PROLIPOSOME AND PROSURFACTOSOME FORMULATIONS FOR PULMONARY DRUG DELIVERY

By

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A thesis submitted in partial fulfilment for the requirements for the degree of PhD at the University of Central Lancashire

November, 2015

Declaration

I declare that while registered as a candidate for the research degree, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution

I declare that no material contained in the thesis has been used in any other submission for an academic award and is solely my own work

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Type of Award **PhD (Doctor of Philosophy)**

School **Pharmacy and Biomedical Science**

Abstract

This study aims to compare the efficiency of conventional liposomes and surfactant-enriched vesicles (surfactosomes) using the hydrophilic drug salbutamol sulphate (SBS) and the hydrophobic drug beclometasone dipropionate (BDP) for pulmonary delivery via nebulisation.

Initially liposomes and surfactosomes with or without cholesterol were prepared using thin film method and were compared for their VMD, span and drug entrapment. Their drug retention on extrusion through 5µm, 2µm, 1µm and 0.4µm polycarbonate membrane using mini-extruder was also studied. It was observed that liposomes were more stable than surfactosome.

Particulate based proliposome technology was also used to study their potential for generating stable and inhalable dispersions. Mannitol was used as the carbohydrate carrier and on hydration; proliposomes and prosurfactosomes have generated liposomes and surfactosomes respectively. The VMD, span and zeta potential of the vesicles, and drug entrapment and drug retention on extrusion were studied. It was seen that lower proportions of SBS were entrapped using proliposome technology; hence, further extrusions through 5µm and 2µm were avoided. In vesicle with BDP, inclusion of cholesterol has decreased the drug entrapment and crystallisation of mannitol was observed.

Nebulisation of liposomes and surfactosomes with and without cholesterol was studied using PARI LC sprint air jet nebuliser, Aeroneb pro and Beurer iH50 vibrating mesh nebulisers. Two stage (Twin) impinger was used to study the potential suitability of the generated vesicles for inhalation. VMD, span and zeta potential of vesicles before and after nebulisation was studied. BDP delivery and retention in both stages of the twin impinger was also studied. It was found that surfactosomes without cholesterol delivered maximum BDP to the twin impinger. Nebulisers suitable for all four formulations were also studied. Beurer iH50 delivered maximum BDP via liposomes with and without cholesterol, Aeroneb Pro delivered maximum BDP via surfactosomes with cholesterol to upper impinger while PARI LC sprint delivered maximum BDP via surfactosomes with cholesterol. VMD and span of aerosols generated from all three nebulisers were also studied.

Stability of liposomes and surfactosomes prepared using proliposome technology was studied. VMD, span, zeta potential and BDP retention before and after spray drying and freeze drying were investigated. It was concluded that liposomes and surfactosomes were equally stable when spray drying was used whereas liposomes were more stable that surfactosomes when freeze drying was conducted. X-ray diffraction, scanning electron microscopy and transmission electron microscopy were used to analyse the characteristics of proliposomes and prosurfactosomes. A reduction in size and crystallinity was observed after spray drying and freeze drying of the formulations. Stability was also studied on storing proliposome and prosurfactosome in different environmental conditions like 5-6°C, room temperature and 40°C for a period of 3 months. It was concluded that both proliposomes and surfactosomes were most stable in 2-8°Cwhereas least stable in 40°C. Proliposomes were more stable than prosurfactosomes regardless of the storage temperature.

Formulation and characterisation of novel prosurfactosomes and comparing it with conventional liposomes for pulmonary drug delivery is the novelty of this thesis.

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Acknowledgements

First and foremost, I would like to thank my supervisor Dr.Abdelbary Elhissi for supporting me throughout my PhD. I appreciate his contribution in terms of time, ideas, motivation and funds. He guided me when I was lost in my experiments and directed me towards the right path. I would also like to thank my supervisors Dr. Mohamed Albed Alhnan and Prof. Waqar Ahmed who helped me in their own ways to make my PhD experience productive. I would specially like to thank Dr. Jai Paul Singh for helping me at every step of PhD right from admissions all the way till thesis submission and for always being around to help in the most difficult situations.

I would also like to thank all the lab technicians who generously helped me when I needed them the most. Without technician's support it would have been very difficult to learn, handle and maintain laboratory equipments. I would also take this opportunity to thank all the office staff of UCLan, in research office as well as in school office without whom it would have been difficult to manage the paper works and formalities required at every stage of PhD. I would like to specially thank David McCarthy from UCL School of pharmacy, UK for helping me with TEM pictures selflessly.

I would like to thank all the members of nanotechnology and drug delivery group for their support. Apart from helping me solved issues related to PhD, this group thought me team work as well as how to take responsibility in a team. It attributed for my overall development. Regular meetings and discussions with the group made big issues small, thus, reducing the stress level. I would also like to all my colleagues in my office MB212 as well in lab MB226, especially Oshadie Korale, Dr.Iftikhar Khan, Tanem Garanti, Sakib Yousaf, Dr.Hunar Omer, Dr.Nozad Hussein and Dr. Basel Arafat for helping me to finish my experiments with ease. Without your support this journey would have been difficult and monotonous. Being in similar situation and together for a considerable time, it was easier to share my personal and academic problems with you all. Thanks.

I would take this opportunity to acknowledge Lipoid, Germany for providing me with soya phosphotidylcholine (SPC) for my experiments. I would also acknowledge Aerogen Inc. for gifting me Aeroneb pro nebuliser to use in my PhD research. I am also grateful to Sigma Aldrich and Fisher Scientific for making available all the chemicals and laboratory equipments and for their quick delivery and customer support.

I would like to express my gratitude towards my parents in India who supported me throughout my PhD emotionally as well as financially. They guided me throughout the journey and encouraged me to pursue my dreams without any hesitations. My relatives in India were also equally involved in motivating me through out and supporting my parents when I was away doing my research. I would also like to thank my friends in India- Sunaina Nair, Saurabh Mahajan, Aman Lohia, Sneha Ramakrishnan and Shravan Ravi who constantly helped me by being available whenever I was homesick and emotionally broken during ups and downs in PhD over a span on 3.5 years. I would specially like to thank my friends in UK- Nikhil Mahajan for being my guide, friend and technical support; Dr. Swati Kumar for being my support at home (in UK) as well as in academics, Oshadie Korale for being my friend to share my happiness and sorrow and Dr. Saurabh Prabhu for guiding me in thesis write-up. Without them my journey of PhD in UK would have been impossible as they filled my life with hope and joy.

I would like to thank my financial supports in UK- University of Central Lancashire and Vinay Mistry from Nisa Local for trusting me and employing me throughout my PhD with maximum flexibility. They did their best not to disturb me from my PhD schedule and decreased my stress and work load as and when required. I would like to specially thank Bank of India for providing me with funds when I required the most.

Last but not the least I would like to thank God for listening to my prayers and filling my soul with positivity and strength. He gave me the right opportunity at right time which helped me complete my PhD without any major obstacles.

Sneha Subramanian

List of abbreviations

Aeroneb pro	Aeroneb Pro vibrating mesh nebuliser
ANOVA	Analysis of variance
Beurer	Beurer iH50 vibrating mesh nebuliser
BDP	Beclometasone dipropionate
BP	British Pharmacopeia
CFC	Chlorofluorocarbon
Chol	Cholesterol
COPD	Chronic obstructive pulmonary disorder
CsA	Ciclosporine A
D_2O	Deuterium Oxide
DMPC	Dimyristoylphosphatidylcholine
DPI	Dry powder inhaler
DPPC	Dypalmitoylphosphatidylcholine
DSC	Differential scanning calorimetry
EPC	Egg phosphotidylcholine
Fig	Figure
FPF	Fine particle fraction
HLB	Hydrophilic/ lipophilic balance
HPLC	High performance liquid chromatography
IFN γ	Interferon gamma
IgE	Immunoglubulin E

L_α	Liquid crystalline phase of phospholipid
$L_ß$	Gel phase of a phospholipid
LUVs	Large unilamellar vesicles
MLVs	Multilammelar vesicles
MVLs	Multivesicular liposomes
NaCl	Sodium Chloride
OLVs	Oligolamellar vesicles
Omron (mesh)	Omron Micro Air NE-U22 vibrating mesh nebuliser
P value	Probability
Pari (jet)	PARI LC sprint air-jet nebuliser
PC	Phosphotidylcholine
PIMs	Pulmonary intravascular macrophages
PM	Physical mixture
pMDI	Pressurised metered dose inhaler
ROS	Reactive oxygen species
SBS	Salbutamol sulphate
SD	Standard deviation
SEM	Scanning electron microscopy
Span	(90% undersize-10% undersize) /Volume mean diameter
Span 80	Sorbitan monooleate
SPC	Soya phosphotidyl choline
SUVs	Small unilamellar vesicles

TEM	Transmission electron microscopy
Tm	Main phase transition temperature
Tween 80	Polyoxyethylene sorbitan monooleate 80
USP	United States Pharmacopeia
UV	Ultraviolet
VMD	Volume median diameter

The mind is everything. What you think you become.

- Gautama Buddha

Chapter 1

Introduction

1.1. Phospholipids

Phospholipids are major components of cell membranes. They are made from a glycerol backbone, phosphate headgroups and fatty acid chains. They act as building blocks of every cell (Cevc and Paltauf, 1995). A phospholipid molecule consists of diglycerides, a phosphate group and an organic molecule like choline as shown in Figure 1.1. Diglyceride is glycerine which has two fatty acid chains and covalently bonded to glycerol via ester linkage. Glycerol is part of the hydrophilic head and it also helps the fatty acid tail to connect to phosphate headgroup. The carbon 3 of glycerol consists of polar headgroup, i.e. water soluble and carbon 1 and 2 consists of fatty acid which forms the non-polar tail i.e. lipid soluble (Berg et al., 2002). Hence, phospholipids are described as amphipathic molecules owing to their polar and non-polar moieties (Lasic, 1988). Phospholipids are made of diverse headgroups, and diverse degree of saturation and length of hydrocarbon chains. It has been found that when phospholipids are combined with water they form hollow spheres (Bangham et al., 1965). Hydrophilic head contacts the aqueous medium forming the outside and inside of the vesicles that are in contact with the aqueous environment. Hydrophobic tails mutually attracts and remain sandwiched in between the polar moieties. This arrangement avoids the contact of hydrophobic tail with water. These phospholipids have a phenomenal property of self-assembling when dispersed in aqueous media that are free from detergents and co-solvents.

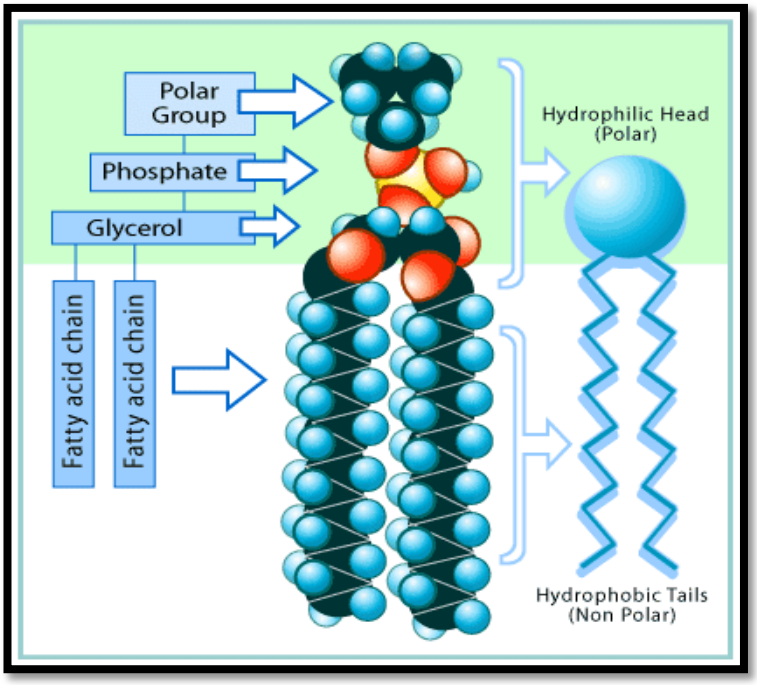

Figure 1.1 Structure of phospholipid molecule (Taken from Ranger, 2007)

1.2. Micelles

Micelles are spherical amphipathic molecule aggregates which arrange themselves in a way that protects their hydrophobic moieties from the aqueous environment. Micelles have very small particle size of about 50nm. Micelles contain polar head moiety on their outer side which contacts with the surrounding water and non-polar tail which is orientated towards the inner side away from the aqueous phase. Micelles create a highly hydrophobic microenvironment within their core which helps in maximum entrapment/solubility of hydrophobic drugs. This may increase the bioavailability of the drug (Wei et al., 2009).

1.3. Vesicles

Vesicles are small membrane enclosed sack which has the ability to transport materials across biological barriers (Honeywell-Nguyen and Bouwstra, 2005). These vesicles are separated from the surrounding with phospholipid layers and can be divided into range of types depending on their morphology such as unilamellar or multilamellar vesicles. Their membrane is similar to the plasma membranes, thus, they tend to fuse with the desired cell and release their contents into the cytoplasm. Vesicles can carry both hydrophilic and lipophilic molecules and, thus they are extensively used for various purposes like drug delivery, drug targeting, protection of proteins against degradation, controlled drug release and protection of drug against metabolism (Sudhamani et al., 2010a). Most commonly described vesicles in pharmacy are niosomes and liposomes (Honeywell-Nguyen and Bouwstra, 2005) .

1.4. Niosomes

Niosomes are non-ionic surfactant vesicles that are used in drug delivery to entrap a solute in a manner analogous to liposomes (Sudhamani et al., 2010b). Niosomes are usually formed using a mixture of non-ionic surfactants of the alkyl or dialkyl polyglycerol ether class and cholesterol followed by hydration in aqueous media. The surfactants used are uncharged single chain molecules, unlike liposomes which may have charged double chained phospholipids. As shown in Figure 1.2, niosomes can entrap both hydrophilic and lipophilic drugs, either in the aqueous core or in vesicular membranes made of lipid materials (Srinivas et al., 2010). They have many properties similar to liposomes in delivering drug to various regions of the body. They are more

stable and made of materials that are cheaper than those made to manufacture liposomes. The size of niosomes is observed using microscopic techniques to be in the range of 10nm to few micrometers. Niosomes have disadvantages like tendency of aggregation, fusion, hydrolysis and leakage of encapsulated drugs from niosomes to the surrounding aqueous environment (Sudhamani et al., 2010b).

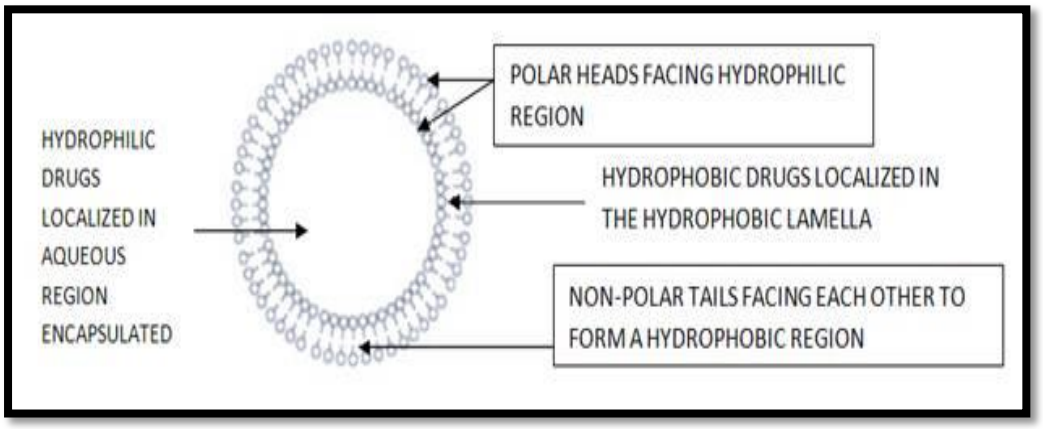

Figure 1.2 Structure of Niosome (Taken from Sudhamani et al., 2010).

1.5. Liposomes

Liposomes are hollow phospholipid vesicles normally dispersed in hydrophilic solvent. Liposomes are formed due to the self-assembly of phospholipids in the presence of an aqueous environment as seen in Figure 1.3. A liposome surrounds an aqueous internal core that may contain drug molecules and sustain their release. Liposomes are regarded as successful carriers for a wide range of drugs having different water solubility (Lopes et al., 2004).

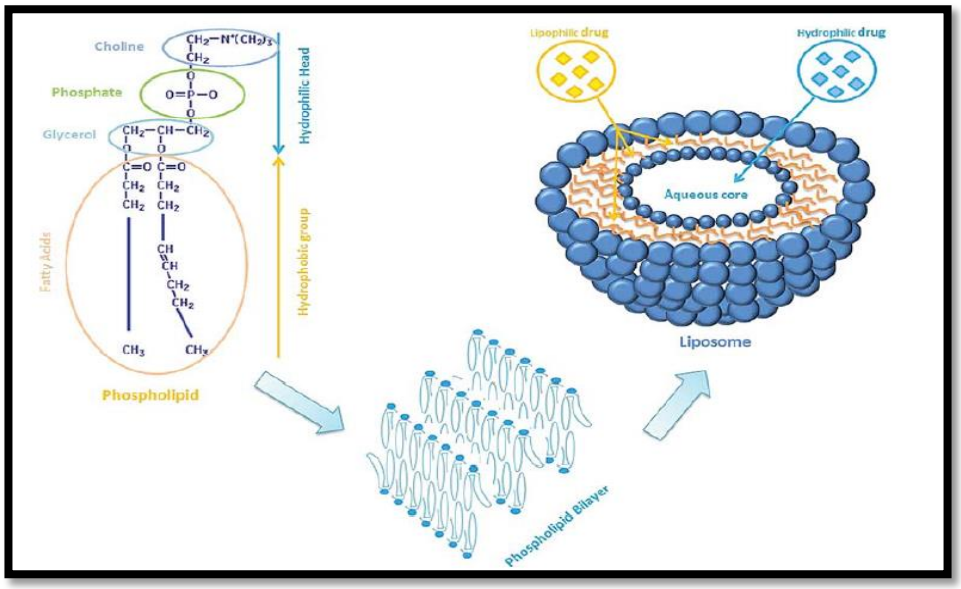

Figure 1.3 Structure of Niosome (Taken from Sudhamani et al., 2010)

Liposomes were first discovered by Alec D Bangham in 1961 in England while he was studying phospholipids and blood clotting (Sharma et al., 2009). Some liposomal drug formulations such as Doxil® (PEGylated liposomal doxorubicin) and Daunoxome® (daunorubicin citrate liposome injection) have already been used in clinic (Huang et al., 2010b). Liposomes have also been extensively investigated as carriers for antimicrobial drugs, steroidal drugs and vaccines. As shown in Figure 1.4, a liposome can be used to deliver many materials into the body. The size of liposomes ranges between 25nm and 20µm (Kozubek et al., 2000). Liposomes act as delivery vehicles for drugs, genetic materials, enzymes and other macromolecules and facilitate the uptake of these materials by living cells (2004). These vesicles have unique structures which are capable of entrapping hydrophilic, lipophilic, amphiphilic and charged hydrophilic drugs (Prajapati et al., 2011). Due to the amphipathic nature of liposome, it can entrap hydrophilic drugs into its aqueous phase and incorporate hydrophobic drugs in its phospholipid bilayers (Lopes et al., 2004, Huang et al., 2010b). Unilamellar liposomes are preferred for entrapping hydrophilic drugs whereas multilamellar liposomes are preferred for entrapping lipophilic drugs. Due to the presence of large range of phospholipids with various combinations and characteristics to prepare liposomes, drug delivery and targeting using liposomes may vary with accordance to the materials used to manufacture the liposomes. Liposomes can have various molecules attached to their surface such as the polymer polyethylene glycol or antibodies. Liposomes may also

have the ability to fuse with cell membranes, thus, releasing the entrapped drug into the internal components of the target cell. This is illustrated in Figure 1.5.

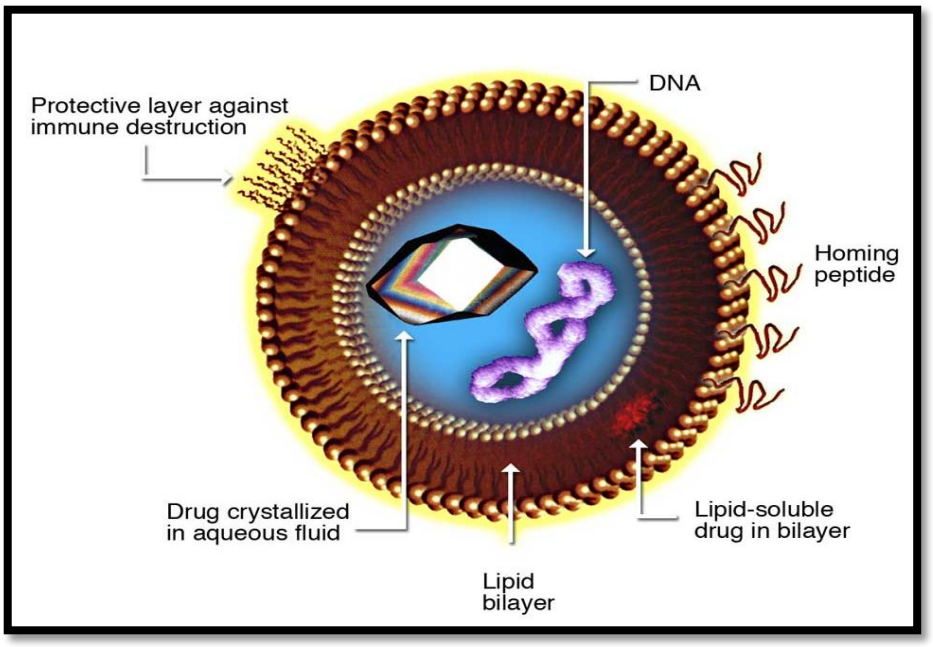

Figure 1.4 Use of liposomes to deliver different materials into the body (Taken from Wikipedia)

http://en.wikipedia.org/wiki/Liposome

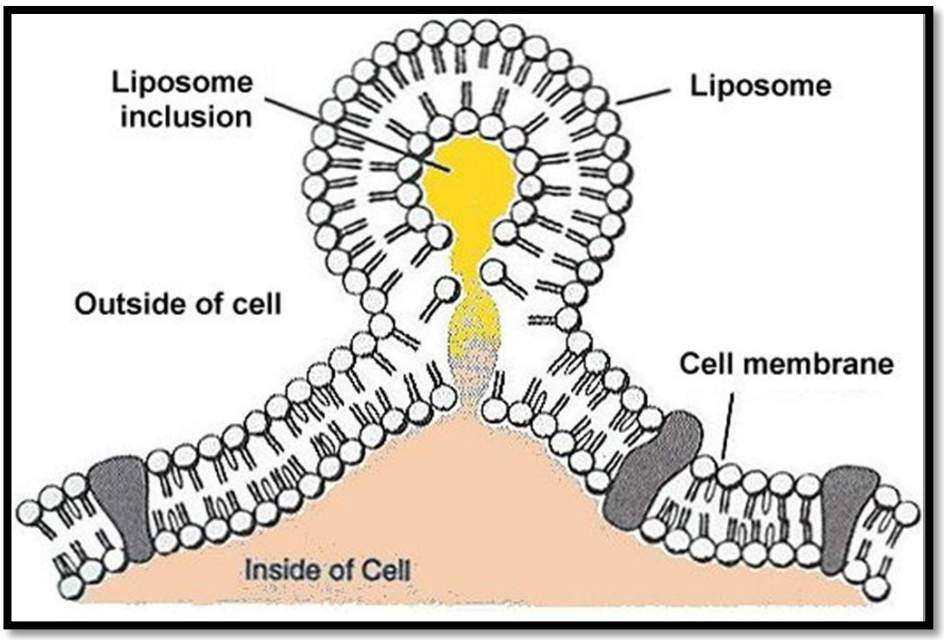

Figure 1.5 Uptake of liposomes into cell (Taken from Sampathkumar et al., 2012)

For liposomes to form, the hydration procedure must be undertaken at a temperature significantly exceeding the temperature at which phospholipid passes from the gel phase ($L_{\beta'}$) to the liquid crystalline phase ($L\alpha$) where its flexibility is higher. This temperature is called the phase transition temperature(T_m) which is a characteristic for each phospholipid depending on the type of its polar head-group and the length and degree of saturation of its hydrophobic alkyl chains. As demonstrated in Figure 1.6, when aqueous phase is added to a thin film of phospholipid, the hydration of the outer monolayer predominates compared to the inner layers. This results in the expansion of the polar headgroups of the phospholipid molecules and formation of blisters. Aqueous phase penetration through these "blisters" results in formation of phospholipid bilayers (Lasic, 1988). The most commonly used liposomes are composed of synthetic lipids (Misra et al., 2009).

