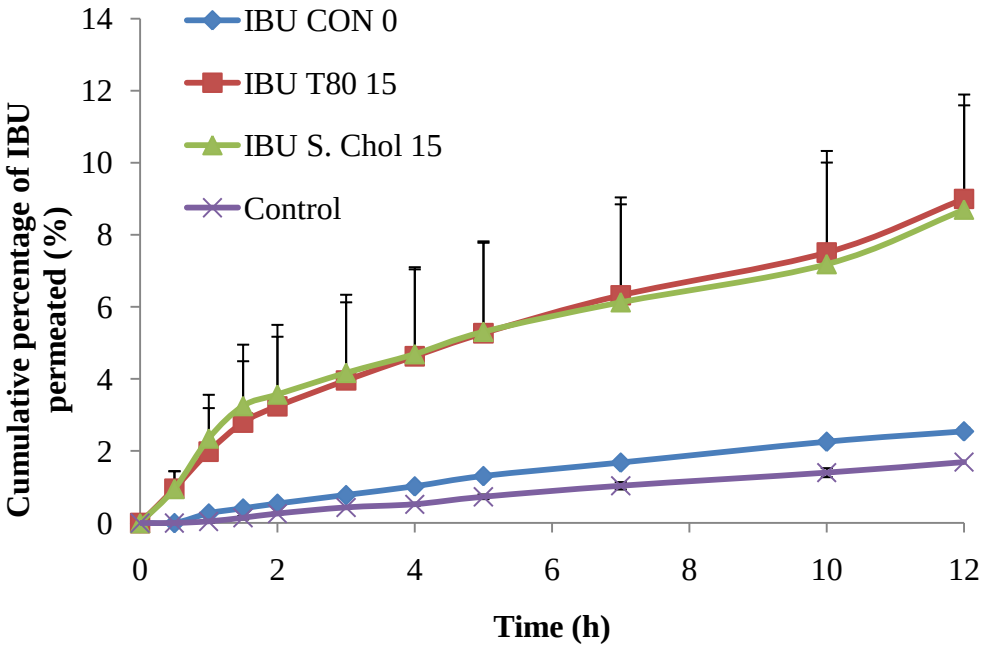


Figure 3.14: Cumulative percentage of Ibuprofen (IBU) permeated across human epidermis from different liposomes and control in PBS pH 7.4 over 12 h. IBU T80 15 and IBU S. Chol 15 represent IBU loaded elastic liposomes containing 15% w/w of Tween 80 and sodium cholate respectively in conjunction with Phosphatidylcholine (PC). IBU CON 0 represents IBU loaded conventional liposome with no cholesterol. Control: 1mg/mL of IBU solution in PBS pH 7.4. Data represents as mean ±S.E.M (n=4-8)

The values of steady-state transepidermal flux from various IBU loaded liposomes were found to be formulation dependent; IBU T80 15 and IBU S. Chol 15 had flux values of 3.0±0.90 µg/cm^2/h and 2.74±1.01 µg/cm^2/h. The flux values obtained from Tween 80 and sodium cholate elastic liposomes were 5.0 and 4.5 fold higher than that of control respectively (0.64±0.04 µg/cm^2/h) and 3.0 and 2.7 fold higher than that of conventional liposome (1.0±0.03 µg/cm^2/h) respectively (Table 3.22). Boinpally *et al*[145] studied the epidermal penetration of diclofenac (weak acid; pKa 4.0) from elastic liposomes (containing sodium cholate and 10% ethanol) and reported an ER of 2 for diclofenac penetration from elastic liposomes compared to conventional liposome.

Table 3.22: Transepidermal permeation parameters for IBU

Donor	J_{ss} (µg/cm^2/h)	K (x10^{-3})±(x10^{-3})	De (µg)	ER^A
IBU CON 0	1.00±0.03	0.97±0.04	3.01±0.51	1.6
IBU T80 15	3.00±0.90	2.90±0.90	13.24±4.02	5.0
IBU S. Chol 15	2.74±1.01	2.70±1.00	14.12±8.11	4.5
Control	0.64±0.04	0.65±0.04	5.82±4.03	-

J_{ss}: steady-state flux; K: permeability coefficient; De: drug deposited in the epidermis; ER^A: Enhancement ratio compared to control

The possible reasons for better transepidermal flux, better enhancement and improved skin deposition of drug from elastic liposomes can be explained by the effect of non-occlusion, surfactants and ethanol.

The osmotic force by which vesicles may enter the skin is defined as xerophobia, or a non-occluded condition, which is of utmost importance in topical/transdermal drug delivery. When phospholipid vesicles are applied on the skin non-occlusively, due to their tendency to avoid dry surrounding to remain in swollen state, they tend to drive themselves towards the hydrated viable tissue by following the transdermal hydration gradient[112]. Elastic liposomes work most effectively under non-occluded condition. Surfactants, the key component of elastic vesicles introduce elasticity to liposomal membrane[246]. Under non-occluded condition, when elastic liposomes are applied on the skin, they can squeeze and drive themselves through the stratum corneum because of the high curvature produced by surfactant molecules. Therefore Tween 80 and sodium cholate had played vital role in the enhanced penetration of IBU from elastic liposomes. Moreover under non-occluded condition, drug partition coefficient may be improved due to the partition of phospholipids in the stratum corneum and epidermis, which is the result of increased diffusion of drug within the stratum corneum[134]. Moreover surfactants not only exert deformability to liposome membrane but also bind with stratum corneum and may extract lipids from it, which finally disrupts the lipid bilayer packing[130], thus increase the pore size of it, hence enhance permeation. Anionic surfactants for example sodium cholate causes alteration in stratum corneum barrier function by strongly interacting with the negatively charged components of the stratum corneum. They probably enhance drug penetration by binding with the epidermal proteins[279].

Another reason of improved transdermal flux from elastic liposomes could be due to the effect of ethanol which generally acts by affecting the intercellular regions of the stratum corneum [120, 121]. Ethanol generally acts by the 'push-pull' mechanism. Elastic liposomes containing ethanol, when applied on the skin, two factors may play roles: firstly, ethanol may permeate though the skin, carrying the drug molecules dissolved in it. This can improve drug partitioning into the stratum corneum. Secondly ethanol evaporated from the system, causing an increase in drug concentration in donor, which changed the thermodynamic activity of drug, and enhanced permeation.

The poor permeation of IBU from conventional liposome was due to less deformability of bilayer membrane. Conventional liposomes when applied on the skin non-occluded, to avoid dry surroundings, they tend to squeeze though the narrow pores of stratum corneum but due to the lack of elasticity, these vesicles cannot squeeze and only remain in the upper layers on the stratum corneum.

From skin extraction data, it was found that the highest IBU deposition in the stratum corneum was from elastic liposomes. As elastic liposomes showed better transepidermal flux, more drug was expected to be found in the stratum corneum due to the penetration enhancing ability of elastic vesicles. These vesicles might have carried the drug into the stratum corneum or travelled through the stratum corneum as intact. Higher drug deposition showed that elastic liposomes may have depot forming ability that could be beneficial to localize IBU to diseased tissue. There was no significant difference found in the level accumulated in the stratum corneum from the control and the conventional liposomes, though marginal better flux was found from conventional liposomes.

Delivery of IBU by passive diffusion and its consequent pharmacological effect are dose dependent; the better is the permeation, the greater the therapeutic effect. Therefore formulation plays a vital role in topical drug delivery as the vehicle composition influences drug partitioning and/or the diffusivity and absolute amount delivered. The results of our *in vitro* release study demonstrated greater effectiveness of dermally applied IBU elastic liposomal formulation in comparison to the conventional liposome and free drug solution. Therefore an optimized elastic

liposomal formulation of IBU may offer a promising means for the non-invasive treatment of local pain and inflammation by topical application. Furthermore, this study also highlighted the potential of elastic liposomes as topical carriers for other therapeutic molecules to improve their skin permeation and local therapeutic activities.

GENERAL DISCUSSION AND CONCLUSION

4.1. General discussion

Due to the unwanted reactions arising from the systemic administration of IBU, a considerable interest has grown in the potential usefulness of the topical application of this drug. However the problem associated with topical application of IBU is its poor permeability[177]. Therefore the primary objective of this project was to develop elastic liposomes of IBU and to evaluate its skin permeability in comparison to that of conventional liposome and free drug solution when applied topically. Conventional and elastic liposomes were developed and characterized in terms of their physicochemical properties, such as particle size distribution, vesicular shape, zeta potential and EE. Two different compounds were chosen for drug loading into liposomes; NaFl, a model hydrophilic compound, and IBU, a poorly water-soluble drug. NaFl was chosen because of its simple and rapid analysis and also to show the difference between physicochemical properties of liposomes loaded with hydrophilic and hydrophobic compound.

4.1.1. Preparation and characterization of liposomes

In this project, all liposomes were prepared by the thin film hydration technique as this technique is very simple and easy for laboratory scale-up procedures. To prepare a stable liposomal suspension, few process variables were investigated for example a suitable solvent system for lipid film formation, hydration medium to hydrate the film and method of size reduction of liposomal particles. After careful evaluation, chloroform-methanol (3:1) was found to be the best solvent system to form a homogeneous and thin film. To minimize the level of chloroform residue, a 12 h or overnight drying of lipid film was conducted with each preparation under vacuum to ensure maximum removal of chloroform.

The selection of a suitable hydration medium was achieved by evaluating three types of hydration media (water, PBS and phosphate buffer saline). The effect of hydration medium on particle size, zeta potential and physical appearance of pure PC liposome was investigated. Results showed that both water and PBS produced stable

suspensions whereas phosphate buffer saline caused a clear phase separation due to the influence of electrolytes on particle aggregation, therefore the latter was found not suitable as hydration medium. The zeta potential of water hydrated liposome was negative, whereas that of PBS was positive. This effect could be explained by the change in the direction of the positive choline head group of PC which orientates to the outer region of bilayer while in PBS, resulting in an overall positive charge[218]. As smaller size and negatively charged liposomes are expected to have better skin penetration, water was selected as the hydration medium to develop all liposomes. Thin film hydration technique produces MLVs which are larger in size. SUVs are advantageous for skin permeation, as has been previously demonstrated: 120 nm vesicles showed better transdermal flux of carboxyfluorescein compared to that of larger ones (>300 nm)[222]. Hence MLVs were downsized to smaller sized particles. The combination of a 30 min bath and a 1 min probe sonication followed by extrusion through 200 and 100 nm filters effectively reduced particle size to approximately 153 nm and data were found to be reproducible. Therefore in a standard formulation process all three techniques were combined to obtain small and constant liposome size.