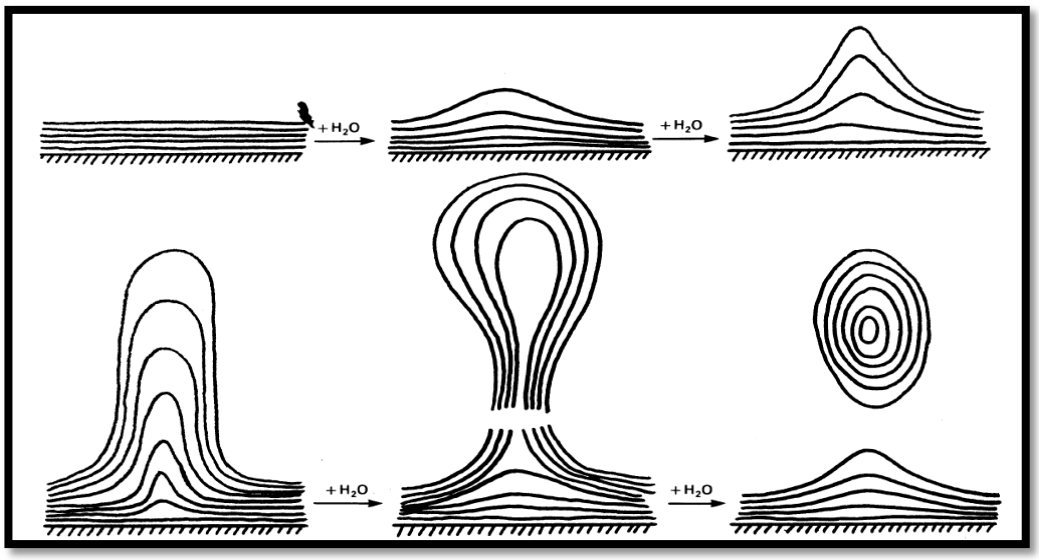

Figure 1.6 A schematic representation for the formation of MLVs on hydration of a dry phospholipid thin film (Taken from Lasic, 1988).

1.6. Role of Cholesterol

Cholesterol can be included in the mixture that forms the bilayers because of its effect on the physical properties, for instance cholesterol may exhibit the following properties when included in liposomes:

1. Acts as a fluidity buffer and intercalates with phospholipid molecules (Charnvanich et al., 2010).

2. Enhances the liposome rigidity and stability *in vitro* and *in vivo* (Benson, 2010, Samad et al., 2007).

It has been studied that cholesterol tends to dry the lipid/water interface of vesicular membranes and enhances close contact and increases van der Waals interactions between adjacent lipid molecules. This contributes to the reduced membrane permeability and prevention of leakage on using cholesterol in the liposomal formulation (Wang et al., 2006).

1.7. Zeta potential for stability of colloids

Zeta potential is an electric charge usually carried by colloidal suspensions or emulsions. This net charge of the particle affects the distribution of counter ion surrounding the interfacial region close to the surface (Attwood and Florence, 2012). The separation of charge that occurs in the interface of two phases in the colloidal system is called electrical double layer as it consists of two layers with opposite charge. This may be due to the ionisation of substance on surface, preferentially absorbed ions of one sign or due to preferential ion adsorption of deliberately added chemicals.

Many important properties of the colloidal system is determined by its electrical charge directly or indirectly. This charge distribution determines the interaction energy between the particles in colloidal system and its aggregative stability (Olton, 2008). Electrostatic repulsion between the particles determined its zeta potential. The greater the zeta potential the more is the repulsion between particles and more stable will be the system. Derjaguin, Landau, Vervey, and Overbeek (DLVO) developed a theory which deals with the stability of colloidal system in 1940s. They suggested that the stability of the colloidal system depends on the total potential energy function when particles are in Brownian motion. When the repulsive power is more than attraction the particles may resist aggregation and flocculation, thus, increasing the stability of the system (Kirby and Hasselbrink, 2004).

1.7.1. Basic theories of double diffusion layer

There are many theories and models proposed by which zeta potential of a colloidal system emerged from.

Helmholtz model (1879) where Helmholtz put the concept of double layer at the surface of metal in contact with electrolyte (Anchordoguy et al., 1987). His model suggested linear potential drop from the surface. However, he did not take into account

ion diffusion, adsorption on the surface and solvent/surface interactions (Gregory, 2006). It was followed by **Gouy-chapman model (1909- 1913)** where the surface was considered flat, infinite, uniformly charged and ions were considered as point charge. This model also considered the exponential potential decrease from the surface (Abdelwahed et al., 2006, Oldham, 2008, Stojek, 2010). It was further modified by **the Stern model (1924)** which was a combination of Helmholtz's theory of rigid layer and Gouy-Chapman's theory of diffuse layer (Anchordoguy et al., 1987). He made assumptions like finite size of ions and ion cannot approach the surface at the distance smaller than the magnitude of ionic radius (Abdelwahed et al., 2006). It also introduced the slipping place at the boundary of diffuse layer (Gregory, 2006). Further developments were made to the stern model by **Graham model (1940)** where he proposed the existence of three regions. The inner Helmholtz plane (IHP), the outer Helmholtz plane (OHP) and the diffuse layer (Grahame, 1947).

1.7.2. Double diffusion layer model

The liquid layer surrounding the charged particle exists in two layers: stern layer (strongly bound region) and diffusion layer (loosely bound region). This is illustrated in Figure 1.7. The stern layer is the inner sub layer that is formed close to the surface and where the counter-ions are specifically adsorbed. The outer part is called the diffusion layer. There is another characteristic distance called slipping plane which is associated with the tangential motion of the liquid relative to the surface. This plane separates the stern charge from the diffuse charge around the particle. When the particle moves due to Brownian motion, ions within the slipping plane moves with the particle whereas the ions beyond the slipping plane do not travel with particle. The potential at this boundary is zeta potential (Attwood and Florence, 2012). The zeta potential of the particle varies with distance from the surface uses the concept of diffusion double layer. The decay increases exponentially with distance from the shear plane. The inverse of the decay constant is a distance called the Debye double layer thickness. The higher the ionic (free salt) concentration, the faster the decay and smaller the double layer thickness (Kirby and Hasselbrink, 2004).

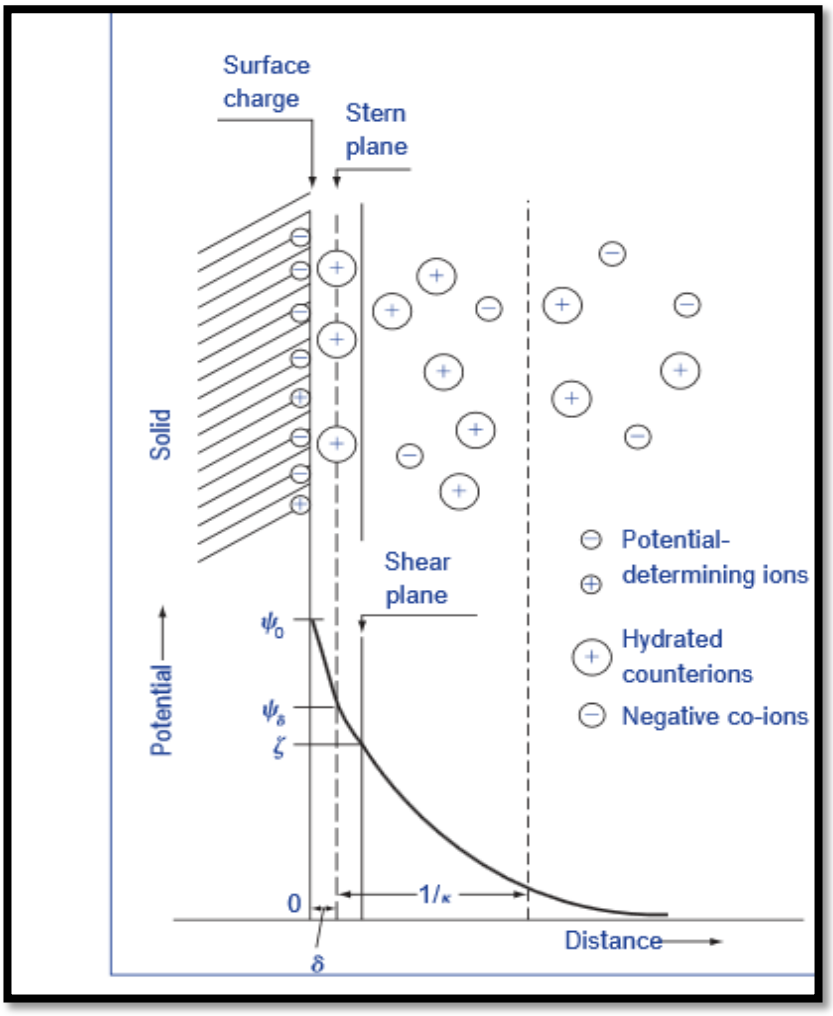

Figure 1.7 Model of double diffusion layer (Attwood and Florence, 2012)

1.7.3. Origin of zeta potential

The origin of zeta potential is due to the following factors

i. Ionisation of surface groups

Dissociation of acidic group on the surface of particle tends to give a negative charge to the particle surface, whereas the basic group tends to give a positive charge to the particle surface. pH at which the net electric is zero is called as iso-electric point. The magnitude of charge depends on pH of the solution as well as the strength of the acidic and basic group.

ii. Differential loss of ions from the crystal lattice

If an ionic compound starts dissolving its ions, the particle may acquire surface charge. If the positive and negative ions dissolve in equal quantity, the particle will be uncharged. However, if the positive ion is dissolved more than the negative ion it will leave a negatively charged surface and vice versa.

iii. Absorption of charged species

Surfactant ions may be specifically adsorbed onto the surface of a particle. Cationic surfactant gives positive charge whereas anionic surfactant gives negative charge to the surface (Everett, 1988, Berg, 2010)

1.7.4. Factors affecting zeta potential

i. pH

pH of the sample is the most important factor affecting the zeta potential in aqueous medium. In Alkaline suspension, the particle tends to aquire more negative charge whereas in acidic suspension the particle aquires positive charge. Therefore a zeta potential versus pH curve will be positive at low pH and lower or negative at high pH. The point in plot where the zeta potential is zero is called the isoelectric point.

ii. Ionic strength

The concentration of ion and its valency in solution determines the thickness of the double layer. The high ionic strength compresses the electric double layer. The ion with higher valency compresses the layer more than ion with low valency.

iii. Concentration of formulation component

The concentration of different individual substance in the formulation can affect the zeta potential of the product. Hence, this can help in formulating a product with maximum stability (Everett, 1988, Hunter and White, 1993).

1.7.5. Different phospholipids affecting zeta potential

The type of phospholipid added does have a major effect on the zeta potential of the formulation. Surface charge is based on the structure of the lipid. Phosphotidyl choline (PC), also called as lecithin, at physiological pH is a neutral zwitter ion. Similarly, phosphotidylethanol amines are neutral zwitter ions at physiological pH 7.4. However, phosphotidyl serine (PS) and phosphotidyl glycerol (PG) were found to have negative charge at physiological pH and tend to increase the negative zeta potential of the vesicle. PG and PS based vesicles usually exhibit a net charge of -1 (Yandrapati, 2012). It was demonstrated that lipid vesicles made of acidic phospholipids (PS, PG) possessed negative surface charges on their dissociative groups (such as PO_4^- and COO^-) at

neutral pH (Yandrapati, 2012). Synthetic lipids like DPPC and DMPC possess neutral to slightly negative surface charge (Wang, 2000). Hence, the surface charge of the vesicles can be altered by changing the lipid or mixing them together.

1.8. Types of liposomes

As shown in Figure 1.8, liposomes can be classified according to their lamellarity into multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), large unilamellar vesicles (LUVs) and oligolamellar vesicles (OLVs). Table 1.1 gives an overall review of the types of liposomes and their approximate size.

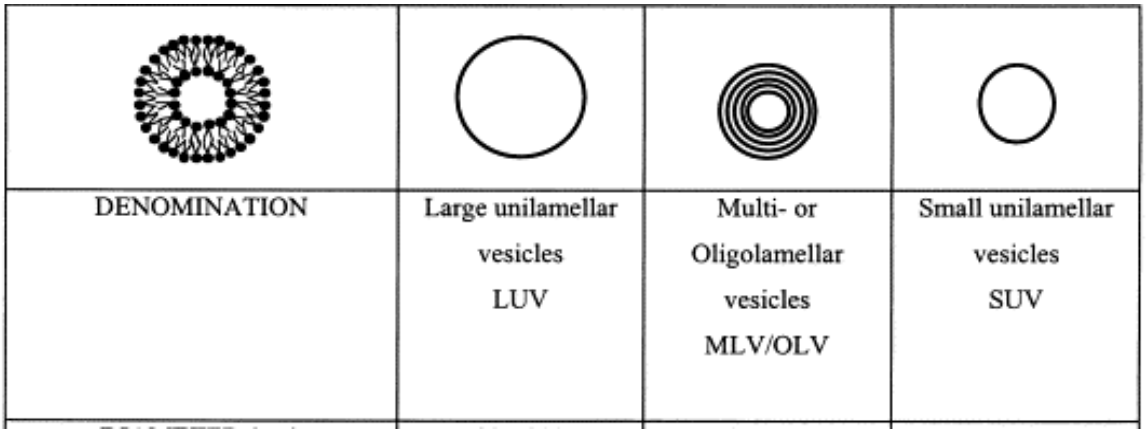

Figure .1.8 Schematic representation of different types of liposomes with different lamellarity (Taken from Bochot et al., 2000)

Table 1.1 Vesicle Types with their Size and Number of Lipid Layers (adapted from Samad et al., 2007)

Vesicle type	**Abbreviation**	**Diameter size**	**Number of lipid bilayers**
Multilamellar vesicles	MLVs	More than 0.5µm	More than 5
Oligolamellar vesicles	OLVs	0.1-1µm	2-3
Multivesicular liposomes	MVLs	More than 1µm	Multi compartmental structure
Small unilamellar vesicles	SUVs	20-100nm	1
Large unilamellar vesicles	LUVs	More than 100nm	1

1.8.1. Multilamellar vesicles (MLVs)

Multilamellar vesicles (MLVs) have several lipid bilayers (up to 14) in "onion-like" layers. Each layer is separated from the next one by a space of aqueous solution as shown in Figure 1.9. An MLV typically has a VMD larger than 0.5µm. MLVs are generally prepared by hydrating a thin film of phospholipids followed by vigorous shaking via vortex mixing, sonication or hand shaking (Lasic, 1988). The aqueous phase added should have a temperature above the phase transition temperature (T_m) of the lipid system used in the formulation. The preparation protocol of MLVs should be controlled to obtain liposomes with relatively narrow size distribution. MLVs can also be prepared from preformed SUVs or LUVs by controlled fusion, freeze-thawing or dehydration-rehydration methods. MLVs are generally preferred for entrapping lipophilic drugs because of the large number of bilayers they have in which lipophilic molecules can be incorporated.

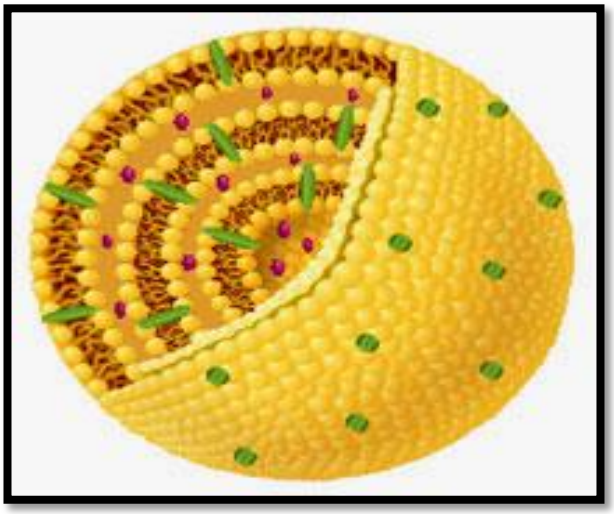

Figure.1.9 Onion like structure of multilamellar liposomes (Taken from Sciences, 2012)

1.8.2. Oligolamellar vesicles (OLVs)

MLVs possessing only two or three phospholipid bilayers may be referred to as oligolamellar vesicles (OLVs). They are 0.1-1µm in size (Samad et al., 2007). OLVs can be made using reverse phase evaporation and are thus, sometimes called reverse phase evaporation vesicles (REVs) (Szoka and Papahadjopoulos, 1978). They are made by formation of water in oil emulsion by brief sonication of an aqueous phase/buffer and phospholipid dissolved in an organic solvent (diethylether or isopropylether or

mixture of isopropyl ether and chloroform). The organic phase is evaporated under reduced pressure to form a viscous gel. This in turn forms a mixture of OLVs and LUVs by removal of residual solvent by continued rotary evaporation under reduced pressure (Dua et al., 2012). The aqueous phase is added at this point.

1.8.3. Small unilamellar vesicles (SUVs)

Small unilamellar vesicles (SUVs) are made of single phospholipid bilayer and have a size range of 20-100nm (Kozubek et al., 2000). SUVs are prepared by injecting an ethanolic solution of phospholipid in an aqueous phase above the phase transition temperature of the phospholipid (Batzri and Korn, 1973b). Alternatively, probe or bath sonication of MLVs is a common technique employed for formation of SUVs (Lasic, 1988). SUVs can also be made by detergent depletion technique in which MLVs are dissolved in detergent which is later removed by dilution, dialysis, chromatography, adsorption, ultrafiltration or centrifugation to form SUVs (Brunner et al., 1976, Lasic, 1988).

1.8.4. Large unilamellar vesicles (LUVs)

LUVs have single lipid bilayer and have a size ranging from 0.1 to 1µm. LUVs can also be of cell size (Kozubek et al., 2000). LUVs can be utilised for provision of higher entrapment of hydrophilic drugs. Injection of ether solutions of phospholipids into water warmed to a temperature above the T_m of the dissolved phospholipid(s) yields LUVs (Batzri and Korn, 1973a). Reverse phase evaporation method causes the production of both OLVs and LUVs (Szoka and Papahadjopoulos, 1978). In this method, a buffer is used for the formation of w/o emulsion of phospholipid. The organic phase is removed using rotary evaporator and a gel containing LUVs is obtained by sonication. It can also be made by ethanol injection of phospholipids in an aqueous phase (Lasic, 1988). Even LUVs can be prepared from preformed MLVs by size extrusion as extrusion decreases the lamellarity of the liposome (Berger et al., 2001a).

1.9. Advantages of using liposomes over traditional drug delivery systems

Liposomal drug delivery systems has many advantages like reduced toxicity, sustained drug release, manipulation of drug pharmacokinetics, targeting specific tissues and protection of encapsulated drugs from enzymatic degradation.

1.9.1. Reduced toxicity

Liposomes are non-toxic, biodegradable and non-immunogenic drug carriers because they are prepared from phospholipids which are very similar to the components of the biological membranes and respiratory tract surfactants (Huang et al., 2010a). Due to advancement in the research of preparation and formulation of liposomes, many liposome preparations can provide greatly enhanced drug encapsulation. Drug encapsulation in liposomes may prevent drug toxicity or attenuate adverse effects by retaining and improving the therapeutic effect of the drug. This is achieved by sustaining the drug release from the liposomes and enhancing the targeting of the drug to the desired tissue.

1.9.2. Sustained delivery system

Following inhalation of liposomal aerosols, they may provide sustained release of the drug, which help in localising the drug action (e.g. in the lung) for prolonged durations in the respiratory tract tissue (Huang et al., 2010a). The drug encapsulated in the liposome's central aqueous core has to pass across each bilayer of the liposome to be released, thus this provides the sustained release property and reduces the need for frequent dose administration (Chrai et al., 2001). It is hence, expected that liposomes made from many bilayers might be desirable for provision of prolonged drug release.

1.9.3. Alteration of the Pharmacokinetics of drugs

Drug encapsulation in liposomes may significantly alter the pharmacokinetic and pharmacodynamic properties of the drug, thus, enhancing drug uptake, delaying drug clearance and avoiding frequent drug administration (Gibbons et al., 2011, Alino et al., 1999).

1.9.4. Avoidance of side effects and local irritation

Liposomes target specific cell type, thus, minimising the uptake of the encapsulated drug by organs other than the targeted one. When given via inhalation, liposomes may reduce drug levels in the systemic circulation, hence minimising the potential of adverse systemic effects by the drug (Perrett et al., 1991).

1.9.5. Protection for the encapsulated drug

Drug or material encapsulated inside liposomes is protected from direct contact with the external environment until it is released from the liposomes; this may minimise or prevent degradation of the drug because its encapsulation in the liposome can protect it from the external enzymatic environment (Samad et al., 2007, Sharma et al., 2009).

1.10. Drawbacks of conventional liposomes

Liposomes have been extensively studied for parenteral, transdermal, nasal and pulmonary drug delivery. Liposomes have been used for potential application in transdermal drug delivery for treatment of various diseases like cardiovascular diseases, Parkinson's disease, Alzheimer's disease, depression, anxiety, skin cancer, female sexual dysfunction, post-menopausal bone loss and urinary incontinence (Patel et al., 2009). The use of liposomes for transdermal delivery is limited because of the barrier provided by the outer most layer of skin epidermis called stratum corneum. The conventional liposomes do not have great flexibility, thus, it becomes difficult for them to pass through small skin openings. The rigid property of liposomes also causes problems in treating pulmonary diseases like asthma, pneumonia, chronic obstructive pulmonary disorder and acute lung injuries, because rigid vesicles may not be adequately aerosolised because of their resistance against nebulisation (Saari et al., 1999, Darwis and Kellaway, 2001). Hence, it became necessary to form a more flexible liposome to overcome these problems which is called elastic vesicles or Transfersomes (Prajapati et al., 2011).

1.11. Transferosomes termed as "surfactosomes"

This new class of liposomes termed Transfersomes was first registered by the German company IDEA AG by Gregor Cevc in 1991 (Benson, 2010, Prajapati et al., 2011). Transferosomes are 'the carrying body'; In Latin, the word 'Transferre' means a "to carry across' and the Greek word 'soma' means 'body'. In this study transferosomes are termed as surfactosomes in order avoid the usage of trademark word. Transferosomes have been previously studied extensively for skin delivery. It was observed to be more elastic than conventional liposome which helps it to pass through stratum cornuem of skin with less leakage of drug. Along with phospholipids, these transferosomes are also composed of surfactants such as sodium cholate, deoxycholate, Span, Tween and dipotassium glycyrrhizinate in appropriate ratios (Trotta et al., 2002). These surfactants

act as ‘edge activators’ to destabilise the lipid bilayers and increases deformability and flexibility of the vesicles (El Maghraby et al., 1999). Due to its flexible nature, transferosome can squeeze through channels that are one-tenth of its diameter (Benson, 2010). The flexibility of transferosome minimises the risk of bilayer rupture and subsequent drug leakage (Prajapati et al., 2011). Incorporation of the ‘edge activator’ in the form of surfactant was the basic principle of Cevc’s original Transferosome approach.Transferosomes have better penetration ability through the skin and is highly adaptable, ultra flexible, stress-responsive, when compared with conventional liposomes (Patel et al., 2009). They are also permeable, having ‘softened’ bilayers. They also have the capability to self-optimize and self-repair themselves, thus, becoming able to squeeze through small gaps between the cells despite their large size (Dubey et al., 2008). This high deformability gives better penetration of intact vesicles as shown in Figure 1.10 (Prajapati et al., 2011), enabling them to cross various cellular barriers efficiently. Since then, huge amount of research has been conducted to investigate on these surfactosomes under different titles like transferosomes, flexible vesicles, ultradeformable vesicles and elastic vesicles.

In this study a similar system for pulmonary drug delivery has been hypothised and studied for its usefulness. Transferosomes are renamed as surfactosomes for pulmonary delivery. Here, surfactosomes are believed to be more elastic/flexible than conventional liposomes, hence, can carry drug without much leakage when passed via nebulisers for pulmonary drug delivery. Like liposomes, surfactosomes possess an aqueous core surrounded by the lipid bilayers..

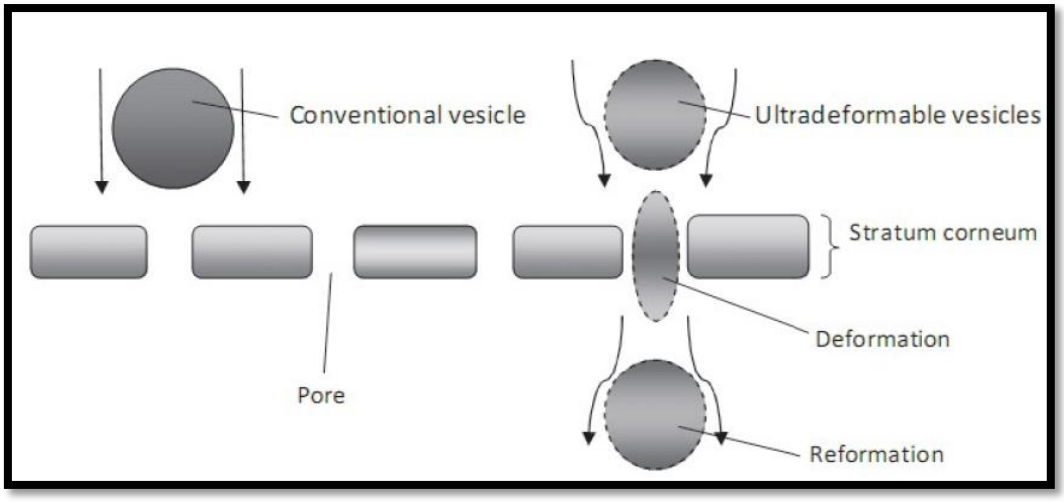

Figure.1.10 Schematic representation of vesicular penetration via two micropores (Taken from Prajapati et al., 2011)

1.12. Surfactants

The term surfactant designates a substance which exhibits some surface and interfacial activity. Surfactants are anionic surfactants, non-ionic surfactants or cationic surfactants. These surfactants, also termed “edge activators”, have been reported to confer elastic properties to lipid vesicles, allowing dramatically improved flexibility and adaptability to deliver agents to the target. In many vesicle-based formulations, non-ionic surfactants have been used (Mahale et al., 2012). The packing characteristics of lipids in the bilayer are affected by the type of surfactant and thus, efficient drug delivery system can be engineered by selecting the right surfactant with the right lipid in the right proportion.

Hydrophilic/ lipophilic balance (HLB) gives a measure of the physicochemical properties of surfactants in terms of their affinity for or solubility in water or lipid. HLB values are 4.3, 15 and 16.7 for Sorbitan monooleate (Span 80), Polyoxyethylene sorbitan monooleate 80 (Tween 80) and sodium cholate respectively (El Maghraby et al., 2004). Based on these HLB values, the affinity for lipids is expected to be in the order of Span 80 > Tween 80 > Sodium cholate (i.e. the surfactant with highest HLB value has the lowest affinity to lipid). Considering the distribution between lipid and aqueous components, there will be an effective molar ratio (Re) of surfactant to lipid. This effective molar ratio describes the actual amount of surfactant in liposomes relative to the lipid concentration (El Maghraby et al., 2004).

1.12.1. Sorbitan Monooleate (Span)

Span 80 is known as commercial name of sorbitan monooleate containing several kinds of esters. Span 80 has a relatively small head-group compared with Tween 80, as it lacks the Polyoxyethylene units. It has an HLB of 4.3, is lipophilic and immiscible with water and thus, its lipid to water distribution coefficient is high (El Maghraby et al., 2004).

1.12.2. Polyoxyethylene sorbitane monooleate (Tween)

Tweens are probably the most commonly used non-ionic surfactants in pharmaceutical industry. Tween 80 is a non-ionic surfactant with a large head-group and an HLB value of 15, is miscible with water, thus, it is expected that Tween 80 will distribute more in the water compared to lipid. Tween 80 comprises a partial oleic acid ester of sorbitol-

derived cyclic ether, condensed with 20 ethylene oxide units per molecule (Simoes et al., 2005).

1.12.3. Sodium cholate

Sodium cholate (cholic acid) is a water-soluble bile-salt. An expansion of the vesicles takes place and momentary defects are induced in liposomal membranes on addition of sodium cholate to lipid bilayers. It leads to enhanced permeability for molecules (Subuddhi and Mishra, 2007). Number of factors like chemical nature and concentration of the bile salt, molecular structure of the lipids, and size and shape, type of buffer and pH and temperature of the dispersion may affect the interaction of bile salts (e.g. sodium cholate) with the phospholipid vesicles (Subuddhi and Mishra, 2007).

1.13. Anti-asthma drugs

There are many anti-asthma drugs. In this review, the discussion is limited to the model bronchodilator salbutamol sulphate (SBS) (Figure 1.10) and model prophylactic steroid beclometasone dipropionate (BDP) (Figure 1.11). These two drugs are probably the most commonly used in the treatment of asthma.

1.13.1. Salbutamol sulphate (SBS)

Salbutamol sulphate (SBS) is a selective β2 adrenoreceptor agonist having bronchodilatory effect (Tanwar, 2007). Its chemical structure demonstrates its hydrophilic characteristics and is shown in Figure 1.11. It is useful in therapeutic management of bronchial asthma, chronic bronchitis and emphysema (Huang et al., 2010a). SBS can be used in various dosage forms like peroral tablets, injections or aerosols. Many side effects are observed when this drug is given in conventional oral or injectable formulations. When taken orally it is readily absorbed from the gastrointestinal tract. It also undergoes first pass metabolism in the liver (Tanwar, 2007). Hence, the preferred route of delivery is via inhalation for its effect on lungs or slow intravenous injections for direct effect on bronchial smooth muscles (Huang et al., 2010a). SBS is widely used as amorphous spray dried product for inhalation (Corrigan et al., 2004). As the plasma half-life of the drug ranges from 4 to 6 hours, the recommended dose frequency is every 4 to 6 hours (Bendas and Tadros, 2007). Thus, its short biological half-life and short duration of action are the main drawbacks (El-Gendy et al., 2009). Hence, it is necessary to formulate a controlled release drug delivery

system for SBS to avoid the frequent administration of the drug and potentially minimise its adverse effects. The use of liposomes for SBS may constitute one form of sustained delivery formulations for SBS (El-Gendy et al., 2009).

Major side effects of SBS are manifested by skeletal muscle tremors, tachycardia and other types cardiac arrhythmias. The side effects are reversible. Some rare side-effects include urticaria, angioedema, hypotension, and collapse have also been reported (Lulich et al., 1986)

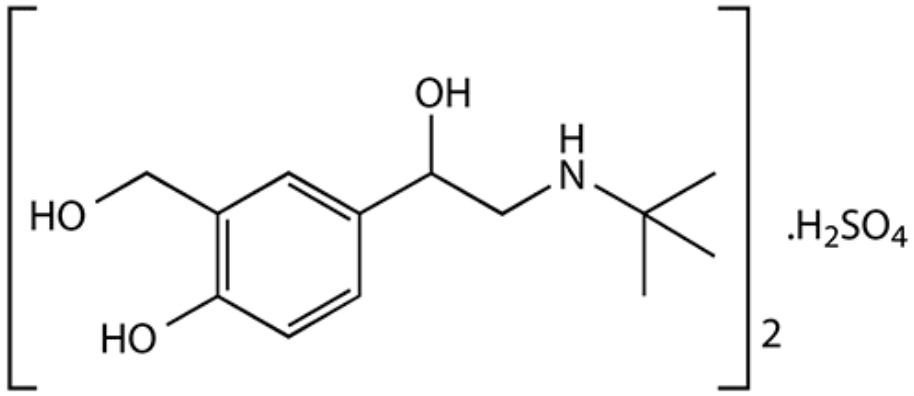

Figure.1.11 Chemical structure of salbutamol sulphate

1.13.2. Beclometasone dipropionate (BDP)

Beclometasone dipropionate (BDP) is a water-insoluble (i.e. hydrophobic) steroid (Figure 1.12). BDP is highly soluble in chloroform, and freely soluble in acetone and in ethanol. However, is only slightly soluble in water (i.e. 49.39mg/L). Thus, when incorporated into the phospholipid of liposomes it is expected to associate more with the lipid bilayers instead of the aqueous spaces. For treatment of asthma, it is convenient to deliver BDP as aerosols from aqueous suspension via nebulisation for the treatment of asthma and other inflammatory lung diseases (Batavia et al., 2001) especially in the initial stage of treatment (Zeng et al., 2000). Direct administration of steroids like BDP to the lung has been found to cause localised side-effects manifested by oral candidiasis and dyspnea. Hence, the use of liposomes formulation of BDP may have an advantage over microcrystalline BDP suspensions since liposomes can provide sustained release and proper solubilisation matrix for this drug. Delivery of BDP to the lung via nebulisation using liposomes has been reported to be highly suitable (Saari et al., 1999, Darwis and Kellaway, 2001).

A proportion of BDP tends to crystallise in liposomal formulations due to the incompatible steric fit between the steroid and the liposome bilayers and its limited solubility in phospholipids. This observation has been reported upon the detection of large amounts of crystalline steroid after extrusion and during storage of the BDP liposome formulations (Batavia et al., 2001). Moreover, there are many concerns about giving corticosteroid drugs like BDP in large doses since systemic side effects of this drug like adrenocortical suppression, skin changes (thinning, bruising) and cataract have been reported (Zeng et al., 2000). Therefore, ideally maximised targeting of the administered dose of BDP to the site of action in the respiratory tract should be achieved in order to obtain a localized therapeutic effect minimised amount gaining access to the systemic circulation (Zeng et al., 2000).

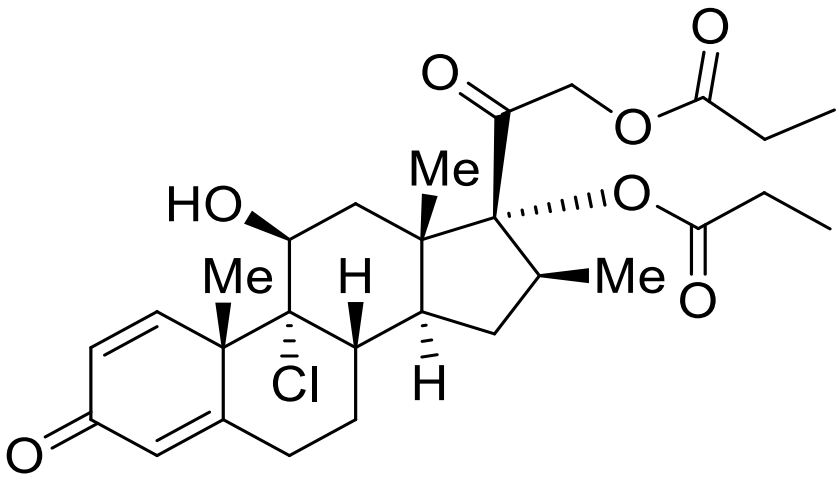

Figure.1.12 Chemical structure of Beclometasone dipropionate (BDP)

1.14. Instability of liposomes and surfactosomes in aqueous media

The chemical and physical properties of liposomes and surfactosomes are critical parameters affecting the performance of drug loaded into the vesicles. These vesicles are very unstable in aqueous media. Instability manifestations are vesicle aggregation, bilayer fusion, and phospholipid hydrolysis and oxidation with concomitant leakage of the originally entrapped drug (Ahn et al., 1995). This could greatly shorten the shelf-life of formulation, or change the pharmacokinetic profile of the encapsulated material. Many formulation ingredients like buffer, solvent and pH modifier may also affect the stability of liposomes. Hydrolysis of ester bonds linking the fatty acids to glycerol backbone and peroxidation of unsaturated acyl chains are the main chemical instabilities

affecting liposomal phospholipids (Shaji and Bhatia, 2013). Hence, storage of these vesicles as dry powders has been considered to improve their stability during storage (Desai et al., 2002a). Many techniques are employed to manufacture dry powders of liposomes like freeze drying (Lu and Hickey, 2005) and spray drying (Lo et al., 2004). Alternatively, proliposome technology is an approach used to manufacture stable phospholipid formulations that can generate liposomes upon constitution with aqueous phase prior to administration by patient (Payne et al., 1986b, Perrett et al., 1991).

1.14.1. Freeze drying (lyophilisation)

Freeze drying is commonly known as "lyophilisation" and is considered a promising means of extending the shelf-life of liposomes. As freeze drying is a low temperature process it is unlikely to cause thermal degradation of liposomes (Pikal, 2006). This process offers protection against various conditions that might cause liposomal instability in aqueous dispersions, thus, yielding a product with a greatly extended shelf-life (Crommelin and Van Bommel, 1984, Bridges and Taylor, 2001). Lyophilisation is a drying process which converts solutions of thermo-labile materials into solids having sufficient stability for distribution, storage and aerosol delivery (Lu and Hickey, 2005, Pikal, 2006). Freeze drying can be viewed as a three-step process consisting of freezing, primary drying and secondary drying. In freeze drying, most of the water is converted into ice during the freezing stage. Ice is subsequently removed by direct sublimation in the primary drying stage. Most of the unfrozen water is then removed in the secondary drying stage by desorption (Pikal, 2006).

Freeze drying equipment is relatively expensive and long processing time is needed to produce dry liposomes (Pikal, 2006). Both freezing and drying may cause instability problems and stress to liposomes as a result of the induced structural or functional damage to the vesicles during freezing and drying. This in turn leads to leakage of the encapsulated drug on rehydration, thus compromising integrity of liposome formulations (Bridges and Taylor, 2001). Liposome bilayers depend for their stability on hydrogen bonding between water molecules and the polar head groups of the phospholipid molecules in liposomes. The process of drying leads to loss of water which leads to changes in the bilayer behavior and loss of liposome integrity. This further leads to bilayer damage, fusion or vesicle aggregation, ultimately leading to loss of the previously entrapped material (Bridges and Taylor, 2001). Freezing may cause phase transition changes, osmotic stress and expansion of the bilayers due to ice

formation (Bridges and Taylor, 2001). To maintain the same particle size distribution of liposomes after lyophilisation and rehydration, cryoprotectants should be added before freeze drying (Bridges and Taylor, 2001).

1.14.1.1. Cryoprotectants and lyoprotectants

Delivery of drugs using colloidal vectors and nanoparticles is very efficient. Physical and chemical instabilities of these vectors are the major obstacles found on their storage in aqueous suspensions for extended periods (Henriksen et al., 1994). To improve the physical and chemical instability of these substances, removal of water molecules is very important (Grahame, 1947). This is most commonly achieved by freeze drying in pharmaceutical field which converts solution/suspensions into solids by vacuum desorption or sublimation, thus, improving stability (Henriksen et al., 1994). This process creates stress during freezing and drying step like solute concentration, formation of ice crystals, pH changes etc. (Wang, 2000). Cryoprotectants are added to protect them from freezing stress and lyoprotectant from drying stress.

Carbohydrates are favoured to be used during lyophilisation because they are chemically non-toxic and can be easily vetrified. Most of them also have transition temperature above -30°C making them more favourable (Gregory, 2006). Cryoprotectants and lyoprotectants were initially studied with proteins. There are two theories regarding the mechanism of stabilisation during freeze drying: ‘vitrification’ and ‘water replacement theory’ (Olton, 2008).

Vitrification theory

Crystallisation of ice during lyophilisation may induce a mechanical stress on particles leading to destabilisation, especially on fragile systems like nanoparticles. It has been suggested in previous studies that cryoprotectants directly interact with the bilayer and is associated with it throughout the freezing process to maintain bilayer integrity (Anchordoguy et al., 1987). Sugars like trehalose, mannitol, sucrose and glucose are the popular cryoprotectants and are known to vetrify at certain temperatures denoted Tg’ (Grahame, 1947). Vitrification is solidification of liquids without crystallization, a state in which it comprises a glassy state as the system is amorphous with no organised structure but possesses the properties of a solid. During vitrification, the viscosity of the solute in increased by concentrating it (critical viscosity) which inhibits the coming together of water molecules to form ice (Grahame, 1947). These cryoprotectants tend to immobilise the nanoparticles with their glassy matrix and protect them against

mechanical stress of ice crystals formed during freezing. The point at which this occurs is called the glass transition temperature (Tg'). Freezing must be carried out below the Tg' of the amorphous sample. Concentration of these sugars play an important part in level of stabilisation afforded. This regime completely eliminates ice formation inside and outside the nanoparticle, thus preserving its shape (Grahame, 1947). During drying when the ice is removed by sublimation, it leaves behind a highly porous glass with nanoparticles embedded it them. Thus, it can be hypothesised that the rigidity of glassy matrix prevents the damage of nanoparticles from ice crystal and also prevents molecular aggregation by inhibiting molecular motion (Olton, 2008).

Water replacement theory

Lyoprotectant helps in stabilisation of nanoparticles by water replacement hypothesis where there is a formation of hydrogen bond between the OH group of lyoprotectant and the polar groups on the surface of nanoparticles at the end of drying process. These bonds serve to replace water when the water is lost during the process of drying, thus, helps to maintain structure (Olton, 2008). This satisfies the hydrogen bonding requirement of nanoparticle. This may keep the nanoparticles in pseudo hydrated state, thus, helping to preserve the native structure of nanoparticles by serving as water substitutes (Grahame, 1947).

1.14.2. Spray drying

Spray drying is a one-step process of drying that can have applications in designing liposome dry powders. Spray drying is a versatile technology which can have multiple applications and employed to manufacture a range of products including pharmaceutical and nutritional (Gasper et al., 2007). This process has the ability to produce spherical micro-particle powders with good flow properties, high porosity and low density. Thus, "respirable" dry powdered liposomes have been manufactured using the spray drying technology (Lo et al., 2004). Spray drying has many benefits like control over particle size, density and degree of crystallinity, improved bioavailability and product stability and rapid drying of thermo-sensitive materials (Gasper et al., 2007). Typical spray drying sequences that occur within fractions of a second are atomization of feed into a spray, spray-air contact, moisture evaporation of the sprayed droplets, and separation of the dried particles from the air (Lo et al., 2004). The spray drying technique dries the drug-loaded liposomes in order to retain their contents during storage (Charnvanich et al., 2010). High temperature process in spray drying may lead to thermal degradation of

protein activity which is a major concern. This activity loss occurs due to protein's sensitive structural alteration by heat (Lo et al., 2004). Thus, optimization of the operating parameters in spray drying is essential. Stabilizing adjuvants can be included to protect the drug integrity during spray drying. In spray drying the most commonly used stabilizing adjuvants are carbohydrates such as sucrose, mannitol, lactose, trehalose, and polyols (Lo et al., 2004).

1.14.3. Particulate based proliposomes

To overcome the instability issue of liposomes, a delivery system called proliposomes was introduced by Payne and co-workers (1986). Proliposome technology represents an economic and convenient alternative to freeze- drying and spray drying to prepare liposome precursors that can generate liposomes with high entrapment efficiencies by addition of aqueous phase and shaking prior to administration. Proliposome of two types: Particulate based proliposomes (Payne et al., 1986) and ethanol based proliposomes (Perrett et al., 1991)

According to Payne et al. (1986a;b), particulate-based proliposomes are the free flowing granular product which are composed of phospholipids, cholesterol (optional), drug and carbohydrate carrier, which on addition of aqueous phase (e.g. buffer solution) gets converted into an isotonic dispersion of MLVs (Payne et al., 1986a). Particulate-based proliposomes has been regarded as potentially most efficient and cost effective in commercially producing precursors for generating liposomes on large scale (Shaji and Bhatia, 2013). In our laboratory, proliposomes have been prepared by mixing lipid, drug and carbohydrate carrier in an organic solvent within a round bottom flask. The flask is then attached to a rotary evaporator under vacuum. On the evaporation of all the organic solvent, particulate proliposomes are formed. This is different from the traditional method introduced by Payne and co-workers (1986a) which relies on coating the carbohydrate carrier with the lipid solution upon injection through a feed-tube line to coat the carrier following evaporation of the organic solvent. The advantage of the method adapted in our laboratory is the avoidance of lipid losses in the tube-line and the risk associated with possible "splash" of the organic solvent during injection into the feed-line. There are different types of carbohydrates used in the preparation of proliposomes such as sorbitol, mannitol, lactose, sodium chloride and glucose (Payne et al., 1986a).

Different types of proliposomes have been prepared and experimented. Katare *et al* formulated an effervescent particulate based proliposomes using fluid bed method. They formulated effervescent proliposomes to produce liposomes with narrow size distribution, and high entrapment of non-steroidal anti-inflammatory drugs such as ibuprofen and indomethacin (Katare et al., 1990, Katare et al., 1991, Katare et al., 1995). Desai *et al* studied the effect of jet-milling following blending carrier and lipid to form proliposomes. Formation of liposomes after dispersing it in water was established (Desai et al., 2002b, Desai et al., 2003). Deshmukh and co-workers studied the efficiency of proliposomal bead formulations for cromolyn (BCS Class III compound). Distearylphosphatidylcholine, cholesterol and the surfactant Tween 80 were spray-coated onto beads of the anti-asthma drug cromolyn sodium. Vesicle formation and drug entrapment efficiency using the beads were evaluated using Caco-2 cells and everted rat intestinal sac model. This study concluded that the phospholipid-surfactant proliposomal beads have offered an effective method for oral delivery of Cromolyn (Deshmukh et al., 2008). Proliposomes were formed in enteric-coated beads and glyburide was used as a model drug. The beads were enteric coated with Eudragit L-100. These proliposome beads proved to be more stable, enhanced drug dissolution and produced liposomes on hydration for oral administration (Kumar 2001). Chen and Alli in 1987 created proliposomes by coating Nonpareil beads (sugar spheres) with phospholipids. On hydration with aqueous phase these beads formed liposomes. These new types of proliposomes were termed as "bead-based proliposomes" (Chen and Alli, 1987).

1.14.4. Prosurfactosomes

Like proliposomes, prosurfactosomes also termed as protransferosomes are made up of lipid and drug along with surfactant coated with carbohydrate carrier. On hydration, prosurfactosomes becomes surfactosomes also termed as transferosomes. Gupta and Trivedi (2012) have prepared cisplatin-loaded protransferosomes for topical drug delivery. These protransferosomes were surface modified using gelling agents and block co-polymers. They were evaluated for stability on storage for 6 months (Gupta and Trivedi, 2012). Ajay and Kumar investigated ketoprofen protransferosomes for sustained and efficient transdermal drug delivery. Sodium cholate was used as surfactant and nine different formulations with or without cholesterol were evaluated for the entrapment efficiency and release profile of the drug (Ajay and Vinit, 2013). Jain *et al i*nvestigated protransferosomes of norgestrel for transdermal delivery. They were

characterised for different parameters like drug loading capacity, vesicular shape, size and size distribution, degree of vesicle deformability, transit time and formulation stability at 40ºC and room temperature (Jain et al., 2003). It was concluded that protransferosomes are better than proliposomes for transdermal drug delivery.