Evaluation of formulation variables (phospholipid, cholesterol and surfactant and type of therapeutic molecule incorporated) in terms of their effect on physicochemical properties of liposomes allow us to better understand factors that influence the properties and eventually the performance of liposomes in vivo. Therefore once the process variables were optimized, a set of NaFl loaded conventional and elastic liposomes were prepared by passive loading technique using cholesterol and surfactants by varying their ratios. Incorporation of cholesterol into conventional liposome reduced the particle size of NaFl loaded liposomes. This effect was in agreement with the published literature[230] and can be explained by the condensation effect of cholesterol on bilayer showing a decrease in effective area per molecule as cholesterol content increases. The zeta potential of cholesterol containing liposomes was more negative than that of cholesterol free liposomes possibly because of the binding of hydroxyl groups of cholesterol with the choline in PC, creating a dipole tropism which increased the surface negative charge[225]. Compared to cholesterol, surfactants (Tween 80 and sodium cholate) had similar effect on particle size and zeta potential of elastic liposomes. Liposomal size was

found to reduce with each inclusion of surfactant; maximum size reduction was observed with the formulation containing 25% w/w surfactant. This effect is in close agreement with published report where it was shown that the higher concentration of surfactants produced micellar structures which are much smaller in size[247]. The zeta potential of elastic liposomes was dependent on the type of surfactant. Tween 80 containing elastic liposomes had less negative surface charge compared to that of sodium cholate containing elastic liposomes. This effect may be attributed to the partial hydrolysis of Tween 80 and the adsorption of cholate anion on PC molecule respectively[226, 228]. The EE however was found to increase in presence of cholesterol containing liposomes whereas decreased in elastic liposomes because cholesterol is a stabilizing agent which causes the bilayer more rigid[236] and surfactants are destabilizing agent, make bilayer flexible and soft. When 40% w/w cholesterol was included in bilayer no liposomes were formed because of the crystallization of cholesterol at high concentration[229] which suggests that cholesterol provides stability only up to a certain concentration.

Bilayer elasticity was determined in all NaFl loaded elastic liposomes and maximum elasticity was found when 15% w/w surfactant was used. Surfactant concentration above this significantly reduced elasticity due to the formation of micelles which are much less deformable in nature[247, 248]. For comparison, the elasticity of two conventional liposomes (containing 30% w/w of cholesterol and pure PC) was determined which as expected, showed very poor elasticity compared to that of 15% w/w surfactant containing elastic liposomes, due to the absence of membrane softening agent 'surfactant' in the former, thereby reducing the probability of squeezing upon extrusion through the 50 nm pores of the filter. It is reported that elastic liposomes, irrespective of their size, can penetrate through very tiny pores of the skin. To support this phenomenon, NaFl level in elastic liposomes was determined after extrusion through 50 nm membrane filters and was found to remain the same indicating that elastic liposomes maintained their structural integrity when squeezing through the narrow pores. This finding is significant as it suggests that drug loaded inside the elastic liposomes will be able to penetrate through small pores to reach the region in the skin where the drug molecule normally cannot reach by itself. Along with cholesterol and surfactant, the type of drug to be incorporated has also influence on the characteristics of liposomes. Thus various empty liposomes

were prepared by hydrating with water and were characterized. The size and zeta potential of NaFl loaded liposomes (both conventional and elastic) were more negative compared to those of the empty liposomes, possibly due to the probe entrapment and the carboxylate anion (from NaFl) adsorption on PC molecule respectively.

Above findings suggest that formulation variables have pronounced influence on the characteristics of liposomes, which should be carefully considered in optimizing liposomal delivery systems. In optimization of conventional liposomes, particle size and zeta potential were chosen as the crucial selection criteria as small[222] and negatively charged particles[223] particles showed better skin penetration. Therefore, NaFl CON 30 (NaFl loaded liposomes containing 30% w/w of cholesterol) was chosen as optimized conventional liposome. In optimization of elastic liposomes, bilayer elasticity is the only selection criteria as it indicates vesicular deformability, a factor directly related to enhanced dermal penetration. Considering the data of deformability index, 15% w/w surfactant containing elastic liposomes were selected as optimized.

Physical stability of liposomes is a very important parameter which should be regularly monitored. Upon storage, vesicles tend to undergo aggregation, fusion and even rupture. Therefore NaFl loaded conventional liposomes were stored for a period of 4 weeks at three different storage conditions and particle sizes were measured. Data obtained in the study showed that cholesterol induced better stability; maximum stability was achieved from NaFl CON 30, which promoted more resistance against particle aggregation and fusion at all storage conditions. Thus cholesterol was evaluated as stabilizing molecule for liposomes.

On completion of characterization of NaFl loaded liposomes, IBU was loaded into both conventional and elastic liposomes with a view to evaluate elastic liposomes in topical delivery of IBU. As water was chosen as suitable hydration medium, pure PC IBU liposomes were prepared with water which showed a very high EE of around 98%. This is not realistic for a compound like IBU. Therefore investigations were carried out and found that IBU crystals were present in liposome, and an EE of 25.3% was achieved after washing pellets with PBS pH 7.4 twice. As it became

evident that IBU was in insoluble form in the formulation, dialysis technique (40 min in PBS pH 7.4 and 40 min in water) was applied to purify IBU liposomes by removing the non-entrapped IBU. However, later it was found that inclusion of 20 and 30% w/w cholesterol in the bilayer required the use of PBS pH 7.4 as hydration medium to form IBU loaded liposomes as water could not fully hydrate the above lipid films. Moreover at pH 7.4, IBU is almost fully ionized so solubility problem in liposomal vehicle was solved. Therefore, PBS was selected as the hydration medium to prepare all IBU loaded liposomes.

IBU loaded conventional and elastic liposomes were prepared and characterized as done in the case of NaFl loaded liposomes. Similar to findings in NaFl loaded liposomes, cholesterol and surfactants showed to reduce the particle size and increase the negative charge on IBU loaded liposomes. The zeta potential of liposomes was found to be dependent on cholesterol, surfactant and drug as well. Maximum size reduction of IBU loaded elastic liposomes was found in 25% w/w surfactant, possibly due to the formation of micellar structures. Compared to the zeta potential of empty pure PC and 30% w/w cholesterol containing liposome in buffer, IBU loaded liposomes had less positive and more negative charge respectively due to the adsorption of anionic IBU on the surface of PC molecule [267]. With each inclusion of additional cholesterol, the zeta potential of IBU conventional liposomes tended to become more negative which could be explained by the reduction in surface binding affinity of cations from PBS on PC molecule in presence of cholesterol[269]. In contrast to NaFl loaded conventional liposomes, cholesterol had a negative effect on the entrapment of IBU loaded liposomes. At PBS pH 7.4, IBU is almost fully ionized and these ionized species tend to be associated with the lipid membrane by an ionic interaction. The higher EE found in pure PC liposomes could be explained by the ionic interaction between the PC and IBU molecule; the anionic portion (carboxylate ion) of IBU associated with the positively charged choline head of PC and the hydrophobic portion of IBU inserted into the acyl regions of PC tails[243]. With each inclusion of cholesterol in liposomal bilayer, the EE reduced, which was possibly due to the competition between IBU and cholesterol to take place in the hydrophobic region of PC, providing a limited space available for IBU[212]. Comparing to the EE of NaFl loaded liposomes, which is freely soluble in water, when loaded into liposomes, readily resides in the aqueous zone. Therefore incorporation of

cholesterol in bilayer had no competition with NaFl, rather it increased bilayer stability, leading to reduced permeation of entrapped NaFl through the bilayer to aqueous exterior. EE in all IBU loaded elastic liposomes reduced when surfactants were included. Maximum reduction in EE was noticed in 25% w/w surfactants containing liposomes, due to the formation of micelles.

Vesicular elasticity was determined in all IBU loaded elastic liposomes and in two selected conventional liposomes. Maximum elasticity was found with 15% w/w surfactant, which dropped when more surfactant was added. Conventional liposomes had overall less deformation compared to elastic liposomes. Cholesterol is reported to reduce the freedom of molecular movement of PC molecule, causing poor elasticity. Results of characteristics of IBU loaded liposomes provided some knowledge on the influence of formulation variables on IBU loaded liposome optimization. Analyzing characterization data, IBU CON 0 was selected as most suitable because of their lowest size and highest EE whereas IBU T80 15 and IBU S. Chol 15 were selected for their highest elasticity. Only optimized liposomes were selected for the *in vitro* drug release study and the *in vitro* epidermal penetration study.

4.1.2. In vitro drug release study

All optimized liposomes were chosen for the *in vitro* drug release study. Drug release from liposomes was found to be dependent on formulation variables and showed a biphasic release pattern. Irrespective of the type of compound entrapped, significant prolongation of release was found from formulations compared to the control (free NaFl or IBU solution). NaFl loaded elastic liposomes showed faster release than that of the conventional liposome because of the presence of surfactant in their structure respectively. Surfactants are destabilizing molecule, causing bilayer soft and flexible for the entrapped drug to diffuse fast. The effect of cholesterol on the release of IBU was studied using different conventional liposomes, which showed a gradual reduction in release up to inclusion of 20% w/w cholesterol. When 30% w/w of cholesterol was incorporated, faster release was observed compared to that of 20% w/w of cholesterol containing liposome suggesting that high concentration of cholesterol might have disrupted the bilayer structure[76]. IBU releases from elastic liposomes were only slightly faster than that of conventional

liposome. Compared to NaFl release, IBU release was faster which could be due to the location of trapped molecule inside liposome. NaFl, being located in the core, is expected to show slow release as it has to cross the bilayer first to diffuse out whereas IBU, being partitioned in the bilayer is close to the vicinity of large receptor.