1.15. Pulmonary drug delivery

Inhalation of drugs for treating local lung diseases such as asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD) has been considered for long decades or even centuries. The advantages of pulmonary drug delivery rather than using oral delivery are

i. High concentration of medication is delivered to the desired site in the lung
ii. Systemic side effects are avoided or minimised
iii. Rapid therapeutic response is achieved
iv. Drugs bypass the therapeutic barriers like first pass liver metabolism and gastric absorption
v. Therapeutic effect is achieved with lower doses
vi. Pulmonary inhalation represent a non-invasive route for drug delivery

The respiratory system as shown in Figure 1.13 starts from the nose and ends in the alveolar sacs. The respiratory tract can be classified into nasopharyngeal and oropharyngeal region which is also called the extrathoracic region which extends from the nose to larynx and is also referred as “upper airways”. This is followed by the tracheo-bronchial region and alveolar region. The tracheo-bronchial region extends from the trachea to terminal bronchioles and is also referred to as “central airways”. The alveolar region consists of bronchioles, alveolar ducts and alveoli; this region is referred as “lower respiratory airways” or “deep lung”. In this region the gas exchange takes place between air and blood via the alveoli (Hillery et al., 2002, Hofmann, 2011).

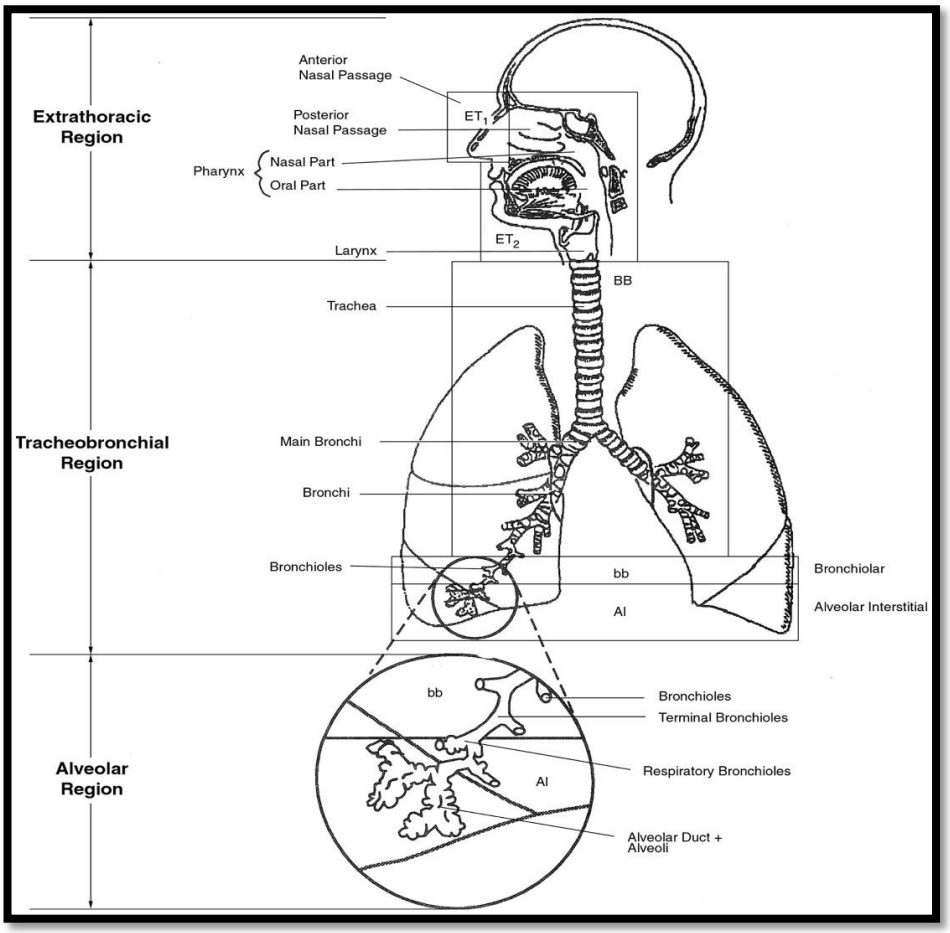

Figure.1.13 A schematic representation of the human respiratory system (Hofmann, 2011)

1.16. Pulmonary drug delivery devices

Inhalation of drugs via aerosols is desirable for drug administration in the treatment of respiratory diseases (Morice et al., 2002). Drug inhalation may allow high concentrations of therapeutic molecules to be targeted to the site of action within the lung, thus systemic adverse effects can be minimised. Drug delivery devices must be chosen according to the specific drug formulation and the region of the lung to be targeted. Modern technology has provided various devices for administration of aerosolised drug to the respiratory tract via inhalation. Current delivery devices available for inhalation therapy are pressurised metered-dose inhalers (pMDIs), dry powder inhalers (DPIs) and nebulisers.

1.16.1. Pressurised metered dose inhalers (pMDIs)

Pressurised metered dose inhalers (pMDIs) were developed in 1955 by Dr. George Maison (Khilnani and Banga, 2008). pMDIs are used to deliver a measured amounts of medication via aerosol to the respiratory airways. Within a canister made from aluminium or stainless steel, the drug is dispersed or dissolved in a liquefied propellant. The main parts of a pMDI device as shown in Figure 1.14 are the canister, a metering valve and an actuator (mouthpiece). The canister contains a mixture of propellants, surfactants, preservatives and the drug (Khilnani and Banga, 2008). The canister is solid and tough to maintain high interior atmospheric pressure and has the capacity of holding 15-30ml of liquefied formulation. The propellant is used for dispersing or dissolving the drug under high pressure within the canister (Ledermuller et al., 2003). The propellant is a liquid of very low boiling point (e.g. < 20°C), hence it is maintained in the liquid status under very high pressure condition within the canister. Liposomal formulations are mostly used after dissolution of phospholipid in a suitable propellant. During the actuation of pMDIs, the propellant is exposed to the atmospheric pressure which leaves the drug in the form of inhalable dry aerosol particles (Khilnani and Banga, 2008). The metered valve helps in the measurement of volume of fluid containing drug to be released from the device for inhalation (Tien et al., 2001).

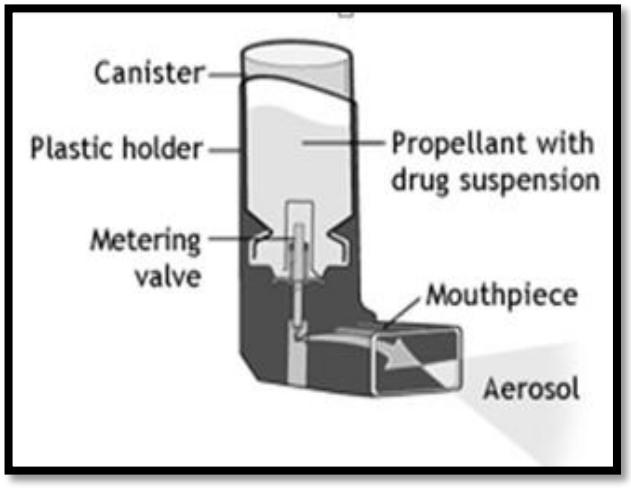

Figure.1.14 Different parts of Pressurised metered dose inhalers (Taken from Asthma society of Canada)

http://www.asthma.ca/adults/treatment/meteredDoseInhaler.php

Major disadvantage of pMDIs is the need for inhalation coordination by the patient. Another disadvantage is the use of propellant like chlorofluorocarbon (CFC) which is found to deplete the ozone layer. This in turn increases the risk of skin cancer, immune suppression and global warming. Another drawback includes nose breathing and coughing by high flow velocity.

1.16.2. Dry Powder inhalers (DPIs)

Dry powder inhalers (DPIs) have been in the market since early 1970s. There are different types of DPIs like Spinhaler, Rotahaler, Diskhaler and Turbuhaler. These devices provide aerosolised drug only when the patient actively inhales (Khilnani and Banga, 2008). Dry liposomes prepared for administration using DPI devices are made by freeze drying or spray drying or by loading micronised drug on carbohydrate carrier particles. Unlike pMDIs, the use of DPIs does not require coordinated patient inhalation (Khilnani and Banga, 2008). The powdered formulation within the device is converted into aerosols by the airflow during active inspiration, resulting in induction of a shear that causes delivery of the powder from the device (Ledermuller et al., 2003). The negative pressure generated by the patient inspiration causes the drug particle to pass through the airflow. As the drug formulation is in powder form, it is physically and chemically more stable compared to corresponding liquid formulations and delivery becomes easy, accurate and reproducible. However, the major disadvantage includes the demand for rapid drug aerosolization which can occur upon strong inspiration. This is difficult to achieve in patients with low inspiratory power or flow rate (e.g. children and old patients). Another disadvantage is provided by the atmospheric humidity and rapid change of temperature. This may lead to aggregation of dried drug particles, thus, (Ledermuller et al., 2003) reducing the delivered fraction of the drug (Khilnani and Banga, 2008). Some constrains in the use of DPIs include size and size distribution of drug particles, shape of carrier, and drug porosity and crystallanity (Telko and Hickey, 2005). Many studies have shown that DPIs are preferred over pMDIs as they are propellant-free and actuation breath synchronization is not required (Morice et al., 2002).

1.16.3. Nebulisers

Nebulisers are neither propellant based nor they need patient coordination during drug inhalation. Nebulisers can be used to deliver high drug doses in a relatively short time

(Khilnani and Banga, 2008). Most of the polydispersed aerosols produced by nebulisers are 1-5µm in diameter; this range is suitable for deposition in the lung (O'Callaghan and Barry, 1997, Ledermuller et al., 2003). Some nebulisers rely on compressed air for atomisation while others use ultrasonic energy and vibrating mesh (Bridges and Taylor, 2001). Nebulisers are preferred devices over pMDIs and DPIs for delivery of liposome formulations. They can provide high doses of drugs to the lung and minimum effort with no coordination are required. Conventional techniques can be used to produce liposomes in nebulisers without any further processing except for removal of unentrapped drug (O'Callaghan and Barry, 1997). However, nebulisation has many disadvantages like inefficient delivery of drug to the lungs, large residual amount of drug in nebuliser and the wastage of drug during exhalation in the surrounding air; however all these disadvantages have been minimised with the revolutionary novel designs of many nebulisers currently available in the market. Some aerosol particles are too large for deposition in the lungs and some are too small to sediment and thus are exhaled (O'Callaghan and Barry, 1997). The effectiveness of drug delivery by nebuliser depends on various factors like particle size, formulation properties, nebuliser type and patient inhalation pattern.

There are three types of nebulisers:

a. Air-jet nebulisers
b. Ultrasonic nebulisers
c. Vibrating-mesh nebulisers

1.16.3.1. Air-jet nebulisers

Air-jet nebulisers consist of mouth piece, medication bottle and source for compressed gas (e.g. gas compressor) to effectively convert liquid medications into “respirable” aerosols as shown in Figure 1.15. Jet nebuliser uses the principle of Bernoulli Effect (O'Callaghan and Barry, 1997). In jet nebulisers, the mouth piece is connected to the medication bottle (i.e. nebuliser reservoir) into which the medication liquid is filled. A gas compressor is used (Ledermuller et al., 2003) which on operation supplies compressed gas through the “venturi” nozzle of the nebuliser reservoir to convert drug into aerosols. The air pressure on top of medication fluid decreases and gas velocity increases. Negative pressure is produced at this point at the other end of the gas feeding tube. This results in the suction of medication liquid via “Bernoulli Effect”, leading to formation of aerosol droplets from the liquid dispersions under the influence of surface

tension (O'Callaghan and Barry, 1997, Elhissi and Taylor, 2005). The viscosity of the drug dispersion is directly proportional to nebulisation time (McCallion et al., 1996). The VMD of droplets is 15-500µm and the output efficiency depends on the baffle, "venturi" orifice and gas velocity and pressure (Newman and Clarke, 1983, Dennis et al.,1990, O'Callaghan and Barry, 1997). Large droplets produced impacts with the baffles within the nebuliser and falls back into the reservoir while the smaller ones can be released from the nebuliser because they are capable of escaping the baffling system of the nebuliser. The VMD of the aerosol droplet is directly proportional to the size of "venturi" nozzle and pressure of compressed gas (Khilnani and Banga, 2008). The use of nebulisers amongst children and infants is simple and easy when compared with DPIs and pMDIs (O'Callaghan and Barry, 1997). During jet nebulisation of liposomal suspensions, disruption of liposomal membranes may occur due to mechanical shearing by the nebuliser. VMD of liposomes, aerosol droplet VMD and gas pressure are the major determinants of vesicle stability during jet nebulisation (Leung et al., 1996).

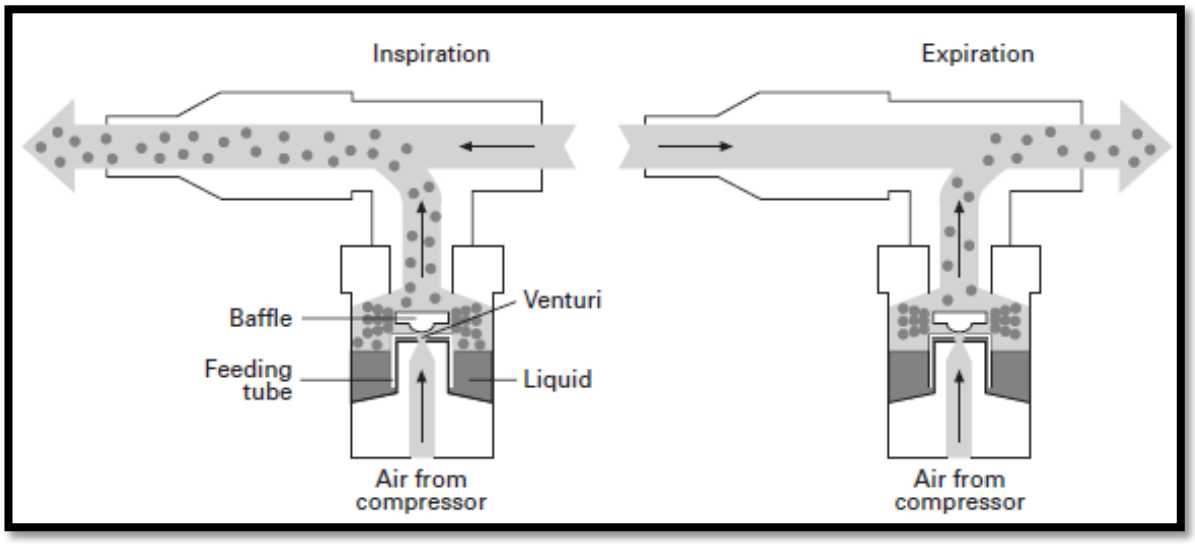

Figure 1.15 Design of conventional Air jet nebuliser. (O'Callagham and Barry, 1997)

Nebulisers can be divided into conventional, continuous open-vent and breath-enhanced open vent nebulisers.

Conventional nebulisers generates a fixed flow of gas containing aerosol. It continuously produces aerosols regardless of patient's inhalation or exhalation, thus, leading to drug losses during delivery (O'Callaghan and Barry, 1997).

In open vent nebulisers, there is a generation of negative pressure by the expansion of compressed air which sucks air and fluid into to the chamber via the vent for atomisation. This pushes more aerosols out to be inhaled by patients. This results in short nebulisation time and reduced droplet VMD due to greater solvent evaporation (O'Callaghan and Barry, 1997). This results in continuous air flow into the chamber pushing more small particles to be inhaled. The nebulisation time is reduced but the total amount of drug inhaled is similar to that using conventional jet nebulisers. Children and patients with low inspiration may fail to adapt to the high flow rate of this nebuliser and this can result in great losses of aerosol during exhalation.

In **breath assisted open vent nebulisers** like Pari LC Plus, there is a vent for air flow in the top and compressed gas flow at the bottom. The vent nebulisers work only during inspiration, thus, reducing aerosol and drug waste during expiration. (O'Callaghan and Barry, 1997).

1.16.3.2. Ultrasonic nebulisers

Ultrasonic nebulisers use ultrasonic energy to convert liquid into aerosol (Leung et al., 1996, Taylor and McCallion, 1997). In ultrasonic nebulisers, the energy required for aerosolisation (i.e. atomisation) of liquid is provided by the high frequency vibrations of a piezoelectric crystal (1-3Mz) that is located at the lower part of the device (Taylor and McCallion, 1997). As the vibrations become intense, they create fountain of droplets as shown in Figure 1.16. Large droplets are created in the apex while the smaller ones are created in the base from which the smallest droplets are converted to aerosol. Air driven by an in situ fitted fan within the nebuliser takes the small aerosol droplets to the mouthpiece side for inhalation by the patient (Elhissi and Taylor, 2005).

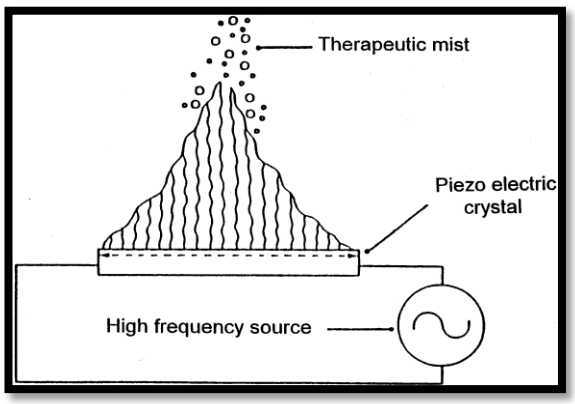

Figure.1.16 Schematic diagram of an ultrasonic nebuliser (Taylor and McCallion, 1997)

Two types of aerosol generation mechanisms are proposed which are the capillary wave theory and cavitation bubble formation (Torchilin and Weissig, 2007). They are diagrammatically represented in Figure 1.17 (A) and (B).

In capillary wave theory, it is proposes that droplets are formed from the capillary waves produced at the surface of the liquid. The formation of capillary jet is directly proportional to the frequency of the sound provided.

In cavitation bubble theory the liquid is atomised by hydraulic shocks which are produced by implosion of cavitation bubbles (Taylor and McCallion, 1997). Low frequency energy is desirable to create bubbles inside the formulation.

Ultrasonic nebulisation increases the temperature of nebuliser fluid by around 15°C. This may lead to chemical degradation of heat-sensitive drugs such as proteins and delicate structures such as liposomes (Leung et al., 1996). However, looking at the positive side, the increase in temperature may promote the solubility of poorly water soluble drugs (Steckel and Eskandar, 2003). This nebuliser also has higher dead volume with larger aerosol VMD and fluid output as compared to jet nebuliser (Taylor and Hoare, 1993)

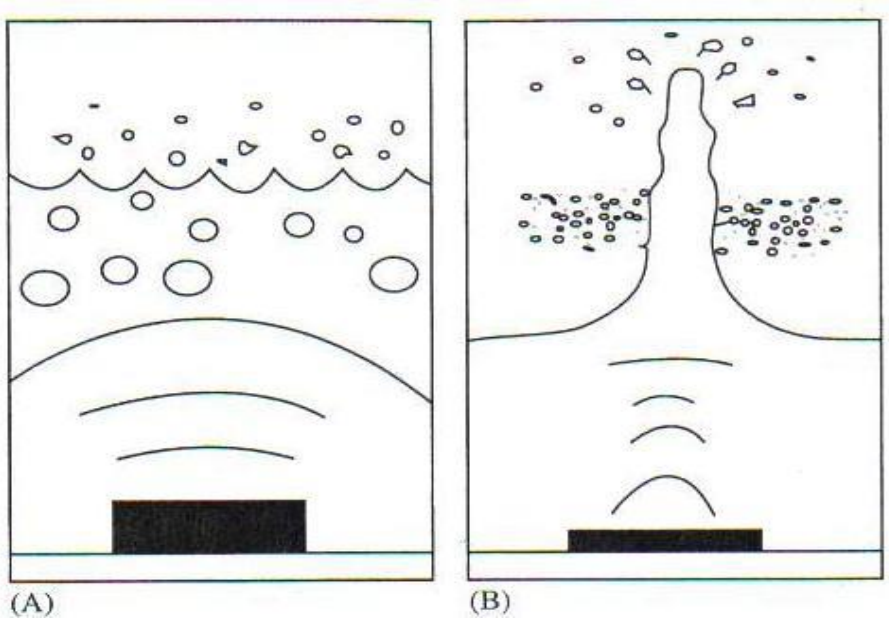

Figure.1.17 Generation of aerosol in ultrasonic nebuliser using (A) Cavitation bubble theory (B) Capillary wave theory (Taylor and McCallion, 2002).

1.16.3.3. Vibrating mesh nebuliser

Vibrating-mesh nebuliser is the most recent type of nebuliser that has become commercially available in market (Dhand, 2002, Elhissi and Taylor, 2005). Vibrating-mesh nebulisers consist of a vibrating mesh with fine multiple apertures thought which the liquid drug solution or suspension is passed through to be atomised into slow moving fine aerosol droplets with narrow size distribution (Dhand, 2002). Vibrating-mesh nebulisers are also highly efficient, portable, user friendly, silent in operation, hand-held and battery operated. In these nebulisers, baffles are not required as the droplet size is controlled by the micro-sized mesh pores of the nebuliser (Newman and Gee-turner, 2005).

Different vibrating-mesh nebulisers employ different mechanisms of aerosol operation, and can be classified into passively vibrating-mesh nebulisers and actively vibrating-mesh nebulisers.

Passively vibrating-mesh nebulisers

In passively (low energy) vibrating-mesh nebuliser, the piezoelectric crystal is attached to a transducer horn to which high frequency ultrasonic waves originated from a piezoelectric crystals are passed and transmitted to the mesh plate. This causes passive movements of the mesh which results in extrusion of the drug fluid through the mesh apertures, resulting in generation of aerosols (Dhand, 2002). The Omron MicroAir NEU22 nebuliser works using this principle (Newman and Gee-turner, 2005).

Actively vibrating-mesh nebulisers

In actively vibrating-mesh nebulisers, a micropump system employs an aerosol generator which produces aerosols. An electric current is applied which leads to the vibration of the ceramic vibrational element. This in turn leads to the upward and downward movement of the mesh. There are around 1,000 micro-pores in the mesh plate and the medication is usually positioned above the domed aperture plate. This ultimately leads to the generation of aerosols by the micropump action of the mesh, which extrudes drug fluid through the apertures. Aeroneb Pro, Aeroneb Go and Aeroneb Solo are examples of nebulisers that use this mechanism of operation. Beurer

iH 50 also uses a similar operating mechanism and is classified as an actively vibrating-mesh nebuliser.

With the Aeroneb Pro nebulisers, shown in Figure 1.18, shorter time is needed to complete nebulisation and almost all the medication fluid present in this nebuliser is aerosolised, ending up with negligible residual volume (Dhand, 2002). Moreover, there is no increase in the temperature of the medication fluid; hence, this nebuliser is potentially appropriate for delivery of peptide and protein drugs. This technology may suffer from a disadvantage like the possible blockage of some apertures and the high price of the nebuliser compared to jet nebulisers.

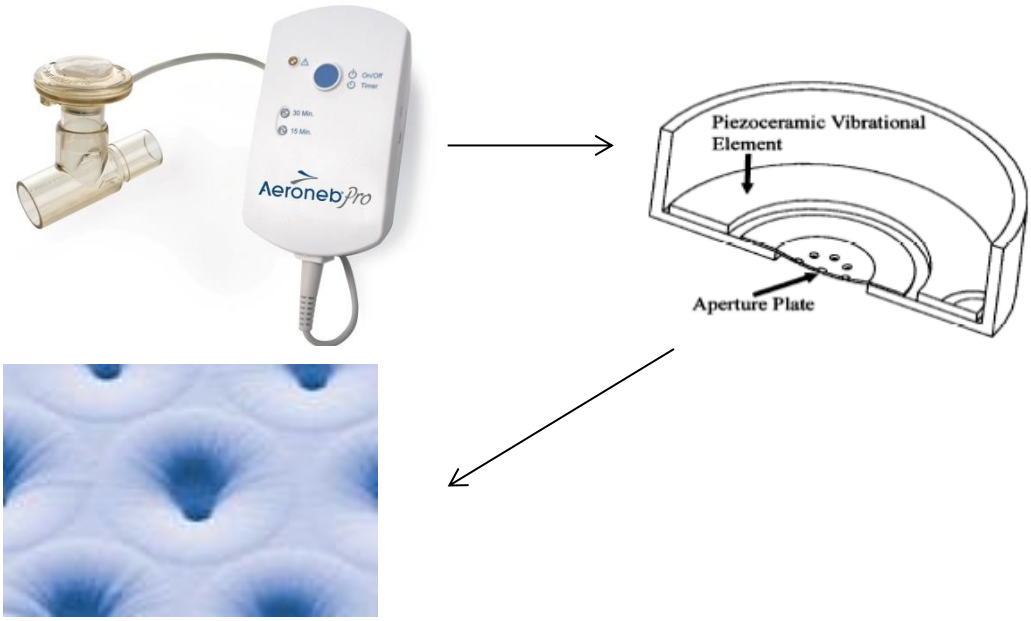

Figure 1.18 Aeroneb pro nebuliser and its vibrating mesh membrane (Fink, 2001) , www.aerogen.com (Bridges and Taylor, 2001)

http://www.aerogen.com/uploads/Instruction%20Manuals/30-012%20Rev%20M%20Aeroneb%20Pro%20DFU%20UK%20(Pro%20DFU).pdf

Beurer iH 50 is another actively vibrating mesh nebuliser utilising a different mechanism of operation. It can be used to treat diseases like asthma and bronchitis. In Beurer iH 50, the medication is atomised using a high tech vibrating membrane. It works with the latest membrane oscillation technology. The vibrating membrane is partially porous, thus, allowing only small particles of medication to pass through. It has

a high nebulization capacity of 0.25 ml/min (Beurer, 2014). This device is small, portable and has been reported to be suitable for use while travelling (Health, 2014) (Figure 1.19).

Figure 1.19 Beurer IH50 vibrating mesh nebuliser (Beurer, 2014)
http://www.beurer.com/web/uk/products/nebulization/nebulization/IH-50

1.17. Apparatus to analyse aerosol characteristics

The pharmacokinetic profile of inhaled drug is affected by its site of deposition in the respiratory airways. Absorption profile of a drug in the upper airways is different from its absorption in the deep lung. To determine the deposition of a drug in various pulmonary regions, pulmonary deposition models have been established and approved officially. For example the Two-stage impinger is an *in vitro* aerosol deposition model designed to study the likelihood of particles to deposit in lungs; this apparatus is approved by the British Pharmacopeia (BP) and United States Pharmacopeia (USP).

1.17.1. Twin (Two-stage) Impinger

Inhaled drug particles via nebulisers are deposited in different areas of the respiratory tract, depending on the aerodynamic size of the particles (Miller et al., 1992). Two-stage impinger, also called Twin impinger, is a two-stage size separation device for assessing

the deposition profile of drug aerosolised using oral inhalation devices (Figure 1.20). Both stages are defined by the aerodynamic particle size cut-off characteristics (Hallworth and Westmoreland, 1987).

The Two-stage impinger consists of a series of glassware through which vacuum is applied to pass air across the two stages of the impinger. The vacuum is applied to draw the air through the instrument and is adjusted at a flow rate of 60L/min. At this flow rate, the cut-off diameter between the two stages is 6.4 µm; hence particles smaller than this size will deposit in the lower stage and be described to be in "fine particle fraction" (FPF) or "respirable" fraction, and this fraction is expected to be therapeutically useful. Thus, the use of the twin impinger helps to determine the fraction of drug particles collected in the first stage of impinge (representing the upper airways) and the second stage (representing the lower airways) (Miller et al., 1992). There are two main disadvantages associated with the use of the twin impinger. Firstly, the sample is divided only in two categories (i.e. "respirable" and "irrespirable"). Secondly, the vacuum applied through the apparatus may result in some solvent evaporation, hence the "respirable" fraction might be overestimated for liquid aerosols (Miller et al., 1992). This impinger, however, is very useful for the routine quality assessment and testing of aerosols produced (Hallworth and Westmoreland, 1987).

Figure 1.20 Twin Impinger (Copley scientific).

http://www.copleyscientific.com/home/inhaler-testing/aerodynamic-particle-size/glass-twin-impinger

1.18. Mechanisms of particle deposition

Lung is a prime organ of exposure for a variety of air-borne particles. On inhalation, these particles may deposit in different regions of the respiratory system. The deposition of inhaled aerosols helps in determining any change in the dimensions of airways and alveoli, thus, could act as a diagnostic approach (Darquenne, 2006). The ability of a particle to reach lower respiratory airways depends on its aerodynamic size. There are three principal mechanisms by which particles are deposited in the lung. These are inertial impaction, gravitational sedimentation and infusion/Brownian diffusion. The mechanism of particle deposition depends on the size of the particle. To a less extent electrostatic precipitation and interception are also additional deposition mechanisms.

1.18.1. Inertial impaction

This mechanism of particle deposition is followed by particles larger than 5µm (Hilman, 1991). This occurs when the particle has to change its pathway due to airway bifurcations (Darquenne, 2006) which occurs when the particle's momentum is too large to change its course according to the airway(Hussain et al., 2011), resulting in particle deposition by impacting the walls of the airways at the point of deflection. Chance of impaction is directly proportional to the size of particle, air velocity and particle density (Heyder, 2004). The main site of deposition by inertial impaction is the upper respiratory tract like nose, mouth and pharynx.

1.18.2. Gravitational sedimentation

In gravitational sedimentation, the settlement of particle depends on the action of gravitational force. Particles reach their terminal settling velocity when the gravitational force equals the opposing viscous resistive forces of the air (Hussain et al., 2011). The probability of particle deposition by sedimentation is directly proportional to the particle size and particle density (Darquenne, 2006) and inversely proportional to the air flow. Gravitational sedimentation generally takes place for particles ranging in size between 0.5µm - 5µm (Darquenne, 2006). This mechanism of particle deposition is dominant in bronchiolar and alveolar-interstitial region as the air flow decelerates (Capstick, 2012), (Hofmann, 2011).

1.18.3. Brownian diffusion

In Brownian diffusion, deposition is due to random collision of particles with gas molecules. Particles having aerodynamic size smaller than 0.5µm may deposit by Brownian diffusion (Hussain et al., 2011) . In airways like bronchioles and alveolar region where the air flow is very low or absent this mechanism of deposition is observed. Deposition of particles by Brownian diffusion is inversely proportional to particle size. Brownian diffusion is dominated in the alveolar regions of the lung due to longer residence time and smaller airways (Darquenne, 2006).

1.18.4. Interception

Deposition via interception is common in particles like fibres where the length to diameter ratio is large. This type of deposition may happen when the edge of particle is in contact with the airway wall while the remaining is in the air space (Darquenne, 2006). The chance of particle interception is inversely proportional to the diameter of respiratory airway.

1.18.5. Electrostatic precipitation

Electrostatic precipitation occurs when charged particles are inhaled. Some surfaces of airways are charged and hence these particles may be electrostatically attracted. This results in a greater deposition of the charged particles rather than the neutral ones. Particle deposition by electrostatic precipitation is not regarded as a common mechanism of deposition (Darquenne, 2006).

1.19. Clearance of deposited particles

There are two mechanisms of particle clearance in the lung; mucociliary clearance in the upper respiratory airways and clearance by alveolar macrophages in lower respiratory airways (Stuart, 1976).

1.19.1. Mucociliary clearance

Mucociliary clearance is the primary mode of particle clearance in nasopharynx and tracheobronchial tree against all types of inhaled particles (Stuart, 1976, Clarke and Pavia, 1980). This region contains goblet cells and ciliated columnar cells. The goblet cells are responsible for the production of mucus that serves for entrapment and

conveyance of deposited particles which is propelled out of the respiratory system by the cilia (Stuart, 1976). Various insoluble materials are cleared using this mechanism. Mucociliary clearance is a continuous process used to eliminate particles immediately following their deposition (Wilkey et al., 1980).

1.19.2. Alveolar clearance

Insoluble particles deposited below ciliated airways are eliminated via slow phagocytosis and conveyance within pulmonary macrophages which is ultimately cleared into the gastrointestinal tract (Brain and Blanchard, 1993). These pulmonary macrophages are the immune cells present mostly in the alveolar region of the lung. They are responsible for the initial clearance of deposited particles, thus, protecting against bacterial and viral infections. They are rich in lysozymes and various enzymes, hence, can engulf and digest invading organisms. The rate of particle clearance by this mechanism depends on the site of particle deposition in the respiratory tract, and total amount, shape, surface properties and size of particle (Stuart, 1976).

1.20. Liposomes for pulmonary delivery

Pulmonary delivery gives a rapid onset of action for the delivered drug. It gives the drug a direct access for the treatment of respiratory diseases and also has large surface area for drug absorption to the systemic circulation. The level of enzymatic activity in the pulmonary system is also low compared to that in the gastro-intestinal tract, hence delivery via inhalation can be particularly advantageous for drugs susceptible to enzymatic degradation such as peptides and proteins (Labiris and Dolovich, 2003). However, one limitation of pulmonary delivery is that the drug may leave the lung rapidly because of rapid absorption to the systemic circulation owing to the thinness of the pulmonary epithelium. Hence, designing novel delivery systems that can prolong the retention of the drug in the lung following inhalation is greatly needed (Zeng and Chong, 1995). Liposomes have been established as a drug delivery system that can entrap drugs and prolong the drug residence in the lung, resulting in enhanced local therapeutic effect in the lung and reduced potential of systemic adverse effects (Chrai et al., 2001, Thomas et al., 1991). Liposomes are made of materials similar to lung surfactants and are biodegradable and non-toxic (Huang et al., 2010a). Thus, extensive research has been conducted on liposomes for pulmonary drug delivery.

1.20.1. Liposomes for acute lung injuries

Acute lung injury is caused by the presence of reactive oxygen species (ROS) which are chemically reactive molecules containing oxygen. They are formed by metabolism of oxygen in the body and known to have important roles to play such as haemostasis and cell signalling. However, over-production of ROS may disrupt cell membranes and cause oxidative stress. Anti-oxidants are required to decrease the number of ROS in the body. Liposomes can be used to entrap and deliver anti-oxidant enzymes to the lung. Some studies show that using liposomes for entrapping antioxidants may provide enhanced prophylaxis against oxidative lung injuries (Tanswell et al., 1990). It may also prolong the retention of the anti-oxidants in the pulmonary cells. Alipour *et al* in 2012 studied the acute toxicity of a single dose of intravenously administered liposomal antioxidant formulation containing *N*-acetylcysteine (NAC) in rats was examined. This study showed no treatment-related toxicity in rats by a single bolus intervenous administration (Alipour et al., 2012). S. D. McClintock *et al* in 2005 published a study on rats with acute lung injuries using liposomes. In this study, 2-chloroethyl ethyl sulphide was installed into the lung to produce acute lung injury in rats in a manner that seems related to the loss of the redox balance in the lung. They concluded that the injury of rat lungs can be substantially diminished by the presence of reducing agents or anti-oxidant enzymes delivered via liposomes (Abraham et al., 1999, McClintock et al., 2006). J. G. Gaca did a study on treating acute lung injuries in swine using liposomal clodronate. Large number of pulmonary intravascular macrophages (PIMs) was found in swine which leads to physiological response in acute lung injuries. It was found that the use of liposomal clodronate significantly decreases the PIM population in the lung (Gaca et al., 2003). This is a promising treatment for acute lung injuries caused by endotoxins.

1.20.2. Liposomes for asthma

Asthma is the most common pulmonary disease. It is characterised by airway hyper responsiveness, chronic inflammation and airway remodelling. Asthma happens when a triggering agent like allergen induces the release of histamine from mast cells, which causes the attraction of many inflammatory cells along with pro-inflammatory cytokines and mediators (Saari et al., 2002). This chronic inflammation often causes an increase in airway hyper responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing (Buist, 2003). The airway inflammation

and lung damage can be prevented by giving steroidal anti-inflammatory drugs such as beclometasone dipropionate (BDP) and budesonide and relieved using bronchodilators such as salbutamol sulphate (SBS). Chen *et al* in their experiment used SBS in liposome formulation to treat asthma using rat models. Effective distribution of liposomes with sustained release of the drug in the lung for 48 hours was reported. Pharmacodynamic studies in guinea pigs where SBS entrapped in liposomes showed anti-asthmatic effect for 18 hours whereas free SBS solution showed only for 8 hours (Chen et al., 2012). Oberoi *et al* in 2012 worked to develop a liposomal dry powder inhaler using doxophylline. This study showed better retention of doxophylline in liposomal formulation as compared to the controlled release formulation (Oberoi et al., 2012). In 2003 K. S. Konduri used budesonide in stealth liposomes to treat asthma. They used mice models to conclude that weekly therapy of budesonide encapsulated in liposomes showed similar effects as daily dosage of budesonide on its own in conventional formulation. The liposomal budesonide proved most efficient of all formulations used in the experiment to decrease the lung inflammation, peripheral blood eosinophil levels and serum IgE levels (Konduri et al., 2003).

1.20.3. Liposomes for Pneumonia

Pneumonia is an inflammatory condition of the lung which is due to the infections caused by bacteria, fungi, parasites or viruses. Common syndrome includes chest pain, cough and difficulty in breathing. Antibiotics are normally useful for their treatment. This treatment can be complicated and less effective due to various factors like unfavourable location of infection, decreased immunity of the host or limited susceptibility of the applied antimicrobial agent (Schiffelers et al., 1999). Use of liposomes for carrying these agents could modify the pharmacokinetics of the drug along with proper tissue distribution. This helps to increase the drug concentration in the desired site and reduce the toxicity in undesired tissues away from the lung (Schiffelers et al., 1999). In various experiments of Schiffelers and co-workers, liposomes have been demonstrated to be an effective carrier of antibiotics (Schiffelers et al., 2000). Liposome-encapsulated Gentamicin proved to be more efficient in the treatment of pneumonia in rat models than free Gentamicin. Liposomes with polyethanol glycol coating (i.e. PEGylated liposomes) have shown to be more beneficial for targeting antibiotics during pneumonia (Schiffelers et al., 2000). Ellbogen and co-workers have shown that ciprofloxacin encapsulated sterically stabilized liposomes increased the pharmacokinetics of drug. They used rat models to conclude that the

efficiency of liposome encapsulated ciprofloxacin better than the free ciprofloxacin for treatment of pneumococcal pneumonia when injected intravenously (Ellbogen et al., 2003). A new liposomal formulation is developed as a delivery system for antibiotics. "Arikace" is a new liposomal formulation of Amikacin for aerosol delivery with potent *Pseudomonas aeruginosa* killing and prolonged lung deposition. It's a registered trademark word of Transave, Inc., Monmouth Junction, NJ. Arikace is being developed for inhalation treatment of gram negative bacteria using nebuliser (Li et al., 2008**),** (Drulis-Kawa and Dorotkiewicz-Jach, 2010). Recently a research was done to prove the tolerability, safety, biologic activity and efficacy of Arikace in patients with Cistic Fibrosis with P aeruginosa infection with a dose of once daily for 28 days (Clancy et al., 2013).

1.21. Delivery of liposomes through nebulisers.

Liposomes can be converted to aerosols in nebulisers before being delivered to the lungs. Various studies has been done to study the fate of liposomes after being delivered by this method

Elhissi *et al* in 2007 studied the effect of nebulisation using vibrating mesh nebuliser on liposomes. They compared the delivery of non-extruded liposomes to the delivery of liposomes extruded through 1µm and 0.4µm using SBS as sample drug. They observed that extrusion did not affect the delivery of drug through jet nebuliser. It was also observed that less disruption was caused by vibrating mesh nebuliser to the liposomes extrude through 1µm membrane than the non-extruded liposomes. However, liposomes extruded by 0.4µm membrane showed the least stability and were unable to withstand the sheer generated by the vibrating mesh (Elhissi et al., 2007).

Ghazanfari *et al* compared the effect of passively and actively vibrating mesh nebuliser on liposomes. It was observed that vibrating mesh nebuliser was inappropriate for nebulizing highly viscous fluids. It was observed that passive vibrating mesh nebuliser was superior to actively vibrating nebuliser in generating very high total aerosol outputs. It was also observed that actively vibrating mesh nebuliser was better than passively vibrating nebuliser by providing shorter nebulisation time and reducing higher aerosol output rates for higher viscosity. Thus, vibrating mesh nebulisers were affected by the fluid properties of liposomal dispersion and nebuliser mechanism of operation (Ghazanfari et al., 2007).

Saari *et al* in 1999 studied that jet nebulisation reduces the liposome size and effectively delivers it to the lungs in human volunteers (Saari et al., 1999). Waldrep *et al.* in 1993 studied the delivery of Ciclosporine A (CsA) to lungs via jet nebuliser using five different phosphotidylcholine formulations. He found that CsA-DLPC was the best formulation for aerosol delivery to the lung. It was also proved that liposomes prepared using phosphotidylcholine with low transition temperature were more efficient for nebulisation than those with higher transition temperature (Waldrep et al., 1993).

Gasper *et al* in 2010 investigated the membrane integrity of liposomes encapsulating a florescent compound calcein using Aeroneb Pro nebuliser. He concluded that vibrating mesh nebulisers are well suited for the pulmonary delivery of drug (Gasper et al., 2007).

1.22. Stability of liposomes to nebulisation

Various studies are done to study the stability of liposomes on nebulisation. Liposomes are delicate vesicles, hence, the shearing forces generated during nebulisations may lead to the leakage of hydrophilic drug (Niven et al., 1991, Taylor et al., 1990b). Taylor et al in 1990 observed the large loss of entrapped drug on passage of multilamellar vesicles through an air jet nebuliser due to vesicle fragmentation. He also observed that nebulisation of liposomes previously extruded through 1µm membrane decreased the drug loss (Taylor et al., 1990b). It is also studied that drug loss is less when the size of liposomes is smaller than the aerosols droplet size generated by nebulisations. This prevents the liposomes from breaking down into smaller vesicle to fit into the aerosol, thus, avoiding drug leakage (Niven et al., 1991).

Bridges and Taylors in 1998 observed that smaller the liposomal size more efficient they are to be delivered from nebulisers. He also observed that the inclusion of cholesterol or DPPC in the liposome bilayers rendered them more resistant to the sheer forces to which they were exposed during nebulisation. This also suggests that inclusion of hign transition temperature lipid or cholesterol incleased the stability of liposomes by increasing the vesicle rigidity (Bridges and Taylor, 1998).

Bridges and Taylor in 2000 studied the effect of freeze drying on the stability of liposomes for nebulisation. He observed that size of liposomes was larger and they aggregated without the addition of cryoprotectant trehalose. Freeze drying of liposomes without cryoprotectant trehalose were proved unstable to nebulisation. He also concluded that freeze drying provided liposomal preparation having long term physical

and chemical stabilitywhich maybe hydrated before its delivery through nebuliser (Bridges and Taylor, 2001).

1.23. Hypothesis

Liposomes are promising carriers for pulmonary drug delivery. However, conventional liposomes when used for pulmonary drug delivery via nebulisers are observed to leak its entrapped drug. This is due to the stress provided by the nebulisers during nebulisation and lack of liposome elasticity.

Transferosomes made up of surfactants like Tween 80 have proven flexibility and they have structure similar to liposomes and useful for skin delivery.In this study it is hypothesised that a similar system can be useful for pulmonary delivery. Surfactosomes like transferosomes can be considered more elastic and flexible than liposomes, hence, expected to minimise the drug leakage during nebulisation. Surfactosomes would be more efficient than conventional liposomes for pulmonary drug delivery.

Hence, surfactosomes made up of surfactant, Tween 80, in addition to liposomal components will be tested for its efficiency in pulmonary delivery.

1.24. Aim of thesis

The Aim of this thesis was to investigate the properties of an elastic vesicle called surfactosome, also termed as transferosome. The stability and efficiency of surfactosome was compared with the conventional liposome for pulmonary drug delivery. Hydrophilic model drug salbutamol sulphate (SBS) and hydrophobic model drug beclometasone dipropionate (BDP) were used for this purpose. The stability and efficiency was initially investigated using extruder of different polycarbonate membranes and later by nebulisers. The vesicles were prepared by thin film method as well as proliposome technology. Formulations with and without cholesterol were investigated to find if inclusion of cholesterol proved an advantage or disadvantage for pulmonary aerosol delivery. A novel formulation called prosurfactosomes was also investigated which on hydration forms surfactosome and compared with liposomes formed from hydration of proliposomes. Here Aeroneb Pro, Beurer iH50 and PARI LC sprint nebulisers were used to compare both the vesicles. The work focused on finding the best formulation for pulmonary drug delivery and to investigate if surfactosomes were better than liposomes to sustain the different forces applied on these vesicles before being delivered to the lungs. Effect of freeze drying and spray drying on these

vesicles were also investigated. This thesis also aimed to investigate the stability of liposomes and surfactosomes in different environment.

The final outcome of this thesis was aimed at formulating a better vesicle for pulmonary drug delivery and to see if prosurfactosomes were better than conventional proliposomes.

Chapter 2

General Materials and Methodology

2.1. Materials

Materials used are shown in Table 2.1

Table 2.1 List of chemicals and their supplier used for the research

Chemical	**Supplier**
Soya Phosphotidylcholine (Lipoid S-100)	A gift from Lipoid, Switzerland
NaCl (ACS, 99.0% min)	Alfa Aesar, UK
Salbutamol Sulphate (99%)	
Sodium 1-hexanesulfonate monohydrate (99%)	
Triton X -100	
Deuterium oxide (For NMR, 99.8% atom %D)	Acros Organics, UK
Chloroform (for HPLC, stabilized with ethanol)	Fisher Scientific, UK
Ethanol (99.8+% absolute duty free for HPLC certified HPLC	
Glacial acetic acid 99+%	
HPLC water (HPLC gradient grade)	
Methanol (HPLC grade)	
Beclometasone dipropionate (BDP)	Sigma, UK
Cholesterol (Sigma grade, ≥99%)	
Mannitol ≥98%	
Polysorbate 80 (Tween 80)	
Ammonium thiocyanate	BDH, UK
Ferric chloride	
Phosphotungstic acid	TAAB Laboratories Equipment Ltd., UK
pH buffers (Phosphate), reference standard 4 ± 0.01, 7± 0.01 and 10 ± 0.01 at 25°C	Sigma life Science, USA

2.2. Methods

2.2.1. Preparation of conventional liposomes

SPC and cholesterol were used in desired ratio as lipid phase. Different ratios of SPC and cholesterol were used in different studies and are specified precisely in the following chapters. The lipid phase was dissolved in ethanol or chloroform (20mg/ml) within a round bottom flask. The size of round bottom flask depended on the quantity of liposomes to be prepared. The organic solvents were removed using a rotary evaporator (R-215, Buchi, Swirzerland) under vacuum (V-700, Buchi, Switzerland) for 1 hour in a water bath (B-491, Buchi, Switzerland) at 38ºC at maximum rotation speed of 280rpm. After 1h the vacuum was turned off, negative pressure as released and the round bottom flask was detached. The resultant thin film was hydrated. Different hydration concentrations were used in different studies and are specified precisely in the following chapters.

2.2.2. Preparation of proliposomes

SPC and cholesterol were used in desired ratio as lipid phase. Different ratios of SPC and cholesterol were used in different studies and are specified precisely in the following chapters. The lipid phase was dissolved in chloroform (20mg/ml) within a round bottom flask. The size of the round bottom flask depended on the quantity of proliposomes prepared. Mannitol was used as the carbohydrate carrier and was added to the lipid phase in desired ratio for preparation of proliposomes with SBS or BDP. Different concentrations of mannitol were used in different studies and are specified precisely in the following chapters. The organic solvent was removed by evaporation using a rotary evaporator as described in section 2.2.1. After releasing the negative pressure and detaching the flask, the proliposomes were collected using a clean spatula. Proliposomes were then stored at room temperature and was used immediately.