4.1.3. In vitro skin penetration study

An *in vitro* penetration study of IBU loaded optimized liposomes across human epidermis was conducted non-occlusively. The study revealed that the transepidermal flux of IBU from Tween 80 and sodium cholate containing elastic liposomes were 5.0 and 4.5 fold higher than that of control (0.64±0.04 μg/cm^2/h) respectively and 3.0 and 2.7 fold higher than that of conventional liposome (1.00±0.03 μg/cm^2/h) respectively. Penetration enhancing property of liposomes was dependent on formulation variable. Surfactants were found to be the key components in enhancing epidermal penetration which exert high deformability on liposome structure. Along with surfactants, another component which is thought to play role in improving transepidermal flux of IBU was ethanol, which might have improved drug partitioning into the stratum corneum. Elastic liposomes showed better drug deposition in the stratum corneum compared to that of conventional liposome and control suggesting that elastic liposomes may show depot forming ability to exert the local therapeutic effect of IBU. No significant difference in transepidermal flux was observed in between the two elastic liposomes. Conventional liposomes were found not suitable delivery system for topical application of IBU because they failed to show better IBU permeation compared to that of control. Result of epidermal permeation study shows that elastic liposomes have potential to be considered as potential delivery system in topical delivery of IBU and other therapeutic molecules.

4.2. Conclusion

In this study elastic liposomes were prepared to evaluate the topical delivery of plain drug solution and conventional liposomes in delivering IBU as a model NSAID. Both conventional and elastic liposomes were developed and characterized with a

view to evaluate formulation variables in terms of their effect on the physicochemical properties of liposomes.

Inclusion of both cholesterol and surfactant into liposomal bilayer was found to reduce particle size and increase the negative charge of liposomes compared to that of liposome without cholesterol and surfactant. The influence of cholesterol on EE of liposomes was found to be dependent on the type of compound entrapped. Cholesterol increased the EE of NaFl loaded liposomes while reduced the same in IBU loaded liposomes suggesting that the design of conventional liposomes should pay careful consideration on the influence of cholesterol on liposomal physicochemical properties. The hydration medium was found to show marked influence on size and zeta potential as well; all liposomes hydrated with water had smaller particle size and more negative zeta potential than those hydrated with PBS pH 7.4. Bilayer elasticity of elastic liposomes was found to be highly dependent on the ratio of PC and surfactant. All elastic liposomes showed maximum deformability at the surfactant content of 15% w/w regardless of the type of surfactant used. Above this content, elasticity was found to drop dramatically suggesting that elastic liposomes that show maximum deformation at 15% surfactant inclusion may be expected to enhance skin penetration . Results of physical stability of liposomes showed that cholesterol incorporation into bilayer has potential stabilization of liposome against particle aggregation and fusion upon long term storage. Vesicular stability improved under refrigeration (4±2ºC). Under TEM, all liposomes were appeared as spherical and regular vesicles.

The *in vitro* dialysis and human epidermal skin release study showed a significant prolongation of drug release from liposomes compared to that of free drug solution indicating that liposomal formulations may act as sustained release delivery vehicle. Incorporation of cholesterol caused the liposomal bilayer more rigid and provided a slow drug release compared to that of elastic liposomes prepared from either Tween 80 or sodium cholate. Moreover release of IBU was faster from all liposomes compared to the release of NaFl concluding that drug entrapped inside the core shows slow release.

Elastic liposomes improved *in vitro* topical delivery of IBU across human epidermis compared to conventional liposomes and free drug solution. Inclusion of surfactants and ethanol into elastic liposomes were found to play major role in enhancing IBU permeation when vesicles were applied under non-occluded condition. No significant difference in enhancement was observed in between the elastic liposomes. Better IBU drug deposition in the stratum corneum was obtained from elastic liposomes indicating that these delivery vehicles may show depot forming ability and localize IBU at the targeted tissue. Conventional liposomes failed to achieve significant improvement in drug permeation compared to control drug solution due to the absence of penetration enhancers in their composition. Results from the skin penetration study indicate that elastic liposomal formulations are better topical delivery system for IBU in comparison to conventional liposomes and the control. This delivery system has a great potential for the delivery of other potential therapeutic molecules to achieve the local pharmacological effects.

4.3. Scope for future work

The current study revealed that elastic liposomes showed better penetration of IBU, but no mechanistic study was drawn. The possible mechanism by which elastic liposomes enhance dermal penetration is still not very clear. Different mechanisms have been proposed and discussed in this thesis. Elastic liposomes work either as drug carrier systems and/or as penetration enhancers. In the case of the former mechanism, intact vesicles are proposed to enter the stratum corneum. Therefore, a skin distribution study needs to be investigated in future to support this mechanism. To evaluate elastic liposomes as penetration enhancers, pre-treatment with empty elastic vesicles followed by drug application and liposomes with drug outside (i.e. non-entrapped) will be worthwhile to investigate the penetration enhancing mechanism.

Some investigators have reported that conventional liposomes work better under occluded condition, as there is no transdermal hydration gradient operating at this condition. Therefore, a separate *in vitro* skin penetration study should be conducted under occluded condition to investigate how conventional liposomes show IBU transport through the stratum corneum and should be compared with that of elastic liposomes applied under non-occluded condition.

In the current study, a single type of phospholipid was used to prepare all liposomes, i.e. PC. Utilization of other lipids, for example, charge imparting agents as well as skin lipids could be used to investigate if they can also be used to prepare elastic liposomes and to enhance the drug skin penetration. Utilization of other surfactants, for example Span series could also be done to see how it influences epidermal delivery of IBU.

REFERENCES

1. Shivhare UD, Ambulkar DU, Mathur VB, Bhusari KP, Godbole MD. Formulation and Evaluation of Pentoxifylline Liposome Formulation. Digest J Nanomaterials and Biostructures. 2009; 4(4):857-862.
2. Williams AC. Theoretical aspects of transdermal drug delivery. In: A.C. W, editor. Transdermal and Topical Drug Delivery: From Theory to Clinical Practice. London: Pharmaceutical Press; 2003.p.27-49
3. Barry BW. Novel mechanisms and devices to enable successful transdermal drug delivery. Eur J Pharm Sci. 2001; 14(2):101-114.
4. Tanner T, Marks R. Delivering drugs by the transdermal route: review and comment. Skin Res Technol. 2008; 14(3):249-60.
5. Sharma S. Topical Drug Delivery Systems : A Review. Latest Rev. 2008; 6(5).
6. Michniak-Kohn BB, Wertz PW, Al-Khalili M, Meidan VM. Skin: Physiology and Penetration Pathways. In Delivery System Handbook for Personal Care and Cosmetic Products: Technology, Applications and Formulations. NY: William Andrew Publishing; 2005.p.78-95
7. Siegenthaler D. Importance of your skin's pH. http://Ezine Articles.com/expert=Danny Siegenthaler; 2005
8. Banker GBS, Rodes CT. Modern Pharmaceutics. 4th ed. NY: Marcel Dekker; 2002.
9. Mezei M. Liposomes and the Skin. In: Gregoriadis G, Florence AT, Patel HM, editors. Liposomes in Drug Delivery. Switzerland: Harwood Academic Publishers; 1993. p. 126-133.
10. Wilkes GL, Brown IA, Wildnauer RH. The biomechanical properties of skin. CRC Crit Rev Bioeng 1973:453-495.
11. Briggaman RA, Wheeler CE, Jr. Epidermal-dermal interactions in adult human skin: role of dermis in epidermal maintenance. J Invest Dermatol. 1968; 51(6):454-65.
12. Smith KM. Academic and Drug-induced Skin Diseases. In: Helms RA, Quan DJ, Herfindal ET, Gourley DR, editors. Textbook of Therapeutics: Drug and Disease Management. 8 ed. Pennsylvania, USA: Lippincott Williams and Wilkins; 2006. p. 183.

13. Powell J, Soon C. Physiology of the Skin. Surgery (Oxford). 2002; 20(6):ii-vi.

14. Fischer TG. Anatomy and Physiology I. Pearson Education Inc.; http://www.osovo.com/diagram/skindiagram.htm. 2004.

15. Smith EW, Maibach HI. Percutaneous penetration enhancers: the fundamentals. Chapter 1.1. In: E.W. Smith and H.I. Maibach, Editors, Percutaneous Penetration Enhancers. Boca Raton: CRC Press; 1995.

16. Potts RO, Francoeur ML. Lipid biophysics of water loss through the skin. Proc Natl Acad Sci U S A. 1990; 87(10):3871-3.

17. Cevc G. Lipid vesicles and other colloids as drug carriers on the skin. Adv Drug Deliv Rev. 2004; 56(5):675-711.

18. Pirot F, Berardesca E, Kalia YN, Singh M, Maibach HI, Guy RH. Stratum corneum thickness and apparent water diffusivity: facile and noninvasive quantitation in vivo. Pharm Res. 1998; 15(3):492-4.

19. Scheuplein RJ. Mechanism of percutaneous absorption. II. Transient diffusion and the relative importance of various routes of skin penetration. J Invest Dermatol. 1967; 48(1):79-88.

20. Wertz PW, Downing DT. Stratum Corneum: Biological and Biochemical Considerations. In: Hadgraft J, Guy RH, editors. Transdermal Drug Delivery: Developmental Issues and Research Initiatives. New York: Marcel Dekker, Inc.; 1989. p. 1-21.

21. Elias PM. Epidermal lipids, barrier function, and desquamation. J Invest Dermatol. 1983; 80(1 Suppl):44-9.

22. Cevc G, Blume G, Schätzlein A, Gebauer D, Paul A. The skin: a pathway for systemic treatment with patches and lipid-based agent carriers. Adv Drug Deliv Rev. 1996; 18(3):349-378.

23. Schatzlein A, Cevc G. Non-uniform cellular packing of the stratum corneum and permeability barrier function of intact skin: a high-resolution confocal laser scanning microscopy study using highly deformable vesicles (Transfersomes). Br J Dermatol. 1998; 138(4):583-92.

24. Plewig G, Scheuber E, Reuter B, Waidelich W. Thickness of corneocytes in stratum corneum. In: Stratum Corneum. Springer, Berlin; 1983.

25. Johnson ME, Blankschtein D, Langer R. Evaluation of solute permeation through the stratum corneum: lateral bilayer diffusion as the primary transport mechanism. J Pharm Sci. 1997; 86(10):1162-72.

26. Iizuka H. Epidermal turnover time. J Dermatol Sci. 1994; 8(3):215-217.

27. Wertz PW. Lipids and barrier function of the skin. Acta Derm Venereol Suppl (Stockh). 2000; 208:7-11.

28. Egbaria K, Weiner N. Liposomes as a topical drug delivery system. Adv Drug Deliv Rev. 1990; 5(3):287-300.

29. Sinico C, Fadda AM. Vesicular carriers for dermal drug delivery. Expert Opin Drug Deliv. 2009; 6(8):813-25.

30. Scheuplein RJ, Blank IH. Permeability of the skin. Physiol Rev. 1971; 51(4):702-47.

31. Siddiqui O, Roberts MS, Polack AE. Percutaneous absorption of steroids: relative contribution of epidermal penetration and dermal clearance. J Pharmacokinet Biopharm. 1989; 17:405-424.

32. Benson HA. Transdermal drug delivery: penetration enhancement techniques. Curr Drug Deliv. 2005; 2(1):23-33.

33. Lauer AC, Ramachandran C, Lieb LM, Niemiec S, Weiner ND. Targeted delivery to the pilosebaceous unit via liposomes. Adv Drug Deliv Rev. 1996; 18(3):311-324.

34. Nemanic MK, Elias PM. In situ precipitation: a novel cytochemical technique for visualization of permeability pathways in mammalian stratum corneum. J Histochem Cytochem. 1980; 28(6):573-8.

35. Karasek MA. Cell culture of human skin epidermal cells. In: Goldsmith LA, editor. Biochemistry and Physiology of the Skin. NY: Oxford University Press; 1983.

36. Menon GK. New insights into skin structure: scratching the surface. Adv Drug Deliv Rev. 2002; 54(Supplement 1):S3-S17.

37. Abraham MH, Chanda HS, Mitchell RC. The factors that influence skin penetration of solutes. J Pharm Pharmacol. 1995; 47:8-16.

38. Roberts MS, Pugh WJ, Hadgraft J. Epidermal permeability: Penetrant structure relationships. 2. The effect of h-bonding groups in penetrants on their diffusion through the stratum corneum. Int J Pharm. 1996; 132(1-2):23-32.

39. Schaefer UF, Hansen S, Schneider M, Contreras JL, Lehr C-M. Models for Skin Absorption and Skin Toxicity Testing. In: Carsten Ehrhardt, Kwang-Jin Kim editor. Drug Absorption Studies: In Situ, In Vitro and In Silico Models. NY: AAPS Press; 2008.

40. Magnusson BM, Walters KA, Roberts MS. Veterinary drug delivery: potential for skin penetration enhancement. Adv Drug Deliv Rev. 2001; 50(3):205-27.

41. Johnson ME, Mitragotri S, Patel A, Blankschtein D, Langer R. Synergistic effects of chemical enhancers and therapeutic ultrasound on transdermal drug delivery. J Pharm Sci. 1996; 85(7):670-9.

42. Tsai JC, Guy RH, Thornfeldt CR, Gao WN, Feingold KR, Elias PM. Metabolic approaches to enhance transdermal drug delivery. 1. Effect of lipid synthesis inhibitors. J Pharm Sci. 1996; 85(6):643-8.

43. Choi EH, Lee SH, Ahn SK, Hwang SM. The pretreatment effect of chemical skin penetration enhancers in transdermal drug delivery using iontophoresis. Skin Pharmacol Appl Skin Physiol. 1999; 12(6):326-35.

44. Williams AC, Barry BW. Penetration enhancer. Adv Drug Deliv 2004; 56:603-618.

45. Hoogstrate AJ, Verhoef J, Brusee, Ijzerman AP, Spies F, Bodde HE. Kinetic, ultrastructural aspects and molecular modeling of transdermal peptide flux enhancement by N-alkylazocylohepton. Int J Pharm 1991; 76:37-47.

46. Barry BW. Dermatological formulation: percutaneous absorption. New York: Marcel Dekker; 1983.

47. Asbill CS, Michniak BB. Percutaneous penetration enhancers: local versus transdermal activity. Pharm Sci Technol Today. 2000; 3(1):36-41.

48. Tupker RA, Pinnagoda J, Nater JP. The transient and cumulative effect of sodium lauryl sulphate on the epidermal barrier assessed by transepidermal water loss: inter-individual variation. Acta Derm Venereol. 1990; 70(1):1-5.

49. Pikal MJ, Shah S. Transport mechanisms in iontophoresis. II. Electroosmotic flow and transference number measurements for hairless mouse skin. Pharm Res. 1990; 7(3):213-21.

50. Preat V, Vanbever R. Skin Electroporation for Transdermal and Topical Drug Delivery. In: Guy RH, Hadgraft J, editors. Transdermal Drug Delivery. 2 ed. New York: Marcel Dekker; 2003. p. 227-254.

51. Vanbever R, Lecouturier N, Preat V. Transdermal delivery of metoprolol by electroporation. Pharm Res. 1994; 11(11):1657-62.

52. Prausnitz MR, Bose VG, Langer R, Weaver JC. Electroporation of mammalian skin: a mechanism to enhance transdermal drug delivery. Proc Natl Acad Sci U S A. 1993; 90(22):10504-8.

53. Henry S, McAllister DV, Allen MG, Prausnitz MR. Microfabricated microneedles: a novel approach to transdermal drug delivery. J Pharm Sci. 1998; 87(8):922-5.

54. Daniels R. Strategies for Skin Penetration Enhancement. Skin Care Forum. 2004; (37).

55. Ainbinder D, Touitou E. Testosterone ethosomes for enhanced transdermal delivery. Drug Deliv. 2005; 12(5):297-303.

56. Vora B, Khopade AJ, Jain NK. Proniosome based transdermal delivery of levonorgestrel for effective contraception. J Control Release. 1998; 54(2):149-165.

57. Choi MJ, Maibach HI. Elastic vesicles as topical/transdermal drug delivery systems. Int J Cosmet Sci. 2005; 27(4):211-21.

58. Bangham AD, Standish MM, Watkins JC. Diffusion of univalent ions across the lamellae of swollen phospholipids. J Mol Biol. 1965; 13(1):238-52.

59. Mezei M, Gulasekharam V. Liposomes--a selective drug delivery system for the topical route of administration. Lotion dosage form. Life Sci. 1980; 26(18):1473-7.

60. Rong (Ron) L, John BC, Sophia YLP. Liposomes in Solubilization. In: Liu R, editor. Water-Insoluble Drug Formulation. 2 ed. London, New York: CRC Press; 2008.p.375-417

61. De Leeuw J, De Vijlder HC, Bjerring P, Neumann HA. Liposomes in dermatology today. J Eur Acad Dermat Venereol 2009; 23(5):509-516.

62. Kshirsagar NA, Pandya SK, Kirodian BG, Sanath S. Liposomal drug delivery system from laboratory to clinic. Journal of Postgraduate Medicine. 2005; 51(5):5-15.

63. Verma DD, Verma S, Blume G, Fahr A. Liposomes increase skin penetration of entrapped and non-entrapped hydrophilic substances into human skin: a skin penetration and confocal laser scanning microscopy study. Eur J Pharm Biopharm. 2003; 55(3):271-7.

64. Fang JY, Hong CT, Chiu WT, Wang YY. Effect of liposomes and niosomes on skin permeation of enoxacin. Int J Pharm. 2001; 219(1-2):61-72.

65. Chang JS, Choi MJ, Kim TY, Cho SY, Cheong HS. Immunogenicity of synthetic HIV-1 V3 loop peptides by MPL adjuvanted pH-sensitive liposomes. Vaccine. 1999; 17(11-12):1540-8.

66. Abra RM, Bankert RB, Chen F, Egilmez NK, Huang K, Saville R, et al. The next generation of liposome delivery systems: Recent experience with tumor-targeted, sterically-stabilized immunoliposomes and active-loading gradients. J Liposome Res. 2002; 12(1-2):1-3.

67. Clemons KV, Stevens DA. Comparative efficacies of four amphotericin B formulations--Fungizone, amphotec (Amphocil), AmBisome, and Abelcet--against systemic murine aspergillosis. Antimicrob Agents Chemother. 2004; 48(3):1047-50.

68. Barenholz Y. Liposome application: problems and prospects. Current Opinion in Colloid & Interface Science. 2001; 6(1):66-77.

69. Honeywell-Nguyen PL, Bouwstra JA. Vesicles as a tool for transdermal and dermal delivery. Drug Discovery Today: Technologies. 2005; 2(1):67-74.

70. Westerhof W. Possibilities of liposomes as dynamic dosage form in dermatology. Med Hypotheses. 1985; 16(3):283-8.

71. Yerushalmi N, Margalit R. Bioadhesive, collagen-modified liposomes: molecular and cellular level studies on the kinetics of drug release and on binding to cell monolayers. Biochim Biophys Acta. 1994; 1189(1):13-20.

72. Moghimi SM, Patel HM. Current progress and future prospects of liposomes in dermal drug delivery. J Microencapsul. 1993; 10(2):155-62.

73. Schmid MH, Korting HC. Therapeutic progress with topical liposome drugs for skin disease. Adv Drug Deliv Rev. 1996; 18(3):335-342.

74. Singh B, Mehta G, Kumar R, Bhatia A, Ahuja N, Katare OP. Design, development and optimization of nimesulide-loaded liposomal systems for topical application. Curr Drug Deliv. 2005; 2(2):143-53.

75. Uhumwangho MU, Okor RS. Current trends in the production and biomedical applications of liposomes: a review. JMBR: A Peer-review J Biomed Sciences. 2005; 4(1):9-21.

76. New RRC. Preparation of Liposomes. In: New RRC, editor. Liposomes: A Practical Approach. New York: Oxford University Press; 1990. p. 33-99.

77. Szoka F, Jr., Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. Proc Natl Acad Sci U S A. 1978; 75(9):4194-8.

78. Daniels R. Liposomes - Classification, Processing Technologies, Industry Applications and Risk Assessment. Skin Care Forum (AzoNanotechnology Article). 2005; (25).

79. Bangham AD, Hill MW, Miller NGA. In: Korn ED, editor. Methods in Membrane Biology. New York: Plenum Press; 1974. p. 1-68.