2.2.3. Preparation of surfactosomes

SPC and cholesterol were used in desired ratio as lipid phase along with Tween 80 (15% w/w of the total lipid) in a round bottom flask. . Different ratios of SPC and Cholesterol were used in different studies and are specified precisely in the following chapters. These were dissolved in ethanol or chloroform (20mg/ml) in a round bottom

flask. The organic solvent was evaporated as described in section 2.2.1 and the resultant thin film was hydrated in a concentration of 10 mg/ml.

2.2.4. Preparation of prosurfactosomes

SPC and cholesterol were used in desired ratio as lipid phase along with Tween 80 (15% w/w of the total lipid) in a round bottom flask. SPC, cholesterol and tween 80 were dissolved in chloroform (20mg/ml) in a round bottom flask. The size of the round bottom glass depended on the quantity of proliposome to be prepared. Mannitol was added to this lipid phase in desired ratios for SBS and BDP ultradeformable vesicular formulations respectively. The organic solvent was evaporated as described in section 2.2.1. After evaporation of the solvent, the flask was detached and the proliposomes were collected using a clean spatula. The proliposomes were used immediately for the experiment.

2.2.5. Addition of drug in the vesicular formulations

Salbutamol sulphate (SBS) or and beclometasone dipropionate (BDP) were included within the liposomal, proliposomal, surfactosomal and prosurfactosomal formulations.

2.2.5.1. Incorporation of salbutamol sulphate (SBS)

For vesicles with salbutamol sulphate (SBS), 1 mg/ml drug was dissolved in isotonic sodium chloride solution (0.9% NaCl) before hydration. A two- step hydration method was used to maximise the drug entrapment. In this method, entire drug was dissolved in 2ml of the isotonic NaCl solution and then added to the formulation for hydration. The preparation was vigorously hand-shaken and vortexed for 5min and was kept aside on the bench for 10 min. The remaining drug-free isotonic NaCl solution was then added to the concentrated vesicles and was left for 2 h at room temperature for annealing before conducting further experiments.

2.2.5.2. Incorporation of beclometasone dipropionate (BDP)

For vesicles with Beclometasone dipropionate (BDP), 2.5 mole% drug was added to the lipid components before addition of chloroform. After the removal of the organic solvent by rotary evaporation, the resultant formulation was hydrated with deuterated water (D_2O) to give liposomes or surfactosomes. The preparation was vigorously hand

shaken for 5 min and left for 2 h at room temperature for annealing before conducting further experiments.

2.2.6. VMD analysis of vesicles

VMD of liposomes and surfactosomes was analysed using laser diffraction via the Malvern Mastersizer 2000, Malvern Instruments, UK. The stirring speed was set at 1,360 rpm and the polydisperse mode of analysis was chosen. The 50% undersize of particles (i.e. volume median diameter) was recorded to represent the size of vesicles. The vesicles were mixed up using vortex (Stuart, SA8) to avoid vesicular aggregation.

2.2.7. Zeta potential (Surface charge) analysis

Zeta potential (surface charge) of liposomes and surfactosomes was analysed using electrophoretic light scattering via Malvern Zetasizer Nano ZS (Malvern instruments, UK). Three measurements each of ten runs were used to analyse the zeta potential of the vesicles. The particle suspension was injected into a "folded capillary cell" which is equipped with electrodes on both sides (supplied by Malvern Instruments Ltd, UK). The particle speed down the applied electric field was monitored with the laser beam.

2.2.8. Separation of entrapped and unentrapped drug

In order to quantify the drug in liposomes and surfactosomes using HPLC (1200 series, Agilent Technologies, UK), the separation of unentrapped drug from the vesicle-entrapped fraction was necessary. The entrapped and unentrapped portions of the drug were separated from each other via centrifugation.

2.2.8.1. Salbutamol sulphate

Liposomes entrapping SBS were separated from the unentrapped drug in the continuous aqueous phase using ultracentrifugation. The centrifugation was performed at 55,000 rpm (277,816 relative centrifugal force) for 35min at 6°C. Liposomal dispersion (7 ml) was loaded into the polycarbonate ultracentrifuge tube (Beckmann, USA) for this purpose. The free (unentrapped) drug in the aqueous phase was separated from the liposome pellet (entrapped drug) sedimented upon centrifugation via careful aspiration of the aqueous phase. Diluted Triton X-100 was added to the liposomal pellet to dissolve the liposome membranes, thus, disrupting the liposome vesicles and releasing the entrapped drug. Analysis was conducted using HPLC which determined the

unentrapped and entrapped drug concentration to calculate the percentage entrapment of SBS.

2.2.8.2. Beclometasone dipropionate

For the separation of excess BDP from the liposomal and surfactosomal dispersions, a technique based on density difference between BDP crystals and liposomes was designed. Deuterated water (D_2O) (density: 1.053g/ml) was selected as a vesicular dispersion medium for this purpose. Vesicles with BDP were separated from BDP crystals using D_2O in a bench centrifuge (Jencons-PLS, Spectrafuge 24D, UK). The centrifugation was conducted at 13,000 rpm (15,300 relative centrifugal force) for 90 min at room temperature. The vesicular dispersion (1 ml) was loaded into an eppendorf tube for this purpose. The vesicles containing entrapped drug and continuous D_2O phase containing unentrapped (free) drug were separated using a Gilson pipette. A small deposition of the unentrapped crystalline BDP forming sediment in the bottom of the eppendorf tube was dissolved using methanol and aspirated via a Gilson pipette. Methanol was added to the separated layer to dissolve the vesicle membrane, thus, releasing the entrapped drug. This was, thus, ready for HPLC analysis to determine entrapped drug concentration. Methanol was also added to the BDP spot sediment and D_2O containing unentrapped drug and were made ready for HPLC

2.2.9. Drug entrapment studies

2.2.9.1. Salbutamol sulphate (SBS)

All samples were prepared and suspended in HPLC water. A concentration of 5mM aqueous solution of sodium 1- hexane sulfonate was mixed with methanol (75:25, v/v) to form the mobile phase. Glacial acetic acid (1%) was added to the mixture. HPLC instrument was set up using C18 column (HPLC column Eclipse XDB-C18, 4.6 x 50mm, Agilent, UK) and UV detection at 276nm. The mobile phase flow rate was set at 1ml/min and 40ºC (Elhissi et al., 2007). The assay was validated by using a calibration curve made by using solutions of different known concentrations of SBS from 5µg/ml to 70µg/ml.

$$\%\ SBS\ entrapment = \frac{amount\ of\ SBS\ entrapped}{amount\ of\ SBS\ added}\ x\ 100$$

2.2.9.2. Beclometasone dipropionate (BDP)

All BDP samples for HPLC analysis were dissolved in methanol. HPLC-grades of Methanol and water (3:1 v/a) constituted the mobile phase. The mobile phase flow rate was set up at 1.7ml/min with a sample injection volume of 50µl and UV detection at 238 nm. The assay was validated by using a calibration curve made by using solutions of different known concentrations of BDP from 5µg/ml to 40µg/ml. This HPLC method was adapted from that designed by Batavia and co-workers (2001).

$$\%\ BDP\ entrapment = \frac{amount\ of\ BDP\ entrapped}{amount\ of\ BDP\ added}\ x\ 100$$

2.2.10. Drug loading

Drug loading is calculated to understand the drug loaded per 100mg of lipid.

2.2.10.1. Salbutamol sulphate (SBS)

For SBS, for 100mg of lipid 6.67mg of SBS was used. Hence,

$$SBS\ drug\ loading\ = \frac{amount\ of\ SBS\ entrapped}{amount\ of\ lipid}$$

2.2.10.2. Beclometasone dipropionate (BDP)

For BDP, for 100mg of lipid 2.23mg of BDP was used (2.5 mole %). Hence,

$$BDP\ drug\ loading\ = \frac{amount\ of\ BDP\ entrapped}{amount\ of\ lipid}$$

2.2.11. Quantification of phospholipid using Stewart assay

Stewart assay is used to determine the quantity of phospholipid in samples prepared in organic solvents such as chloroform by exploiting the capacity of phospholipid molecules to develop colour on reaction with ammonium ferrothiocyanate (Stewart, 1980). In this study, phospholipid concentration was determined in liposomal and

surfactosomal samples. Initially ammonium ferrothiocyanade solution was made by dissolving 27.03g of ferric chloride and 30.4 g of ammonium thiocyanate in 1000ml distilled water. A volume of 1ml vesicular dispersion was taken in a 15ml centrifuge tube. To this, 1ml ethanol was added in excess to form an ethanolic solution of liposomes and surfactosomes. This solution was kept at 90ºC overnight in an oven to evaporate the solvent, thus, forming a dry lipid film on the inner walls of the tube. To the resultant dry film, 2ml of chloroform and equal quantity of ammonium ferrothiocyanate were added. The tube was then vortexed for 20 seconds and centrifuged at 4,000 rpm using the bench centrifuge (Jouan B4i, France) for 10 min at 4ºC. Chloroform formed the lower layer in the centrifuge while ammonium ferrothiocyanate with dissolved phospholipid formed the upper layer. This was due to chloroform being heavier than ferrothiocyanate. The lower (i.e. chloroformic) layer was separated using a Pasteur pipette and the concentration of phospholipid was estimated using spectrophotometry (UV detector) (Jenway, 7315 Spectrophotometer, UK) at 485nm. The standard calibration curve was used to find the lipid concentration in the test sample.

2.2.12. Visualization of samples using light microscopy

Light microscopy (Novex B-Range, Holland) was used to analyse different layer after centrifugation. Light microscopy was also used to study the behaviour of mannitol in water. A drop of sample was placed on a glass slide and covered with a cover slip. Eyepiece magnification of 10x and objective magnification of 10x and 40x were used. Total magnifications of 100x and 400x were used and samples were viewed and analysed using the software Imagefocus v 3.0.

2.2.13. Scanning electron microscopy (SEM)

Proliposomes and prosurfactosomes before and after spray drying and freeze drying were analysed using Scanning electron Microscopy (SEM) (FEI Quanta 200, USA) and vacuum pump (Edwards PV25MK, UK) was used for this purpose. Initially the carbontab (agar, UK) was attached to Aluminium specimen tub (agar, UK) and small amount of sample was carefully kept on specimen tub. Air duster was used to blow away the excess particles. Loaded aluminium stub was sputter coated with gold sputter coater (Quorum technologies Emitech K550X, UK) and vacuum pump. The samples

were then placed on sample stage to be analysed. Samples were viewed and photographed in desired magnification using xT microscope control software.

2.2.14. Transmission electron microscopy (TEM)

A drop of SBS and BDP entrapped in liposome and surfactosome was placed on separate carbon coated grids (TAAB Laboratories Equipment Ltd., UK) and stained negatively using 1% phosphotungstic acid. It was observed and photographed using a Philips CM 120 Bio-Twin TEM (Philips Electron Optics BV, the Netherlands).

2.2.15. Statistical analysis of data

All data were processed using SPSS (statistical package for the social sciences) software by IBM. Data were presented as mean ±standard deviation (SD) with n=3 observations or experiments. Data were processed using either the student t test or One-way ANOVA to compare between 2 sets of groups or more than 2 respectively. A p value of <0.05 indicates that difference between the groups is statistically significant.

Chapter 3

Comparison between liposomes and surfactosomes formed using thin film method for entrapment of drug before and after extrusion

3.1. Introduction

In this chapter liposomes and surfactosomes are prepared using thin film method. Their VMD (size), span (size distribution), drug entrapment and drug retention after extrusion was studied. Formation of liposomes using thin-film method is the classic method of liposome preparation which was first described by Bangham *et al* (Bangham et al., 1965). In this method all the lipid components (lipid, cholesterol and lipid soluble drug if any) were dispersed in an organic solvent (chloroform) which was evaporated in a round bottom flask using rotary evaporator and reduced pressure. For surfactosomes, Tween 80 was included in the lipid phase. A thin lipid layer was thus formed on the inner wall of the round bottom flask. This layer was hydrated using HPLC water (with dissolved drug SBS) for SBS formulations and D_2O for BDP formulations and was agitated above the phase transition temperature for the thin film to disperse appropriately. The vesicles were formed after annealing for 2 h in room temperature. This is above the phase transition temperature of the lipid which is below 0°C.

Multi-lamellar vesicles (1-5µm) were generated and were then reduced in size using mini-extruder. To obtain LUVs extrusion through polycarbonate membrane is preferred as extrusion decreases the lamellarity of the liposomes (Berger et al., 2001b). This gives uniformly sized vesicles. Thin film method is not considered appropriate for the large scale production of liposomes.

In this study, liposomes and surfactosomes were produced using thin film method and characterised for VMD and drug entrapment. Extrusion of the produced vesicles was employed using Avestin mini-extruder and polycarbonate membranes having pore size of 5, 2, 1 and 0.4µm. This investigation was conducted to study and compare the effect of stress on liposomes and surfactosomes. This stress was compared to the shear stress provided by nebulisers during aerosol generation. Retention of the hydrophilic drug salbutamol sulphate (SBS) and the hydrophobic drug beclometasone dipropionate (BDP) in liposomes and surfactosomes with and without cholesterol was studied before and after extrusion.

In this chapter, all four formulations were studied for their physical stability, drug retention and delivery characteristics. This study will help us conclude if surfactosomes are better than conventional liposomes in delivering hydrophilic and hydrophobic drug

to the pulmonary system. Extrusion will help us conclude if surfactosomes can entrap and retain more drug than liposomes after undergoing a stress similar to nebulisation.

3.2. Methodology

3.2.1. Preparation of liposomes for thin-film method

SPC and cholesterol (1:1 mole ratio) were dissolved in ethanol or chloroform (20mg/ml) within a round bottom flask. The organic solvent was evaporated using rotary evaporator as described in chapter 2 (section 2.2.1). For liposomes with SBS, 1mg/ml drug was dissolved in the saline solution (0.9% w/v) before hydration. In case of BDP, 2.5 Mole% drug was added to the lipid components before addition of chloroform.

3.2.2. Surfactosomes

Lipid phase (SPC and cholesterol, 1:1 mole ratio) along with Tween 80 (15% w/w of the total lipid) were placed in a round bottom flask. These were dissolved in ethanol or chloroform (20mg/ml) in a round bottom flask. The organic solvent was evaporated and the resultant thin film was hydrated as described in section 2.2.4. For liposomes with SBS, 1mg/ml drug was dissolved in the isotonic water before hydration. In case of BDP, 2.5 mole% drug was added to the lipid components before addition of chloroform.

3.2.3. Extrusion

Avestin Liposofast mini extruder was used to extrude the liposomes/ surfactosomes through different polycarbonate membranes. Ten ml of samples were extruded at a time. The different sizes of Nucleopore Track-etched membranes used were 5, 2, 1 and 0.4µm. The sample was passed through 5µm membrane 11 times and 5 times through 2µm, 1µm and 0.4µm polycarbonate membranes. All samples were first passed through 5µm membrane and then they were passes through the remaining smaller pore sized membranes. Sample extruded through 1µm polycarbonate membrane was extruded through 0.4µm.

3.2.4. Solubility of BDP in presence and absence of Tween 80

For checking the solubility of BDP in water, excess BDP (three times the normal concentration, 2.5 mole% to lipid, was used) was added to 1ml water within an Eppendorf tube. The mixture was shaken using a shaker water bath for 24 h at 40°C. If

the solution became clear after shaking for 24 h, more BDP was added to ensure that the amount added was in excess. BDP was added till the solution remained milky even after shaking it for 24 h. The eppendorf tube was centrifuged for 20 min and the supernatant was tested for drug concentration using HPLC as described in Chapter 2 section 2.2.9 (b). The same procedure was repeated to analyse the solubility of BDP in the presence of Tween 80. One ml of Tween 80 and water (15:85 v/v) was used for this purpose.

3.2.5. Stability of liposomes and surfactosomes upon extrusion

To test the stability, all formulations including liposomes and surfactosomes with and without cholesterol were prepared. They were centrifuged using a bench centrifuge and the entrapped liposomal and surfactosomal part was separated as described in section 2.2.8. The vesicular (floating) layer with entrapped drug was aspirated and re-suspended in fresh HPLC water. They were extruded 51 times using 1µm polycarbonate membranes as described in section 3.2.3. After extrusion, the drug entrapped in the vesicle was analysed using HPLC as described in section 2.2.8 and 2.2.9. The stability was further analysed by centrifuging the extruded samples using the bench centrifuge for 90 min at 13,000 rpm (15,300 relative centrifugal force at room temperature. The vesicular layer with entrapped drug was separated as described in section 2.2.8. This vesicular layer which contains the entrapped drug was again re-suspended in fresh HPLC water. These 1µm vesicles were further extruded 51 times through the extruder with 1µm polycarbonate membrane filters. Again the drug entrapped was analysed using HPLC as described in chapter 2 section 2.2.9.

3.3. Results and discussion

Liposomes and surfactosomes made from soya phosphotidylcholine (SPC) were measured for their VMD and span. In this study, thin film was formed by the evaporation of chloroform or ethanol in which the lipids were dissolved prior to evaporation using rotary evaporator. Hence, liposomes and surfactosomes prepared by evaporation of chloroformic and ethanolic solutions were studied and compared.

3.3.1. VMD (size) and size distribution (span) of liposomes and surfactosomes with cholesterol prepared from following chloroform evaporation

The median size and span values of liposomes and surfactosomes with cholesterol were studied as shown in Figure 3.1 and Figure 3.2 respectively. It was observed that VMD of liposomes prior to extrusion was around 6.5µm and for surfactosomes it was around 4.8µm. Thus, VMD of surfactosomes was significantly smaller than that of liposomes ($p<0.05$). Following extrusion through 5µm polycarbonate membranes, the VMD of the liposome decreased to around 3.6µm and surfactosomes to around 3.2µm. There is no significant difference in the VMD of both types of vesicles ($p>0.05$). When the vesicles were further extruded through 2µm polycarbonate membranes, the liposomal VMD was decreased to 1.9µm and surfactosomal VMD to 1.95µm. On further extrusions with 1µm and 0.4µm the VMD decreased to 0.89µm and 0.35µm respectively. Surfactosomal VMD was decreased to 0.97µm and 0.37µm respectively. There was no significant difference in the VMD of both formulations upon extrusion using the same VMD polycarbonate membrane ($p>0.05$). VMD reduction of the liposomes and surfactosomes on extrusion suggested that vesicles prepared from conventional components like SPC and cholesterol dissolved in chloroform followed by organic solvent evaporation and lipid hydration exhibited no apparent aggregation or fusion during extrusion.

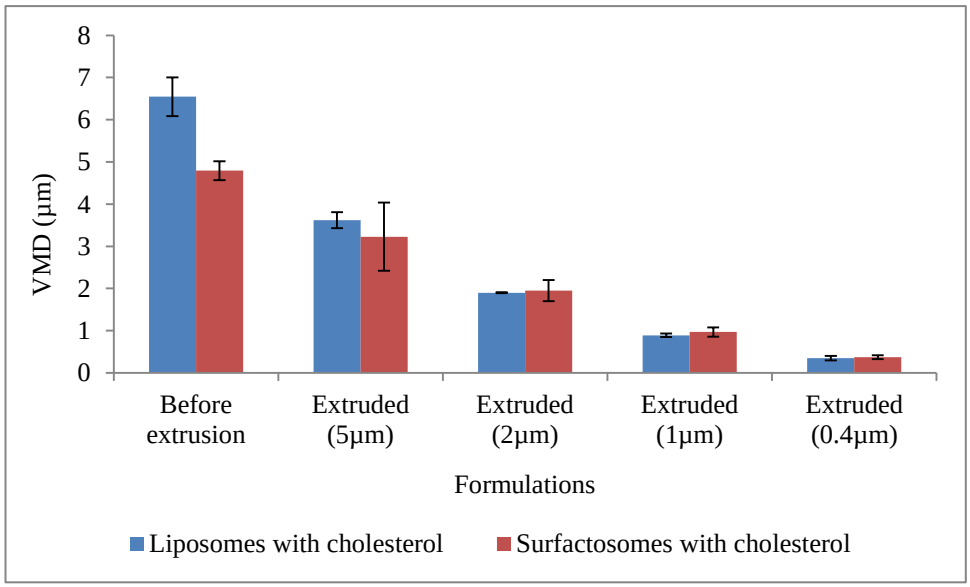

Figure 3.1 VMD of liposomes and surfactosomes with cholesterol made from solvent evaporation of chloroformic solution. Data are mean ±SD, n=3.

It was observed that the span of liposomes prior to extrusion was around 2.06 and span of surfactosomes was around 1.54 with no significant difference between the two formulations. Following extrusion through 5µm polycarbonate membranes, the span of liposomes was reduced to 1.29 and surfactosomes to 0.86. The span of surfactosome was significantly lower than the span of liposomes ($p<0.05$). When the vesicles were further extruded through 2µm polycarbonate membranes, the span of liposome was reduced to 0.87 and span of surfactosome was reduces to 0.66. On further extrusions with 1µm and 0.4µm the liposomal span was reduced to 0.84 and 0.79 respectively, whilst for surfactosomal the span was reduced to 0.63 and 0.53 respectively. For each membrane VMD there was no significant difference in the span of liposomes and surfactosomes, suggesting that the behaviour of both types of vesicles after extrusions with 2µm, 1µm and 0.4µm membranes was similar. The reduced span values suggest that as the vesicles were extruded through the polycarbonate membranes, their size distribution has become more uniform (i.e. with lower polydispersity) and the standard deviation was decreased markedly.

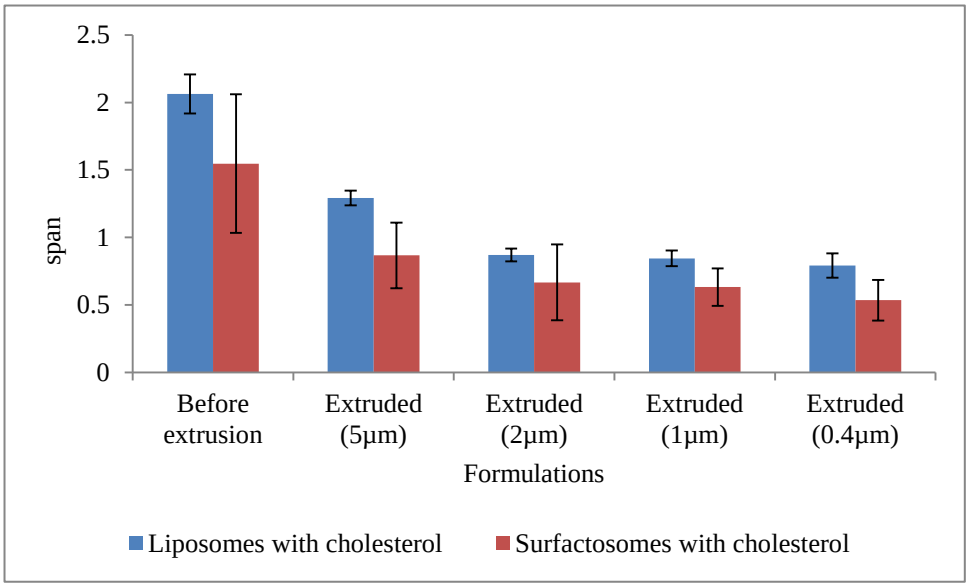

Figure 3.2 Span of liposomes and surfactosomes with cholesterol made from solvent evaporation of chloroformic solution. Data are mean ±SD, n=3.

3.3.2. VMD (size) and span (size distribution) of liposomes and surfactosomes without cholesterol prepared following chloroform solution evaporation

The median VMD and the span values of liposomes and surfactosomes without cholesterol were studied as shown in Figure 3.3 and Figure 3.4. It was observed that the VMD of liposomes prior to extrusion was around 5.82µm and for surfactosomes it was around 5.62µm, with no significant difference between the VMD of both types of formulations ($p>0.05$). Following extrusion through 5µm polycarbonate membranes, the VMD of the liposome decreased to around 3.46µm and surfactosomes to around 3.87µm. When the vesicles were further extruded through 2µm polycarbonate membranes, the liposomal VMD was decreased to 1.74µm and surfactosomal VMD to 2.08µm. On further extrusions with 1µm and 0.4µm the liposomal VMD decreased to 0.89µm and 0.43µm respectively. Surfactosomal VMD was decreased to 1.15µm and 0.38µm respectively. For each membrane size, there was no significant difference between the size of liposomes and surfactosomes ($p>0.05$). Size reduction of the liposomes and surfactosomes on extrusion suggests that vesicles prepared from conventional component like SPC in absence of cholesterol made by the solvent evaporation of chloroform had no apparent aggregation or fusion during extrusion.