80. Woodle MC, Papahadjopoulos D. Liposome preparation and size characterization. Methods Enzymol. 1989; 171:193-217.

81. Vemuri S, Rhodes CT. Preparation and characterization of liposomes as therapeutic delivery systems: a review. Pharmaceutica Acta Helvetiae. 1995; 70(2):95-111.

82. Touitou E, Junginger HE, Weiner ND, Nagai T, Mezei M. Liposomes as carriers for topical and transdermal delivery. J Pharm Sci. 1994; 83(9):1189-203.

83. Liu J, Hu G. Advances in studies of phospholipids as carriers in skin topical application. J Nanjing Medical University. 2007; 21(6):349-353.

84. Kulkarni VS. Liposomes in Personal Care Products. In: Rosen MR, editor. Delivery System Handbook for Personal Care and Cosmetic Products Technology, Application and Formulations. New York: William Andrew, Inc.; 2005. p. 296.

85. Yokomizo Y, Sagitani H. Effects of phospholipids on the percutaneous penetration of indomethacin through the dorsal skin of guinea pigs in vitro. J Control Release. 1996; 38(2-3):267-274.

86. Kadir R, Stempler D, Liron Z, Cohen S. Delivery of theophylline into excised human skin from alkanoic acid solutions: a "push-pull" mechanism. J Pharm Sci. 1987; 76(10):774-9.

87. Valenta C, Wanka M, Heidlas J. Evaluation of novel soya-lecithin formulations for dermal use containing ketoprofen as a model drug. J Control Release. 2000; 63(1-2):165-73.

88. Kato A, Ishibashi Y, Miyake Y. Effect of egg yolk lecithin on transdermal delivery of bunazosin hydrochloride. J Pharm Pharmacol. 1987; 39(5):399-400.

89. Mahjour M, Mauser B, Rashidbaigi Z, Fawzi MB. Effect of egg yolk lecithins and commercial soybean lecithins on in vitro skin permeation of drugs. J Control Release. 1990; 14(3):243-252.

90. Valjakka-Koskela R, Kirjavainen M, Mönkkönen J, Urtti A, Kiesvaara J. Enhancement of percutaneous absorption of naproxen by phospholipids. Int J Pharm. 1998; 175(2):225-230.

91. McMullen TP, McElhaney RN. Differential scanning calorimetric studies of the interaction of cholesterol with distearoyl and dielaidoyl molecular species of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine. Biochemistry. 1997; 36(16):4979-86.

92. Aggarwal G, Goel A, Dhawan S, Sharma A. Carriers/Vesicle based approaches for penetration enhancement in transdermal drug delivery system. Latest Rev. 2010; 18(1).

93. Schreier H, Bouwstra J. Liposomes and niosomes as topical drug carriers: dermal and transdermal drug delivery. J Control Release. 1994; 30(1):1-15.

94. Kirjavainen M, Urtti A, Jaaskelainen I, Suhonen TM, Paronen P, Valjakka-Koskela R, et al. Interaction of liposomes with human skin in vitro--the influence of lipid composition and structure. Biochim Biophys Acta. 1996; 1304(3):179-89.

95. Kirjavainen M, Urtti A, Valjakka-Koskela R, Kiesvaara J, Mönkkönen J. Liposome-skin interactions and their effects on the skin permeation of drugs. Eur J Pharm Sci. 1999; 7(4):279-286.

96. Bouwstra JA, Honeywell-Nguyen PL. Skin structure and mode of action of vesicles. Adv Drug Deliv Rev. 2002; 54(Supplement 1):S41-S55.

97. El Maghraby GM, Williams AC, Barry BW. Can drug-bearing liposomes penetrate intact skin? J Pharm Pharmacol. 2006; 58(4):415-29.

98. Elsayed MMA, Abdallah OY, Naggar VF, Khalafallah NM. Lipid vesicles for skin delivery of drugs: Reviewing three decades of research. Int J Pharm. 2007; 332(1-2):1-16.

99. Mezei M, Gulasekharam V. Liposomes--a selective drug delivery system for the topical route of administration: gel dosage form. J Pharm Pharmacol. 1982; 34(7):473-4.

100. Foldvari M, Gesztes A, Mezei M. Dermal drug delivery by liposome encapsulation: clinical and electron microscopic studies. J Microencapsul. 1990; 7(4):479-89.

101. Fresta M, Puglisi G. Application of liposomes as potential cutaneous drug delivery systems. In vitro and in vivo investigation with radioactively labelled vesicles. J Drug Target. 1996; 4(2):95-101.

102. du Plessis J, Ramachandran C, Weiner N, Müller DG. The influence of particle size of liposomes on the deposition of drug into skin. Int J Pharm. 1994; 103(3):277-282.

103. du Plessis J, Weiner N, Müller DG. The influence of in vivo treatment of skin with liposomes on the topical absorption of a hydrophilic and a hydrophobic drug in vitro. Int J Pharm. 1994; 103(2):R1-R5.

104. Hofland HEJ, Bouwstra JA, Spiesb F, Bodda HE, Nagelkerkec JF, Cullanderd C, et al. Interactions Between non-ionic Surfactant Vesicles and human stratum corneum in vitro. J Liposome Research. 1995; 5(2):241-263.

105. Abraham W, Downing DT. Interaction between corneocytes and stratum corneum lipid liposomes in vitro. Biochim Biophys Acta. 1990; 1021(2):119-25.

106. du Plessis J, Egbaria K, Ramachandran C, Weiner N. Topical delivery of liposomally encapsulated gamma-interferon. Antiviral Res. 1992; 18(3-4):259-65.

107. El Maghraby GM, Williams AC, Barry BW. Skin hydration and possible shunt route penetration in controlled estradiol delivery from ultradeformable and standard liposomes. J Pharm Pharmacol. 2001; 53(10):1311-22.

108. Yu H-Y, Liao H-M. Triamcinolone permeation from different liposome formulations through rat skin in vitro. Int J Pharm. 1996; 127(1):1-7.

109. Liu H, Pan WS, Tang R, Luo SD. Topical delivery of different acyclovir palmitate liposome formulations through rat skin in vitro. Pharmazie. 2004; 59(3):203-6.

110. Deo MR, Sant VP, Parekh SR, Khopade AJ, Banakar UV. Proliposome-based transdermal delivery of levonorgestrel. J Biomater Appl. 1997; 12(1):77-88.

111. Aguilella V, Kontturi K, Murtomäki L, Ramírez P. Estimation of the pore size and charge density in human cadaver skin. J Control Release. 1994; 32(3):249-257.

112. Cevc G, Blume G. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochim Biophys Acta. 1992; 1104(1):226-232.

113. Cevc G. Transfersomes, liposomes and other lipid suspensions on the skin: permeation enhancement, vesicle penetration, and transdermal drug delivery. Crit Rev Ther Drug Carrier Syst. 1996; 13(3-4):257-388.

114. Honeywell-Nguyen PL, Bouwstra JA. The in vitro transport of pergolide from surfactant-based elastic vesicles through human skin: a suggested mechanism of action. J Control Release. 2003; 86(1):145-156.

115. Cevc G, Gebauer D, Stieber J, Schätzlein A, Blume G. Ultraflexible vesicles, Transfersomes, have an extremely low pore penetration resistance and transport

therapeutic amounts of insulin across the intact mammalian skin. Biochim Biophys Acta. 1998; 1368(2):201-215.

116. El Maghraby GM, Williams AC, Barry BW. Skin delivery of oestradiol from deformable and traditional liposomes: mechanistic studies. J Pharm Pharmacol. 1999; 51(10):1123-34.

117. El Maghraby GMM, Williams AC, Barry BW. Oestradiol skin delivery from ultradeformable liposomes: refinement of surfactant concentration. Int J Pharm. 2000; 196(1):63-74.

118. El Maghraby GMM, Williams AC, Barry BW. Skin delivery of oestradiol from lipid vesicles: importance of liposome structure. Int J Pharm. 2000; 204(1-2):159-169.

119. Trotta M, Peira E, Carlotti ME, Gallarate M. Deformable liposomes for dermal administration of methotrexate. Int J Pharm. 2004; 270(1-2):119-125.

120. Magnusson BM, Runn P, Karlsson K, Koskinen LOD. Terpenes and ethanol enhance the transdermal permeation of the tripeptide thyrotropin releasing hormone in human epidermis. Int J Pharm. 1997; 157(1):113-121.

121. Komatsu H, Okada S. Ethanol-enhanced permeation of phosphatidylcholine/phosphatidylethanolamine mixed liposomal membranes due to ethanol-induced lateral phase separation. Biochim Biophys Acta. 1996; 1283(1):73-79.

122. Panchagnula R, Salve PS, Thomas NS, Jain AK, Ramarao P. Transdermal delivery of naloxone: effect of water, propylene glycol, ethanol and their binary combinations on permeation through rat skin. Int J Pharm. 2001; 219(1-2):95-105.

123. Morimoto Y, Wada Y, Seki T, Sugibayashi K. In vitro skin permeation of morphine hydrochloride during the finite application of penetration-enhancing system containing water, ethanol and l-menthol. Biol Pharm Bull. 2002; 25(1):134-6.

124. Berner B, Mazzenga GC, Otte JH, Steffens RJ, Juang RH, Ebert CD. Ethanol: water mutually enhanced transdermal therapeutic system II: skin permeation of ethanol and nitroglycerin. J Pharm Sci. 1989; 78(5):402-7.

125. Obata Y, Takayama K, Maitani Y, Machida Y, Nagai T. Effect of ethanol on skin permeation of nonionized and ionized diclofenac. Int J Pharm. 1993; 89(3):191-198.

126. Chen G-S, Kim D-D, Chien YW. Dual-controlled transdermal delivery of levonorgestrel and estradiol: enhanced permeation and modulated delivery. J Control Release. 1995; 34(2):129-143.

127. Pershing LK, Lambert LD, Knutson K. Mechanism of ethanol-enhanced estradiol permeation across human skin in vivo. Pharm Res. 1990; 7(2):170-5.

128. Kurihara-Bergstrom T, Knutson K, DeNoble LJ, Goates CY. Percutaneous absorption enhancement of an ionic molecule by ethanol-water systems in human skin. Pharm Res. 1990; 7(7):762-6.