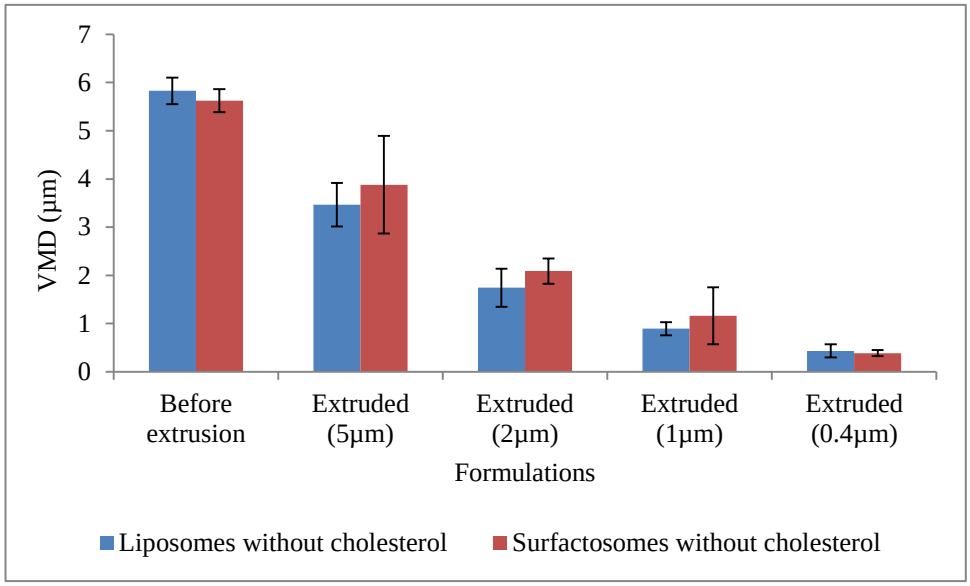

Figure 3.3 VMD of liposomes and surfactosomes without cholesterol made from solvent evaporation of chloroformic solution. Data are mean ±SD, n=3.

It was observed that the span of liposomes prior to extrusion was around 2 and for surfactosomes it was around 1.68, with no significant difference between the two types of vesicles. Following extrusion through 5µm polycarbonate membranes, the span of liposomes was lowered to 1.2 and for surfactosomes it was reduced to 1.01. When the vesicles were further extruded through 2µm polycarbonate membranes, the span of liposome was reduced to 1.06 and for surfactosome it was lowered to 1.23. On further extrusions with 1µm and 0.4µm the span for liposomes was decreased to 0.86 and 0.98 respectively. By contrast, for surfactosomes, the span was reduced to 0.93 and 0.97 respectively. No significant difference was seen between the span values of liposomes and surfactosomes for each membrane size, indicating that both types of vesicles exhibited similar behavior upon facing the stress of extrusion through 5µm, 2µm, 1µm and 0.4µm membranes.

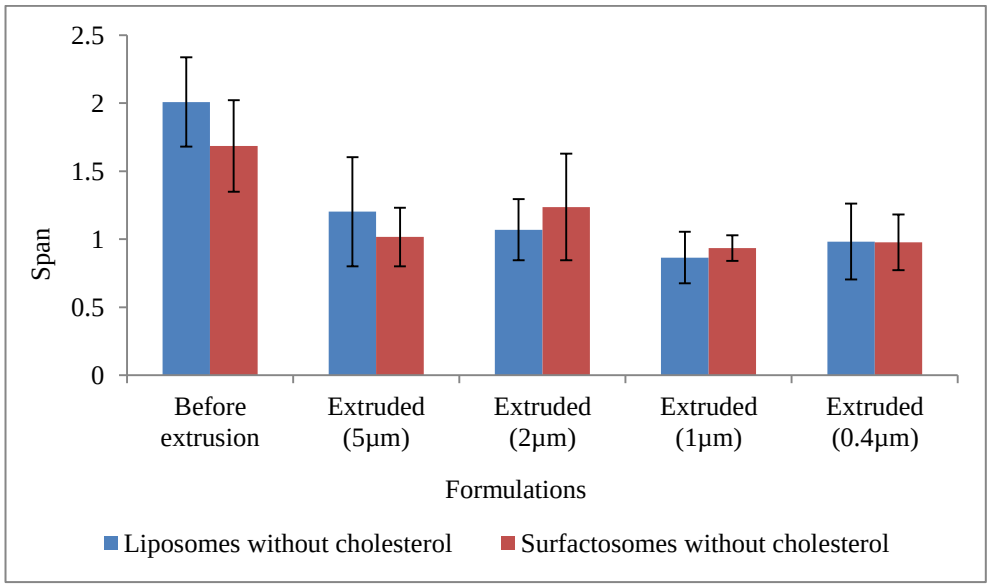

Figure 3.4 Span of liposomes and surfactosomes without cholesterol made from solvent evaporation of chloroformic solution. Data are mean ±SD, n=3.

Hence, from section 3.3.1 and 3.3.2 it can be concluded that inclusion of cholesterol has no significant effect on the VMD and span of liposomes and surfactosomes. This suggests that cholesterol inclusion can be desirable since it may give more vesicular rigidity with no effect in VMD and span.

3.3.3. Liposomes and surfactosomes with cholesterol prepared following evaporation of ethanol

After studying the characteristics of liposomes and surfactosomes prepared following the evaporation of chloroform, ethanol was used as an organic solvent instead of chloroform. The VMD and span of the resultant vesicles were recorded as shown in Figure 3.5 and Figure 3.6. The VMD of liposomes prepared following evaporation of ethanol without extrusion was 5.58µm and for surfactosomes it was 5.08µm, with no significant difference between the formulations ($p>0.05$). Here the VMD was consistent with that of vesicles prepared using chloroform as organic solvent, as discussed in section 3.3.1 and 3.3.2. When extruded with 5µm polycarbonate membranes, unexpectedly, the VMD was increased dramatically to 47.7µm for liposomes and to 110.7µm for surfactosomes. When vesicles were further extruded using 2µm polycarbonate membranes, the VMD remained much larger than expected, being 62.4µm 43.8µm for conventional liposomes and surfactosomes respectively. On further extrusion of liposomes with 1µm and 2µm membranes the VMD was markedly increased to 73µm and 92.3 respectively. Surfactosomes also exhibited very large VMD which was 64.3µm and 132.2µm respectively. For each membrane size, the size measured was formulation-dependent ($p<0.05$). This increase in VMD possibly indicates that liposomes and surfactosomes have undergone aggregation, which was attributed to the use of ethanol as organic solvent for the preparation of the lipid thin film used in manufacturing the liposomes and surfactosomes. The standard deviation for formulations was very large which indicated that the size varied with every repetition performed. This overall indicates that formulations were very unstable and extrusion forced them to aggregate or fuse.

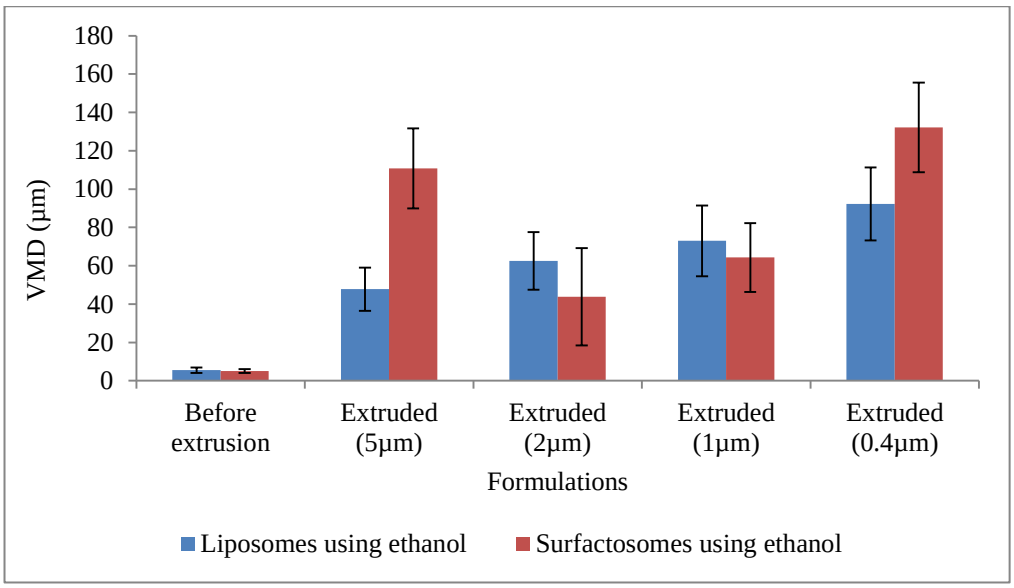

Figure 3.5 VMD of liposomes and surfactosomes made from solvent evaporation of ethanolic solution. Data are mean ±SD, n=3.

Size distribution study confirmed that formulations particularly surfactosomes exhibited aggregation or fusion. For instance, the span measurements of liposomes and surfactosomes prepared following evaporation of ethanol without extrusion were 4.07µm and 7.4µm respectively. These values are too high when compared to those measured using chloroform as lipid solvent as shown in Figure 3.6. When extruded with 5 µm polycarbonate membranes the span of liposomes was lowered to 1.9 and for surfactosomes it was reduced to 1.2, showing that extrusion through 5µm membranes has reduced the polydispersity of the formulations. However, on extrusion with 2µm polycarbonate membrane the span of liposomes was further reduced to 1.39 but for surfactosomes it was increased to 2.94. On further extrusion with 2µm and 1µm membranes the span of liposomes was 1.99 and 1.73 respectively. The span value of surfactosomes was 3.3µm and 4.38µm respectively. The surfactosomes had slightly but significantly larger span than liposomes ($p>0.05$). The standard deviation was very large which indicates that the surfactosomal span varied with every repetition performed.

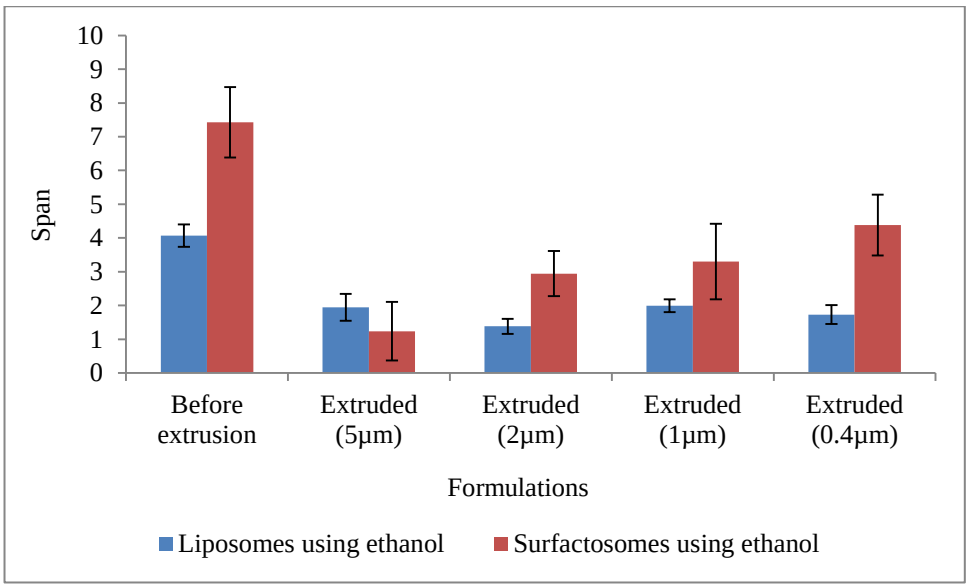

Figure 3.6 Span of liposomes and surfactosomes made from solvent evaporation of ethanolic solution. Data are mean ±SD, n=3.

It can be concluded that ethanol was not appropriate at making a thin lipid film for subsequent hydration into stable liposomes. Compared to chloroform, the use of ethanol increased the size of liposomes and surfactosomes significantly, indicating aggregation and/or fusion of the vesicles with highly variable VMD and span measurements amongst the different batches investigated; this is evident by the high standard deviations. Hence, chloroform as an organic solvent has shown to be more appropriate than ethanol in preparation of liposomes using the thin film method.

After studying the characteristics of liposomes and surfactosomes with regard to their VMD and span, it was concluded that ethanol is not suitable for the formation of thin films. Hence, further experiments employed chloroform in the preparation of liposomes and surfactosomes. It was also observed that there was no significance difference in the size of liposomes with and without cholesterol on using chloroform as solvent ($p>0.05$). Surfactosomes with cholesterol were slightly but significantly smaller than surfactosomes without cholesterol ($p<0.05$). Hence, inclusion of cholesterol can be considered useful in both liposomes and surfactosomes.

After VMD and size distribution investigations of empty liposomes and surfactosomes were conducted, drug entrapment studies were carried out. Salbutamol Sulphate (SBS), a model hydrophilic drug and beclometasone dipropionate (BDP), a model hydrophobic

drug were used to evaluate the influence of drug solubility on the entrapment in liposomes and surfactosomes.

To compare and study the characteristics of liposomes and surfactosomes, drug entrapment in these vesicles were studied using HPLC. These vesicles were also extruded through various polycarbonate membranes using the mini extruder to study the effect of stress and shearing on the drug retention.

3.3.4. Entrapment efficiency of SBS by liposome and surfactosomes with and without cholesterol

SBS entrapment was studied using HPLC and the results are shown in Figure 3.7. It was observed that there was no significant difference between the initial SBS entrapment efficiencies of all four formulations ($p>0.05$). The low entrapment of this drug in liposomes or surfactosomes may be due to the losses during the high rotational energy exerted during ultracentrifugation (Bendas and Tadros, 2007). It has been previously observed that the entrapment of hydrophilic drugs in liposomes is generally low (Taylor et al., 1990a, Shivhare et al., 2012). Stability of liposomes has been a concern since chemical decomposition of the lipid components and physical aggregation of the vesicles may alter the packing patterns of the bilayers, resulting in drug losses (Darwis and Kellaway, 2001). However, the entrapment values obtained in this study (Table 1) are higher than those found in other studies for the same drug using the thin film hydration method (Elhissi et al., 2006, Elhissi et al., 2007), which is possibly attributed to the two-step hydration protocol used in the present work.

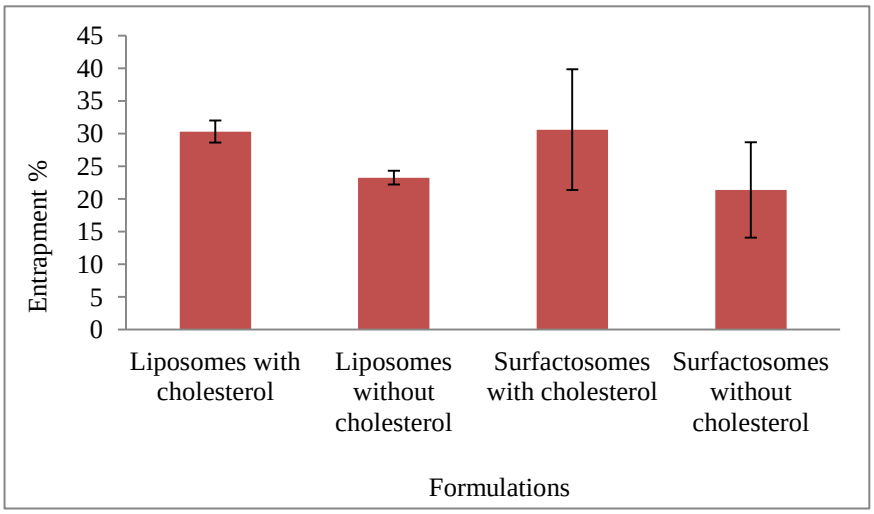

Figure 3.7 Entrapment of salbutamol sulphate in liposomes and surfactosomes before extrusion in the presence or absence of cholesterol. . Data are mean ±SD, n=3.

3.3.5. Drug loading of SBS in liposomes and surfactosomes

The drug loading of SBS in liposomes and surfactosomes were calculated. This was calculated to give the quantity of drug entrapped by 100mg of lipid. This study will help to analyse if formulation is economically feasible for drug entrapment. As shown in Figure 3.8 there was no significant difference in the SBS loading capacity in all four formulations ($p>0.05$). The highest loading of SBS was obtained using liposomes without cholesterol where the loading capacity was 2.38mg per 100mg of lipid.

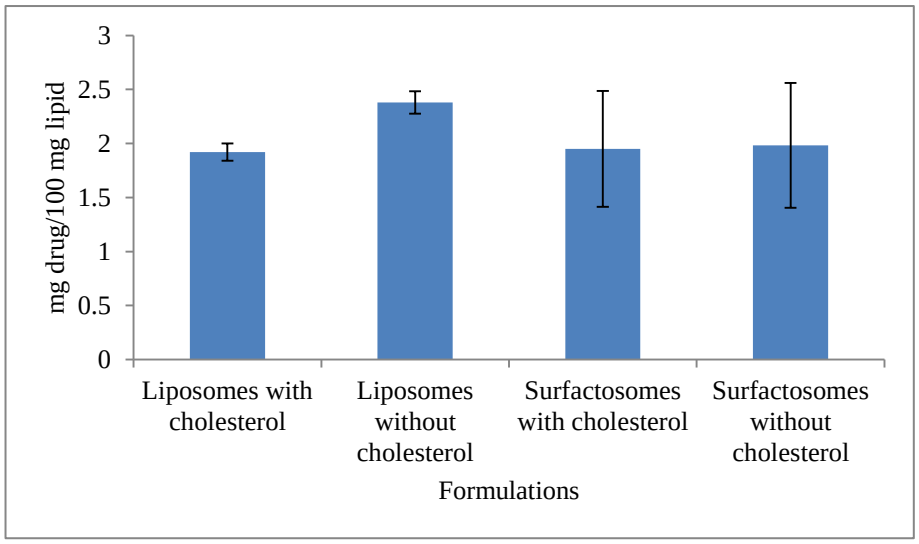

Figure 3.8 Drug loading of SBS in liposomes and surfactosomes. Data are mean ±SD, n=3.

3.3.6. Effect of extrusion on SBS entrapment in liposomes and surfactosomes prepared with or without cholesterol

The liposomes and surfactosomes entrapping SBS were separated by centrifugation and were re-suspended in fresh drug-free HPLC water. The resultant dispersions were then extruded using various polycarbonate membranes which are 5µm, 2µm, 1µm and 0.4µm. The stability of vesicles to the external stress was studied by measuring the drug entrapment following extrusion (Figure 3.9). It was observed that without any extrusion the drug leaked from both liposomes and surfactosomes. In surfactosomes, the proportion of drug leaked was greater than those using liposomes. Without any extrusion liposomes with cholesterol retained 88.6% while those without cholesterol retained 87.8% with no significant difference ($p>0.05$); suggesting that the large number of bilayers prior to extrusion has reduced the leakage of drug from the liposomes. By contrast, surfactosomes with cholesterol retained 54% whilst without cholesterol only 39% of the drug was retained with no significant difference ($p>0.05$); However the

slight increase in BDP retention in formulation with cholesterol indicates that inclusion of cholesterol has reduced the leakage of drug from the surfactosomes. Thus, although formulations were not extruded they leaked certain proportions of SBS in a magnitude that was dependent on formulation. This is because during annealing the liposomes/surfactosomes may undergo physical and chemical changes which may lead to vesicle alteration leading to drug leakage via "burst effect" (Darwis and Kellaway, 2001, Chandy and Sharma, 1996). When extruded through 5µm polycarbonate membranes, the SBS retention decreased further. Liposomes with cholesterol and without cholesterol retained 84.6 % and 72.9 % respectively with no significant difference ($p>0.05$); whereas surfactosomes with and without cholesterol retained 43.2 % and 17.8 % respectively with the formulation with cholesterol retaining significantly more than the one without cholesterol. When extruded with 2µm polycarbonate membranes, the percentage entrapment continued to decrease. Liposome with cholesterol and without cholesterol retained 79.4 % and 60 % of SBS respectively which is significantly different ($p<0.05$) whilst surfactosomes with and without cholesterol retained 33.9 % and 16 % respectively without any significant difference ($p>0.05$). When these vesicles were extruded through 1µm polycarbonate membrane, there was further loss of the drug. Liposomes with cholesterol and without cholesterol retained 73.7 % and 58 % of SBS respectively with no significant difference ($p>0.05$) and surfactosomes with and without cholesterol retained 27.1% and 12.47% respectively which has a significant difference ($p<0.05$). Finally these vesicles were extruded through 0.4µm polycarbonate membranes. The final entrapment of SBS in liposomes and surfactosomes further decreased. Liposome with cholesterol and without cholesterol retained 64.8 % and 45.1 % respectively with a significant difference ($p<0.05$), whereas surfactosomes with and without cholesterol retained 20.9 % and 12.11 % respectively with no significant difference ($p>0.05$).

From Figure 3.9 it can be observed that as the pore size of polycarbonate membrane was smaller, the SBS retention decreased. It can also be observed that liposomes with cholesterol retained greater proportions of the drug than liposomes without cholesterol. The same trend was observed for surfactosomes. This observation shows that cholesterol makes the vesicles more stable since the drug leakage was decreased. Both the vesicles without cholesterol lost significant amount of the drug after being extruded through 0.4µm as compared to vesicles with cholesterol. Cholesterol has been reported to improve the *in vivo* and *in vitro* stability of liposomes (Kirby and Gregoriadis, 1980).