129. Huang CM. Topical vaccination: the skin as a unique portal to adaptive immune responses. Semin Immunopathol. 2007; 29(1):71-80.

130. Santus GC, Baker RW. Transdermal enhancer patent literature. J Control Release. 1993; 25:1-20.

131. Walters KA. In: Swarbick JW, editor. Encyclopedia of Pharmaceutical Technology. New York: Marcel Dekker. Inc; 2002. p. 945-59.

132. Sarpotdar PP, Zatz JL. Percutaneous absorption enhancement by nonionic surfactants. Drug Devel Ind Pharm. 1986; 12.

133. Cappel MJ, Kreuter J. Effect of nonionic surfactants on transdermal drug delivery: I. Polysorbates. Int J Pharm. 1991; 69(2):143-153.

134. Walters KA, Bialik W, Brain KR. The effects of surfactants on penetration across the skin. Int J Cosmet Sci. 1993; 15(6):260-71.

135. Raykar PV, Fung MC, Anderson BD. The role of protein and lipid domains in the uptake of solutes by human stratum corneum. Pharm Res. 1988; 5(3):140-50.

136. Cevc G, Blume G, Schätzlein A. Transfersomes-mediated transepidermal delivery improves the regio-specificity and biological activity of corticosteroids in vivo. J Control Release. 1997; 45(3):211-226.

137. Cevc G, Blume G. New, highly efficient formulation of diclofenac for the topical, transdermal administration in ultradeformable drug carriers, Transfersomes. Biochim Biophys Acta. 2001; 1514(2):191-205.

138. Cevc G, Schätzlein A, Richardsen H. Ultradeformable lipid vesicles can penetrate the skin and other semi-permeable barriers unfragmented. Evidence from double label CLSM experiments and direct size measurements. Biochim Biophys Acta. 2002; 1564(1):21-30.

139. Paul A, Cevc G, Bachhawat BK. Transdermal immunization with large proteins by means of ultradeformable drug carriers. Eur J Immunol. 1995; 25(12):3521-4.

140. Paul A, Cevc G, Bachhawat BK. Transdermal immunisation with an integral membrane component, gap junction protein, by means of ultradeformable drug carriers, transfersomes. Vaccine. 1998; 16(2-3):188-95.

141. van den Bergh BA, Bouwstra JA, Junginger HE, Wertz PW. Elasticity of vesicles affects hairless mouse skin structure and permeability. J Control Release. 1999; 62(3):367-79.

142. Elsayed MM, Abdallah OY, Naggar VF, Khalafallah NM. Deformable liposomes and ethosomes: mechanism of enhanced skin delivery. Int J Pharm. 2006; 322(1-2):60-6.

143. Fang JY, Hwang TL, Huang YL, Fang CL. Enhancement of the transdermal delivery of catechins by liposomes incorporating anionic surfactants and ethanol. Int J Pharm. 2006; 310(1-2):131-8.

144. El Maghraby GM, Williams AC, Barry BW. Skin delivery of 5-fluorouracil from ultradeformable and standard liposomes in-vitro. J Pharm Pharmacol. 2001; 53(8):1069-77.

145. Boinpally RR, Zhou SL, Poondru S, Devraj G, Jasti BR. Lecithin vesicles for topical delivery of diclofenac. Eur J Pharm Biopharm. 2003; 56(3):389-92.

146. Guo J, Ping Q, Sun G, Jiao C. Lecithin vesicular carriers for transdermal delivery of cyclosporin A. Int J Pharm. 2000; 194(2):201-7.

147. Oh YK, Kim MY, Shin JY, Kim TW, Yun MO, Yang SJ, et al. Skin permeation of retinol in Tween 20-based deformable liposomes: in-vitro evaluation in human skin and keratinocyte models. J Pharm Pharmacol. 2006; 58(2):161-6.

148. Trotta M, Peira E, Debernardi F, Gallarate M. Elastic liposomes for skin delivery of dipotassium glycyrrhizinate. Int J Pharm. 2002; 241(2):319-27.

149. Song YK, Kim CK. Topical delivery of low-molecular-weight heparin with surface-charged flexible liposomes. Biomaterials. 2006; 27(2):271-80.

150. Dubey V, Mishra D, Asthana A, Jain NK. Transdermal delivery of a pineal hormone: melatonin via elastic liposomes. Biomaterials. 2006; 27(18):3491-6.

151. Sharma A, Sharma US. Liposomes in drug delivery: Progress and limitations. Int J Pharm. 1997; 154(2):123-140.

152. Rowe ES. Lipid chain length and temperature dependence of ethanol-phosphatidylcholine interactions. Biochemistry. 1983; 22(3299-3305).

153. Simon SA, McIntosh TJ. Interdigitated hydrocarbon chain packing causes the biphasic transition behavior in lipid/alcohol suspensions. Biochim Biophys Acta. 1984; 773(1):169-72.

154. Rowe ES, Campion JM. Alcohol induction of interdigitation in distearoylphosphatidylcholine: fluorescence studies of alcohol chain length requirements. Biophys J. 1994; 67(5):1888-95.

155. Lobbecke L, Cevc G. Effects of short-chain alcohols on the phase behavior and interdigitation of phosphatidylcholine bilayer membranes. Biochim Biophys Acta. 1995; 1237(1):59-69.

156. Lichtenberg D, Barenholz Y. Liposomes: preparation, characterization, and preservation. Methods Biochem Anal. 1988; 33:337-462.

157. Lasch J, Weissig V, Brandl M. Preparation of Liposomes. In: Torchilin VP, Weissig V, editors. Liposomes: A Practical Approach. 2 ed. New York: Oxford University Press; 2003. p. 2-23.

158. Betageri GV, Jenkins SA, Parsons DL. Preparation of Liposomes. In: Liposome Drug Delivery System. Pennsylvania, USA: Technomic Publishing Company, Inc.; 1993. p. 1-22.

159. Martin FJ. Pharmaceutical Manufacturing of Lipids. In: Tyle P, editor. Specialized Drug Delivery Systems: Manufacturing and Production Technology. New York: Marcel Dekker; 1990. p. 267-316.

160. Szoka F, Jr., Papahadjopoulos D. Comparative properties and methods of preparation of lipid vesicles (liposomes). Annu Rev Biophys Bioeng. 1980; 9:467-508.

161. Deamer D, Bangham AD. Large volume liposomes by an ether vaporization method. Biochim Biophys Acta. 1976; 443(3):629-634.

162. Batzri S, Korn ED. Single bilayer liposomes prepared without sonication. Biochim Biophys Acta. 1973; 298(4):1015-1019.

163. Talsma H, Cormmelin DJA. Liposomes as Drug Delivery Systems, Part I: Preparation. Liposomes as drug delivery systems, Part-II : Characterization, Pharmaceutical Technology. 1992; 16:96-106.

164. Olson F, Hunt CA, Szoka FC, Vail WJ, Papahadjopoulos D. Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes. Biochim Biophys Acta. 1979; 557(1):9-23.

165. Talsma H, Crommelin DJA. Liposomes as Drug Delivery Systems Part II: Characterization. Pharmaceutical Technology. 1992; 16:52-58.

166. Vyas SP, Khar RK. Targeted and controlled drug delivery: Novel carrier systems. In: Liposomes: CBS Publishers & Distributors 2006. p. 196-197.

167. Patel SS. Liposome: A versatile platform for targeted delivery of drugs. Latest Rev. 2006; 4(5).

168. Lasic DD, Papahadjopoulos D. In: Medical applications of liposomes. New York: Elsevier; 1998. p. 9-12.

169. Hauser H. Phosphplipid vesicles. In: Cevc G, editor. Phospholipids Handbook. New York: Marcel Dekker; 1993. p. 603-637.

170. Crommelin DJ. Influence of lipid composition and ionic strength on the physical stability of liposomes. J Pharm Sci. 1984; 73(11):1559-63.

171. Hadgraft J, du Plessis J, Goosen C. The selection of non-steroidal anti-inflammatory agents for dermal delivery. Int J Pharm. 2000; 207(1-2):31-7.

172. Avdeef A, Box KJ, Comer JEA, Hibbert C, Tam KY. pH -metric log P 10. Determination of liposomal membrane water partition coefficients of ionizable drugs. Pharm. Res. 1998; 15:209-215.

173. Watkinson AC, Brain KR, Walters KA. The penetration of ibuprofen through human skin in vitro: vehicle, enhancer and pH effects. In: Brain, K.R., James, V. and Walters, K.A., Editors. Prediction of Percutaneous Penetration 3B. Cardiff: STS Publishing; 1993.

174. Herkenne C, Naik A, Kalia YN, Hadgraft J, Guy RH. Ibuprofen transport into and through skin from topical formulations: in vitro-in vivo comparison. J Invest Dermatol. 2007; 127(1):135-42.

175. Adams SS, McCullough KF, Nicholson JS. Some biological properties of ibuprofen, an anti-inflammatory, analgesic and antipyretic agent. Arzneim.-Forsch. Drugs Res. 1975; 25:1786-1791.

176. Arendt-Nielsen L, Drewes AM, Svendsen L, Brennum J. Quantitative assessment of joint pain following treatment of rheumatoid arthritis with ibuprofen cream. Scand J Rheumatol. 1994; 23(6):334-7.

177. Cordero JA, Alarcon L, Escribano E, Obach R, Domenech J. A comparative study of the transdermal penetration of a series of nonsteroidal antiinflammatory drugs. J Pharm Sci. 1997; 86(4):503-8.

178. Cordero JA, Camacho M, Obach R, Domenech J, Vila L. In vitro based index of topical anti-inflammatory activity to compare a series of NSAIDs. Eur J Pharm Biopharm. 2001; 51(2):135-42.

179. Singh P, Roberts MS. Skin permeability and local tissue concentrations of nonsteroidal anti-inflammatory drugs after topical application. J Pharmacol Exp Ther. 1994; 268(1):144-51.

180. Babar A, Solanki UD, Cutie AJ, Plakogiannis F. Piroxicam release from dermatological bases: in vitro studies using cellulose membrane and hairless mouse skin. Drug Dev. Ind. Pharm. 1990; 16:523-540.

181. Muktadir A, Babar A, Cutie AJ, Plakogiannis FM. Medicament Release from Ointment Bases: III. Ibuprofen: In Vitro Release and In-Vivo Absorption in Rabbits. Drug Devel Ind Pharm. 1986; 12(14):2521-2540.

182. Chang SY, Park SB, Jung JH, Shon SI, Yoon HJ. Iburpofen heterocyclic esters as dermal prodrugs: in vitro evaluation. S.T.P. Pharma Sci. 1997; 7:315-319.

183. Heard CM, Gallagher SJ, Harwood J, Maguire PB. The in vitro delivery of NSAIDs across skin was in proportion to the delivery of essential fatty acids in the vehicle--evidence that solutes permeate skin associated with their solvation cages? Int J Pharm. 2003; 261(1-2):165-9.

184. Iervolino M, Cappello B, Raghavan SL, Hadgraft J. Penetration enhancement of ibuprofen from supersaturated solutions through human skin. Int J Pharm. 2001; 212(1):131-41.

185. Park KM, Kim CK. Preparation and evaluation of flurbiprofen-loaded microemulsion for parenteral delivery. Int J Pharm. 1999; 181(2):173-9.

186. Perioli L, Ambrogi V, Angelici F, Ricci M, Giovagnoli S, Capuccella M, et al. Development of mucoadhesive patches for buccal administration of ibuprofen. J Control Release. 2004; 99(1):73-82.

187. Stott PW, Williams AC, Barry BW. Transdermal delivery from eutectic systems: enhanced permeation of a model drug, ibuprofen. J Control Release. 1998; 50(1-3):297-308.

188. Walde P, Ichikawa S. Enzymes inside lipid vesicles: preparation, reactivity and applications. Biomol Eng. 2001; 18(4):143-77.

189. K-i Og, J. S. Safety of sodium fluorescein for in vivo study of skin. Skin Res Technol. 2006; 12(3):166-61.

190. Veiro JA, Cummins PG. Imaging of skin epidermis from various origins using confocal laser scanning microscopy. Dermatology. 1994; 189(1):16-22.

191. Watkinson RM, Herkenne C, Guy RH, Hadgraft J, Oliveira G, Lane ME. Influence of ethanol on the solubility, ionization and permeation characteristics of ibuprofen in silicone and human skin. Skin Pharmacol Physiol. 2009; 22(1):15-21.

192. Sherri Farmer PA, Philip Burns, and Ranga Velagaleti. Forced Degradation of Ibuprofen in Bulk Drug and Tablets and Determination of Specificity, Selectivity, and the Stability-Indicating Nature of the USP Ibuprofen Assay Method. Pharmaceutical Technology 2002:28-42.

193. Lasic DD. Liposomes in Gene Delivery. CRC Press LLC; 1997.

194. Cevc G, Schätzlein A, Blume G. Transdermal drug carriers: Basic properties, optimization and transfer efficiency in the case of epicutaneously applied peptides. J Control Release. 1995; 36(1-2):3-16.

195. Ross-Murphy SB. Dynamic Light Scattering. B. J. Berne and R. Pecora, John Wiley, New York, 1976, pp. 376.

196. Lopez-Pinto JM, Gonzalez-Rodriguez ML, Rabasco AM. Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. Int J Pharm. 2005; 298(1):1-12.

197. Jain S, Jain P, Umamaheshwari RB, Jain NK. Transfersomes--a novel vesicular carrier for enhanced transdermal delivery: development, characterization, and performance evaluation. Drug Dev Ind Pharm. 2003; 29(9):1013-26.

198. van den Bergh BA, Wertz PW, Junginger HE, Bouwstra JA. Elasticity of vesicles assessed by electron spin resonance, electron microscopy and extrusion measurements. Int J Pharm. 2001; 217(1-2):13-24.

199. Nounou MM, El-Khordagui LK, Khalafallah NA, Khalil SA. In vitro release of hydrophilic and hydrophobic drugs from liposomal dispersions and gels. Acta Pharm. 2006; 56:311-324.

200. Schaefer H, Loth H. An ex vivo model for the study of drug penetration into human skin. Pharm. Res. 1996; 13:366.

201. Michel C, Purmann T, Mentrup E, Seiller E, Kreuter J. Effect of liposomes on percutaneous penetration of lipophilic materials. Int J Pharm. 1992; 84(2):93-105.

202. Bronaugh RL, Stewart RF, Congdon ER, Giles AL, Jr. Methods for in vitro percutaneous absorption studies. I. Comparison with in vivo results. Toxicol Appl Pharmacol. 1982; 62(3):474-80.

203. Kligman AM, Christophers E. Preparation of Isolated Sheets of Human Stratum Corneum. Arch Dermatol. 1963; 88:702-5.

204. Zhao K, Singh J. In vitro percutaneous absorption enhancement of propranolol hydrochloride through porcine epidermis by terpenes/ethanol. J Control Release. 1999; 62(3):359-66.

205. Weichers JW. Measuring Skin Delivery. In: Wiechers JW, editor. Science and Applications of Skin Delivery Systems: Culinary and Hospitality Industry Publications Services; 2008.

206. Shantha Kumar TR, Chawla S, Nachaegari SK, Singh SK, Srinivas NR. Validated HPLC analytical method with programmed wavelength UV detection for simultaneous determination of DRF-4367 and Phenol red in rat in situ intestinal perfusion study. J Pharm Biomed Anal. 2005; 38(1):173-9.

207. Rathore R, Jain JP, Srivastava A, Jachak SM, Kumar N. Simultaneous determination of hydrazinocurcumin and phenol red in samples from rat intestinal permeability studies: HPLC method development and validation. J Pharm Biomed Anal. 2008; 46(2):374-380.

208. Coderch L, Oliva M, Pons M, de la Maza A, Manich AM, Parra JL. Percutaneous penetration of liposomes using the tape stripping technique. Int J Pharm. 1996; 139(1-2):197-203.

209. Shotton E, Habeeb AF. Research papers; the estimation of fluorescein in dilute solutions. J Pharm Pharmacol. 1955; 7(7):456-62.

210. Havener WA. Fluorescein and other dyes. In: Havener WH, editor. Ocular Pharmacology. 4 ed. C.V. Mosby, St. Louis: Saint Louis : C. V. Mosby Co.; 1978. p. 413-434.

211. Zhu H, Derksen RC, Krause CR, Fox RD, Brazee RD, Ozkan HE. Fluorescent Intensity of Dye Solutions under Different pH Conditions. J ASTM Int. 2005; 2(6).

212. Mohammed AR, Weston N, Coombes AG, Fitzgerald M, Perrie Y. Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability. Int J Pharm. 2004; 285(1-2):23-34.

213. Al-Saidan SM. Transdermal self-permeation enhancement of ibuprofen. J Control Release. 2004; 100:199-209.

214. Barenholz Y, Crommelin DJA. Liposomes in Pharmaceutical Dosage Forms. In: Swarbick JW, Boylan JC, editors. Encyclopedia of Pharmaceutical Technology. New York: Marcel Dekker; 1994.

215. Costa CAMd, Moraes ÂM. Encapsulation of 5-fluorouracil in liposomes for topical administration. Acta Scientiarum. Technology. 2003; 25(1):53-61.

216. Bangham AD. Preparation of liposomes and methods for measuring their permeabilities. In Techniques in the Life Sciences. 1977:B4/11, B420/1-B420/25.

217. Instruments M. Electrophoretic Light Scattering Overview. Malvern Instruments Technical Support Library. 2008.

218. Makino K, Yamada T, Kimura M, Oka T, Ohshima H, Kondo T. Temperature- and ionic strength-induced conformational changes in the lipid head group region of liposomes as suggested by zeta potential data. Biophys Chem. 1991; 41(2):175-83.

219. Tiyaboonchai W, Limpeanchob N. Formulation and characterization of amphotericin B-chitosan-dextran sulfate nanoparticles. Int J Pharm. 2007; 329(1-2):142-9.

220. Handy RD, Owen R, Valsami-Jones E. The ecotoxicology of nanoparticles and nanomaterials: current status, knowledge gaps, challenges, and future needs. Ecotoxicology. 2008; 17(5):315-25.

221. Sager TM, Porter DW, Robinson VA, Lindsley WG, Schwegler-Berry DE, Castranova V. Improved method to disperse nanoparticles for in vitro and in vivo investigation of toxicity. Nanotoxicology. 2007; 1(2):118-129.

222. Verma DD, Verma S, Blume G, Fahr A. Particle size of liposomes influences dermal delivery of substances into skin. Int J Pharm. 2003; 258(1-2):141-151.

223. Ogiso T, Yamaguchi T, Iwaki M, Tanino T, Miyake Y. Effect of Positively and Negatively Charged Liposomes on Skin Permeation of Drugs. J Drug Targeting. 2001; 9(1):49 - 59.

224. Mayer LD, Tai LC, Ko DS, Masin D, Ginsberg RS, Cullis PR, et al. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res. 1989; 49(21):5922-30.

225. Xia S, Xu S. Ferrous sulfate liposomes: preparation, stability and application in fluid milk. Food Res Int. 2005; 38:289-296.

226. Yang T, Cui F-D, Choi M-K, Cho J-W, Chung S-J, Shim C-K, et al. Enhanced solubility and stability of PEGylated liposomal paclitaxel: In vitro and in vivo evaluation. Int J Pharm. 2007; 338(1-2):317-326.

227. Schwarz C, Mehnert W, Lucks JS, Müller RH. Solid lipid nanoparticles (SLN) for controlled drug delivery. I. Production, characterization and sterilization. J Control Release. 1994; 30(1):83-96.

228. Essa EA, Bonner MC, Barry BW. Electrically assisted skin delivery of liposomal estradiol; phospholipid as damage retardant. J Control Release. 2004; 95(3):535-546.

229. Epand RM, Epand RF, Maekawa S. The arrangement of cholesterol in membranes and binding of NAP-22. Chem Phys Lipids. 2003; 122(1-2):33-9.

230. Rogerson A, Cummings J, Florence AT. Adriamycin-loaded niosomes: drug entrapment, stability and release. J Microencapsul. 1987; 4(4):321-8.

231. Semple SC, Chonn A, Cullis PR. Influence of cholesterol on the association of plasma proteins with liposomes. Biochemistry. 1996; 35(8):2521-5.

232. Devaraj GN, Parakh SR, Devraj R, Apte SS, Rao BR, Rambhau D. Release studies on niosomes containing fatty alcohols as bilayer stabilizers instead of cholesterol. J Colloid Interface Sci. 2002; 251(2):360-5.

233. Mishra D, Garg M, Dubey V, Jain S, Jain NK. Elastic liposomes mediated transdermal delivery of an anti-hypertensive agent: propranolol hydrochloride. J Pharm Sci. 2007; 96(1):145-55.

234. Heurtault B, Saulnier P, Pech B, Proust J-E, Benoit J-P. Physico-chemical stability of colloidal lipid particles. Biomaterials. 2003; 24(23):4283-4300.

235. Barratt G. Characterization of colloidal drug carrier systems with zeta potential measurements. Pharm Tech Eur. 1999:25-32.

236. Gregoriadis G, Davis C. Stability of liposomes in vivo and in vitro is promoted by their cholesterol content and the presence of blood cells. Biochem Biophys Res Commun. 1979; 89(4):1287-93.

237. Demiel RA, Guerts van Kessel WS, van Deenen LL. The properties of polyunsaturated lecithins in monolayers and liposomes and the interactions of these lecithins with cholesterol. Biochim Biophys Acta. 1972; 266(1):26-40.

238. Papahadjopoulos D, Jacobson K, Nir S, Isac T. Phase transitions in phospholipid vesicles. Fluorescence polarization and permeability measurements

concerning the effect of temperature and cholesterol. Biochim Biophys Acta. 1973; 311(3):330-48.

239. Cable C. An examination of the effects of surface modifications on the physicochemical and biological properties of non-ionic surfactant vesicles thesis. Glasgow, UK: University of Strathclyde; 1989.

240. Hao Y, Zhao F, Li N, Yang Y, Li K. Studies on a high encapsulation of colchicine by a niosome system. Int J Pharm. 2002; 244(1-2):73-80.

241. Jaafar-Maalej C, Diab R, Andrieu Vr, Elaissari A, Fessi H. Ethanol injection method for hydrophilic and lipophilic drug-loaded liposome preparation. J Liposome Research. 20(3):228-243.

242. Lee EH, Kim A, Oh YK, Kim CK. Effect of edge activators on the formation and transfection efficiency of ultradeformable liposomes. Biomaterials. 2005; 26(2):205-10.

243. Yang Y, Bai G, Zhang X, Ye C, Liu M. 1H NMR spectroscopic evidence of interaction between ibuprofen and lipoproteins in human blood plasma. Anal Biochem. 2004; 324(2):292-7.

244. Tasi L-M, Liu D-Z, Chen W-Y. Microcalorimetric investigation of the interaction of polysorbate surfactants with unilamellar phosphatidylcholines liposomes. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2003; 213(1):7-14.

245. Hofer C, Hartung R, Gobel R, Deering P, Lehmer A, Breul J. New ultradeformable drug carriers for potential transdermal application of interleukin-2 and interferon-alpha: theoretic and practical aspects. World J Surg. 2000; 24(10):1187-9.

246. Jain S, Tiwary AK, Jain NK. Sustained and targeted delivery of an anti-HIV agent using elastic liposomal formulation: mechanism of action. Curr Drug Deliv. 2006; 3(2):157-66.

247. Lasch J, Hoffmann J, Omelyanenko WG, Klibanov AA, Torchilin VP, Binder H, et al. Interaction of Triton X-100 and octyl glucoside with liposomal membranes at sublytic and lytic concentrations. Spectroscopic studies. Biochim Biophys Acta. 1990; 1022(2):171-80.

248. Lopez O, de la Maza A, Coderch L, Lopez-Iglesias C, Wehrli E, Parra JL. Direct formation of mixed micelles in the solubilization of phospholipid liposomes by Triton X-100. FEBS Lett. 1998; 426(3):314-8.

249. Lichtenberg D, Robson RJ, Dennis EA. Solubilization of phospholipids by detergents. Structural and kinetic aspects. Biochim Biophys Acta. 1983; 737(2):285-304.

250. Grit M, Crommelin DJ. The effect of aging on the physical stability of liposome dispersions. Chem Phys Lipids. 1992; 62(2):113-22.

251. Mouritsen OG. Theoretical models of phospholipid phase transitions. Chem Phys Lipids. 1991; 57(2-3):179-94.

252. Lasic DD. Liposomes: From Physics to Applications. Elsevier Science, Amsterdam. 1993.

253. Samuni AM, Barenholz Y. Stable nitroxide radicals protect lipid acyl chains from radiation damage. Free Radic Biol Med. 1997; 22(7):1165-74.

254. Samuni AM, Barenholz Y, Crommelin DJ, Zuidam NJ. Gamma-irradiation damage to liposomes differing in composition and their protection by nitroxides. Free Radic Biol Med. 1997; 23(7):972-9.

255. Tirosh O, Kohen, R., Alon, A., Katzhendler, J. and Barenholz, Y. Novel synthetic phospholipid protects lipid bilayers against oxidative damage: role of hydration layer and bound water. J. Chem. Soc. Perkin Trans. 1997; 2:383-389.

256. Priev A, Samuni AM, Tirosh O, Barenholz Y. The role of hydration in stabilization of liposomes: resistance to oxidative damage of PEG-grafted liposomes. In: Gregoriadis G, McCormack B, editors. Targeting of Drugs 6: Strategies for Stealth Therapeutic Systems. New York: Plenum Press; 1998. p. 147-167.

257. Bereza UL, Brewer GJ, Hill GM. Effect of dietary cholesterol on erythrocyte peroxidant stress in vitro and in vivo. Biochim Biophys Acta. 1985; 835(3):434-40.

258. Clemens MR, Waller HD. Lipid peroxidation in erythrocytes. Chem Phys Lipids. 1987; 45(2-4):251-68.

259. Parasassi T, Giusti AM, Raimondi M, Ravagnan G, Sapora O, Gratton E. Cholesterol protects the phospholipid bilayer from oxidative damage. Free Radic Biol Med. 1995; 19(4):511-6.

260. Samuni AM, Lipman A, Barenholz Y. Damage to liposomal lipids: protection by antioxidants and cholesterol-mediated dehydration. Chem Phys Lipids. 2000; 105(2):121-34.

261. Simon SA, McIntosh TJ, Latorre R. Influence of cholesterol on water penetration into bilayers. Science. 1982; 216(4541):65-7.

262. Parasassi T, Di Stefano M, Loiero M, Ravagnan G, Gratton E. Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence. Biophys J. 1994; 66(1):120-32.

263. Parasassi T, Di Stefano M, Loiero M, Ravagnan G, Gratton E. Cholesterol modifies water concentration and dynamics in phospholipid bilayers: a fluorescence study using Laurdan probe. Biophys J. 1994; 66(3 Pt 1):763-8.

264. Parasassi T, Giusti AM, Raimondi M, Gratton E. Abrupt modifications of phospholipid bilayer properties at critical cholesterol concentrations. Biophys J. 1995; 68(5):1895-902.

265. Fan M, Xu S, Xia S, Zhang X. Effect of Different Preparation Methods on Physicochemical Properties of Salidroside Liposomes. J. Agric. Food Chem. 2007; 55(8):3089-3095.

266. Gruner SM, Lenk RP, Janoff AS, Ostro MJ. Novel multilayered lipid vesicles: comparison of physical characteristics of multilamellar liposomes and stable plurilamellar vesicles. Biochemistry. 1985; 24(12):2833-42.

267. Paavola A, Kilpelainen I, Yliruusi J, Rosenberg P. Controlled release injectable liposomal gel of ibuprofen for epidural analgesia. Int J Pharm. 2000; 199(1):85-93.

268. Minghui F, Shiying X, Shuqin X, Xiaoming Z. Preparation of salidroside nano-liposomes by ethanol injection method and in vitro release study. Eur Food Res Technol. 2008 227:167-174.

269. Liu D-Z, Chen W-Y, Tasi L-M, Yang S-P. Microcalorimetric and shear studies on the effects of cholesterol on the physical stability of lipid vesicles. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2000; 172(1-3):57-67.

270. Foradada M, Estelrich J. Encapsulation of thioguanine in liposomes. Int J Pharm. 1995; 124(2):261-269.

271. Avdeef A, Box KJ, Comer JE, Gilges M, Hadley M, Hibbert C, et al. PH-metric log P 11. pKa determination of water-insoluble drugs in organic solvent-water mixtures. J Pharm Biomed Anal. 1999; 20(4):631-41.

272. Yoshida A, Hashizaki K, Yamauchi H, Sakai H, Yokoyama S, Abe M. Effect of Lipid with Covalently Attached Poly(ethylene glycol) on the Surface Properties of Liposomal Bilayer Membranes. Langmuir. 1999; 15(7):2333-2337.

273. Nagarsenker MS, Londhe VY. Preparation and evaluation of a liposomal formulation of sodium cromoglicate. Int J Pharm. 2003; 251(1-2):49-56.

274. Peschka R, Dennehy C, Szoka FC, Jr. A simple in vitro model to study the release kinetics of liposome encapsulated material. J Control Release. 1998; 56(1-3):41-51.

275. Henriksen I, Sande SA, Smistad G, Ågren T, Karlsen J. In vitro evaluation of drug release kinetics from liposomes by fractional dialysis. Int J Pharm. 1995; 119(2):231-238.

276. Wissing SA, Kayser O, Muller RH. Solid lipid nanoparticles for parenteral drug delivery. Adv Drug Deliv Rev. 2004; 56(9):1257-72.

277. Cócera M, López O, Coderch L, Parra JL, de la Maza A. Permeability investigations of phospholipid liposomes by adding cholesterol. Colloids and Surfaces A: Physicochemical and Engineering Aspects. 2003; 221(1-3):9-17.

278. Win PP, Shin-ya Y, Hong K-J, Kajiuchi T. Formulation and characterization of pH sensitive drug carrier based on phosphorylated chitosan (PCS). Carbohydrate Polymers. 2003; 53(3):305-310.

279. Breuer MM. The interaction between surfactants and keratinous tissues. J. SOC. Cosmet. Chem. 1979; 30(414).