Previous studies have shown that cholesterol helped the phosphotidylcholine (PC) vesicles to withstand shear stress (Tseng et al., 2007b). In the present study, it is shown that cholesterol enhanced the retention of drug in the vesicles. Moreover, it can also be observed that liposomes retained significantly greater drug proportions than surfactosomes ($p<0.05$). This suggests that liposomes have advantages over surfactosomes at entrapment of hydrophilic dugs like SBS. There is a significant decrease in the SBS entrapment percentage before extrusion and after extrusion though smallest pore size membrane used i.e. 0.4µm for all four formulations ($p<0.05$). The greater drug losses from surfactosomes upon extrusion might be attributed to the presence of surfactant which affected the bilayers packing and made them leakier. Surfactant increases the fluidity by increasing the gaps in bilayer through which SBS may leak to outside environment. Tween 80 may also increase the permeability of liposomes by interacting with the bilayers and affecting their packing (Young et al., 1983, Tasi et al., 2003)

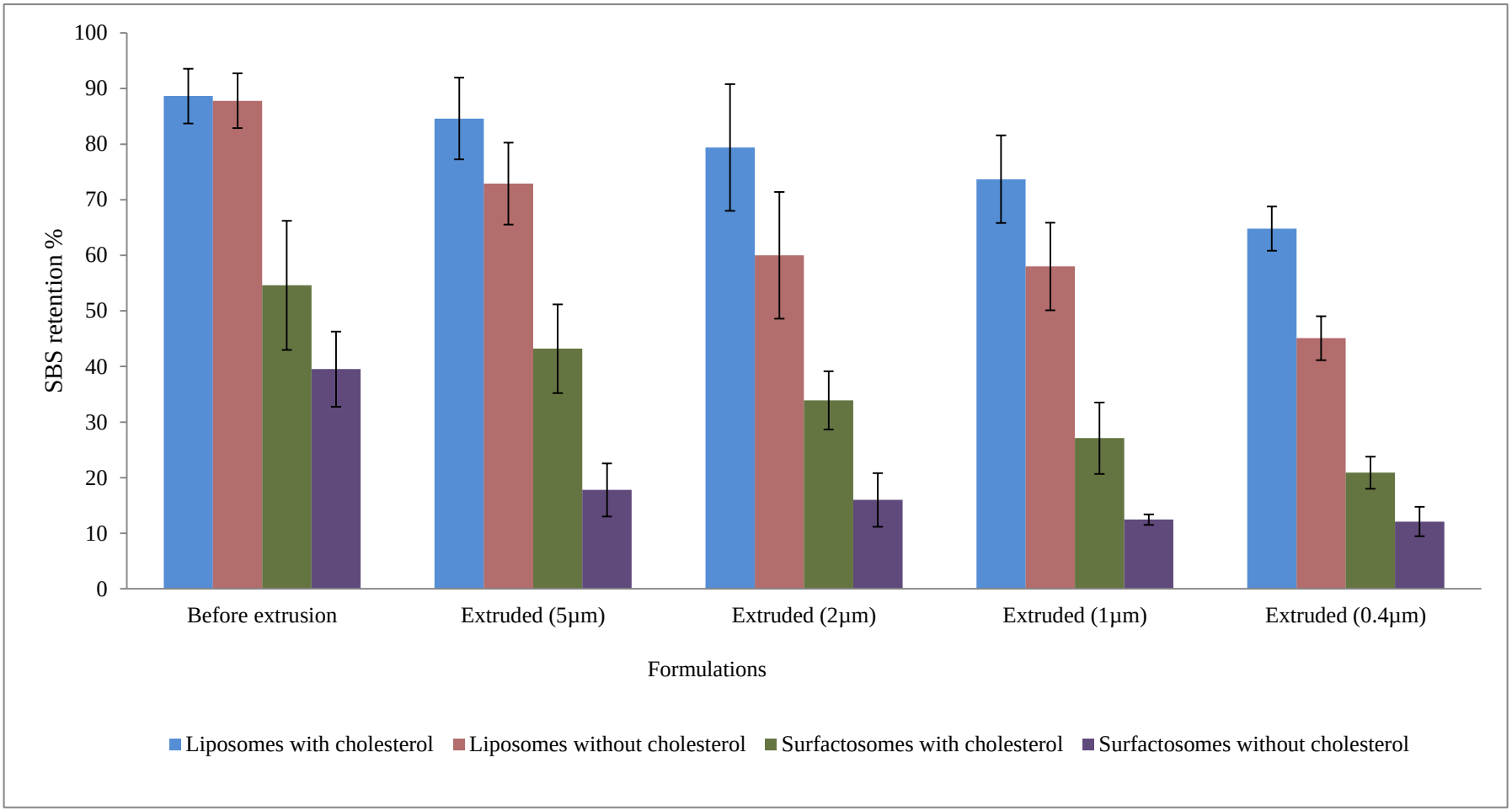

Figure 3.9 SBS retention in liposomes and surfactosomes with and without cholesterol before and after passing the vesicles through various sized polycarbonate membranes: 5µm, 2µm, 1µm and 0.4µm. In this experiment the originally entrapped liposomes and surfactosomes are re-suspended in fresh HPLC water. Data are mean ± SD, n=3.

Surfactosomes are more elastic than liposomes (Patel et al., 2009) which made the pressure required for extrusion very low. Surfactosomal formulations were forced though the polycarbonate membranes more easily with less effort in an air tight extruder. However, the leaky nature of surfactosomes indicates that they lack the ability to withstand pressure and tend to leak easily. The packing of phospholipid in transferosomal bilayers were possibly looser, resulting in larger pores for drug leakage from the vesicles. Inclusion of cholesterol enhanced the packing of the bilayers, hence, drug release decreased in vesicles with cholesterol. When being extruded, the internal pressure physically destabilised the surfactosomes more than liposomes forcing them to release the drug entrapped in their internal aqueous spaces.

Hence, these results suggest that for hydrophilic drugs like SBS liposomes with cholesterol are physically more stable than surfactosomes as they tend to retain greater drug proportions upon extrusion.

3.3.7. Stability of SBS entrapped liposomes and surfactosomes using excessive extrusion

The stability of liposomes and surfactosomes with or without cholesterol with SBS was studied. Formulations were extruded 51 times with the mini-extruded using 1µm polycarbonate membranes. This experiment was designed to simulate the shearing environment within the reservoir of a jet-nebuliser where the vesicles undergo stress multiple times during the generation of aerosols. From Table 3.1 it can be seen that when liposomes with or without cholesterol were extruded 51 times through 1µm polycarbonate membrane, liposomes with cholesterol retained significantly more SBS than liposomes without cholesterol ($p<0.05$). Similarly for surfactosomes, the vesicles with cholesterol retained significantly more drug than the vesicles made without cholesterol ($p<0.05$). It can also be observed that liposomes retained significantly more SBS than surfactosomes, thus, surfactants have contributed to make the vesicles less capable of retaining the hydrophilic drug originally entrapped ($p<0.05$).

It can also be observed that when the 1µm vesicles after separation of the unentrapped drug (i.e. with theoretically 100% drug entrapment efficiency) were again extruded using 1µm polycarbonate membrane, SBS retention was better than the extrusion of un-extruded sample. There was no significant difference in the drug retention in liposomes with and without cholesterol ($p>0.05$). Similarly in surfactosomes also there was no

significant difference in SBS retention by vesicles with and without cholesterol ($p>0.05$). However, the drug retention by surfactosomes was significantly low as compared to liposomes ($p<0.05$).

When un-extruded vesicles were extruded 51 times through 1µm membrane, the leakage of SBS was significantly more than the leakage of SBS after 51 times extrusion of previously extruded vesicles ($p<0.05$). This applies for all four formulations. Hence, it can be observed that leakage of SBS is significantly more in larger vesicles than from small vesicles after excessive extrusion. When a small vesicle is extruded it undergoes fragmentation leading to leakage of SBS. During fragmentation only the external bilayer is destroyed while the internal core still has drug and water. Hence the leakage is less. However when a large un-extruded vesicles is extruded the vesicle is cut through leading to leakage of all drug. Similar observation was concluded by Niven in his studies (Niven et al., 1991).

Hence it can be concluded that only small vesicles survive on extensive extrusion and size correlates to leakage for hydrophilic drug like SBS.

Table 3.1 The stability of liposomes and surfactosomes using excessive extrusion through 1µm polycarbonate membrane. Data are mean ±SD, n=3.

Formulations	**SBS retention after 51 times extrusion through 1µm membrane (%)**	**SBS retention after 51 times extrusion of previously extruded vesicles through 1µm membrane (%)**
Liposomes with cholesterol	60.1 ± 3.67	67.27 ± 1.86
Liposomes without cholesterol	45.06 ± 2.95	63.77 ± 1.65
Surfactosomes with cholesterol	14.6 ± 1.04	52 ± 1.17
Surfactosomes without cholesterol	6.6 ± 0.98	47.9 ± 3.3

Thus, it can be concluded that vesicles with cholesterol are more stable than the one without cholesterol for the retention of SBS. Liposomes are more stable than surfactosomes for SBS as sample drug. It can also be concluded that for hydrophilic drug like SBS smaller vesicles tend to retain more drug than larger vesicles after extensive extrusion.

To compare and study the characteristics of liposomes and surfactosomes, drug entrapment of these vesicles was studied using HPLC. These vesicles were also extruded through various polycarbonate membranes using the mini-extruder to study the effect of stress and shearing on the retained drug entrapment in liposomes.

3.3.8. Entrapment of BDP by liposomes and surfactosomes with and without cholesterol

To study the entrapment of BDP, deuterated water (D_2O) was used instead of HPLC water. This is because on using HPLC water, BDP being insoluble in water was sedimented in the bottom of centrifuge tube upon centrifugation along with liposomes/surfactosomes-entrapped BDP. This made it impossible to separate unentrapped BDP from liposome-entrapped BDP. Hence, high density water (D_2O) was used to separate the unentrapped BDP from the liposome-entrapped fraction of the drug. In this case, upon centrifugation, liposomes containing the entrapped drug will float at the top whilst the unentrapped (free) drugs will sediment as crystals (spot) at the bottom of the tube. The middle clear aqueous layer between the floating layer and the crystal spot adds to the fraction of unentrapped drug (Figure 3.10).

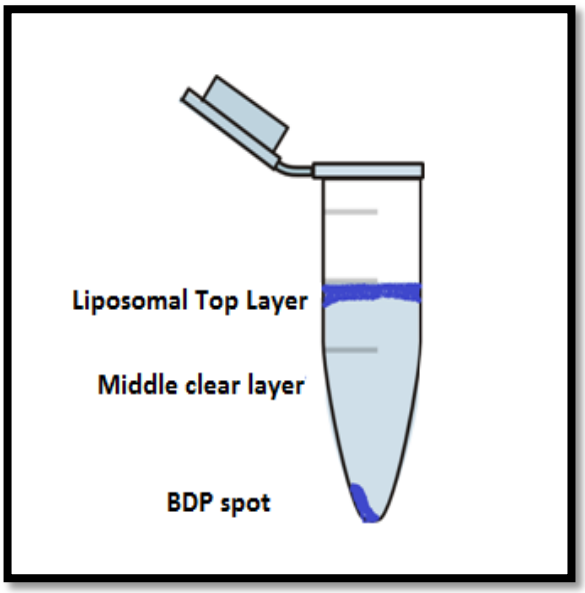

Figure 3.10 The three layers formed in the Eppendorf tube with D_2O upon centrifugation.

3.3.9. Microscopic analysis of the creamy veicular layer and BDP spot in the eppendorf tube

It was observed that in an eppendorf tube used for centrifugation, 3 layers were formed. These included the top thick layer containing liposomes/surfactosomes with entrapped BDP, a middle clear layer with unentrapped BDP and a spot at the bottom with BDP crystals (Figure 3.10). This was concluded after a microscopic analysis of all two layers in the eppendorf after 90 min centrifugation at 13,000 rpm and 15,300 relative gravity (Figure 3.11 and 3.12). BDP tends to crystallise due the incompatible steric fit between the steroid and the liposome bilayers, resulting in formation of large amounts of BDP crystals on storage (Batavia et al., 2001, Radhakrishnan, 1991). This crystallisation further leads to minimised incorporation of this drug into the bilayers, hence drug entrapment is reduced.

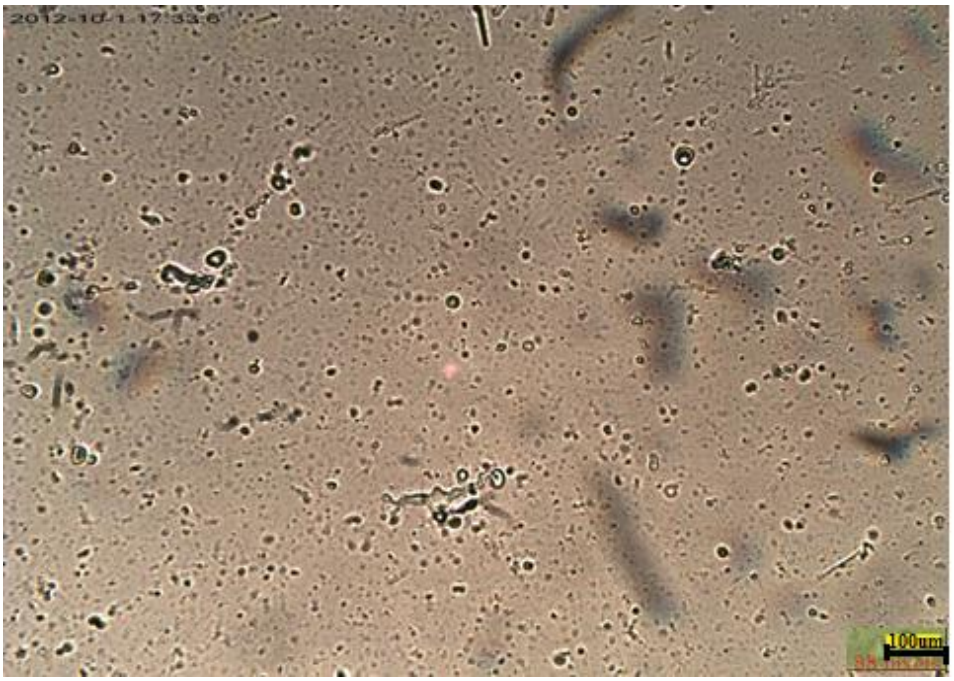

Figure 3.11 A photograph showing liposomes suspended in the top layer of an eppendorf after 90 min centrifugation observed under 40X magnification. This is typical of 3 such different experiments.

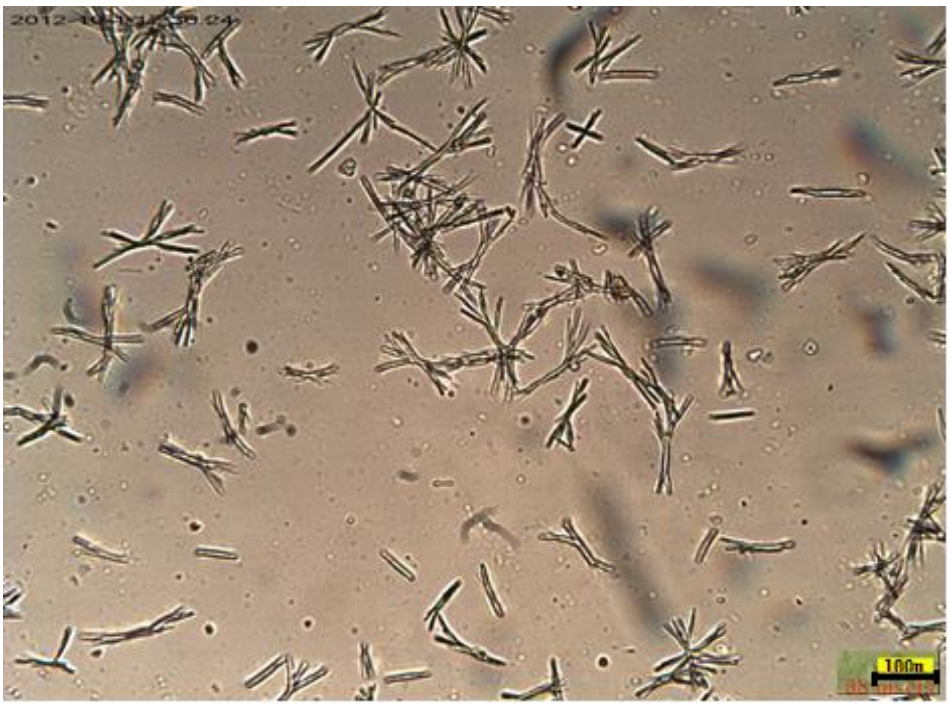

Figure 3.12 A photograph showing BDP crystals deposited in the bottom of centrifuge tube after 90min centrifugation observed under 40X magnification. This is typical of 3 such different experiments.

3.3.10. Stewart assay

Stewart assay was performed to analyse the quantity of phospholipid (SPC) present in each layer after centrifugation. Figure 3.13 and 3.14 graphically represent the amount of lipid in each layer of liposome and surfactosome, respectively.

From Figure 3.13 and 3.14 it was possible to observe that the top layer (i.e. the layer containing liposomal suspension) had maximum amount of lipid. Thus, this top layer contained almost 93% of liposomes and 94% of surfactosomes in the centrifuged eppendorf. This confirmed that separation was highly efficient. Hence, by conducting HPLC of BDP from the top layer the drug entrapment percentage can be calculated. The middle layer had around 5.5% of the lipid and 2.3% in case of surfactosomes. As 5.5% and 2.3% was minimal amount of phospholipid, middle layer can be considered for calculating unentrapped BDP. The sedimented spot was confirmed to be mainly made of BDP crystals since it had less than 3% of the total lipid used. Hence, no appreciable presence of liposomes/surfactosomes was detected in the bottom spot.

The amount of lipid in top layer is significantly more than lipid present in middle layer and spot sediment. Thus, Stewart assay made has confirmed that top layer contained

most of the vesicles; hence BDP present in this layer was quantified to calculate the entrapped drug proportion, while the middle layer and BDP spot were used to calculate the unentrapped proportion of the drug.

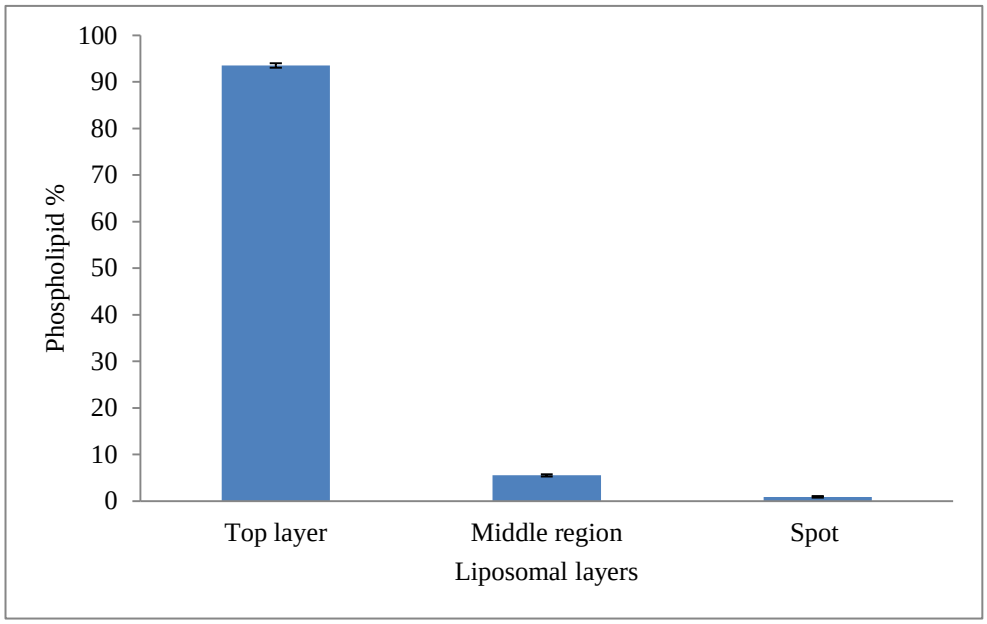

Figure 3.13 Lipid present in each liposomal layer formed after centrifugation. Data are mean ± SD, n=3; for middle layer and spot compared to top layer

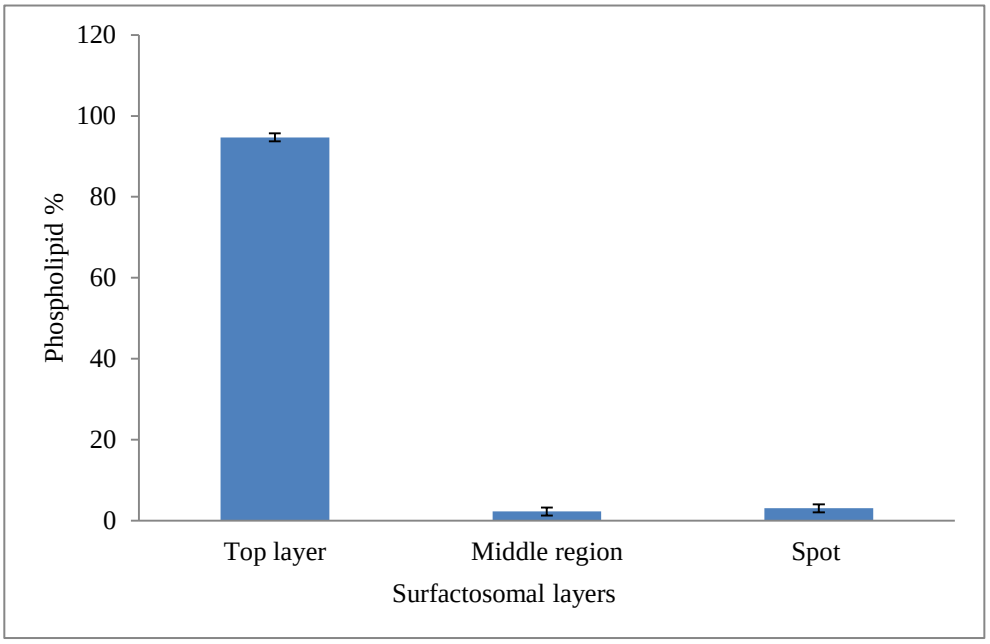

Figure 3.14 Lipid in each surfactosomal layer formed after centrifugation. Data are mean ± SD, n=3; for middle layer and spot compared to top layer

3.3.11. Entrapment of BDP by liposomes and surfactosomes with or without cholesterol

BDP entrapment was studied using HPLC and results were presented in Figure 3.15. It was observed that BDP entrapment in liposomes and surfactosomes with cholesterol was 31.6% and 30.6% respectively while those in liposome and surfactosome without cholesterol was 24.6% and 22.6% respectively. It was observed that there is no significant difference between the entrapment of BDP by all four vesicles ($p>0.05$). The difference in drug entrapment as a result of including cholesterol in the formulations was not statistically significant ($p>0.05$).

This low entrapment is due to the geometric structure of BDP molecules which has possibly prevented them from robustly fitting within tahe lipid bilayers of liposomes and surfactosomes. It is also believed that some BDP may interact with lipid headgroups to form complexes (Darwis and Kellaway, 2001). Steroid drugs have limited solubility in phospholipid, hence the maximum entrapment is low (Fildes and Oliver, 1978). Hence, perhaps there is initial rapid release of BDP leading to low drug entrapment. BDP also tends to crystallise in liposomal formulations due to the incompatible steric fit of the drug with the lipid bilayers (Batavia et al., 2001). Hence, using light microscopy, large amounts of this crystalline steroid were observed after storage or upon centrifugation. Hence, cholesterol is important for providing stability and rigidity to the vesicle, thus reducing drug leakage from liposomes (Kirby et al., 1980). The relatively low drug entrapment may also be due to centrifugation, physical and chemical instability of liposomes and surfactosomes.

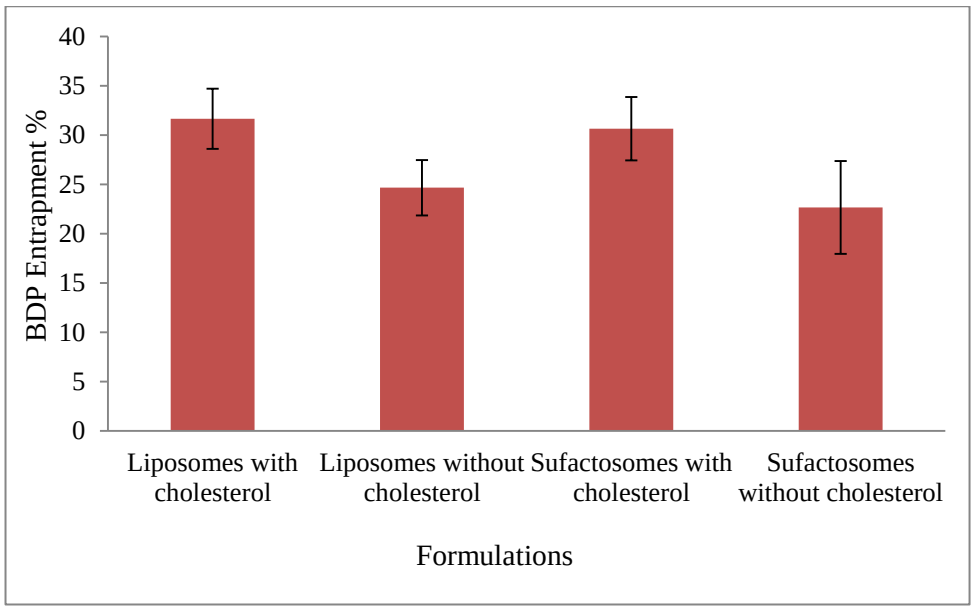

Figure 3.15 Initial entrapment of BDP in liposomes and surfactosomes in the presence and absence of cholesterol. Data are mean ± SD, n=3.

3.3.12. Drug loading of BDP in liposomes and surfactosomes

The drug loading of BDP in liposomes and surfactosomes was calculated as the quantity of drug present in 100mg of lipid. This study will help to analyse if formulations are economically feasible for drug entrapment. As shown in Figure 3.16 there is no significant difference in the BDP loading efficiency in all four formulations ($p>0.05$). The liposomes with cholesterol have the maximum loading efficiency of 0.43mg of drug per 100mg of lipid.

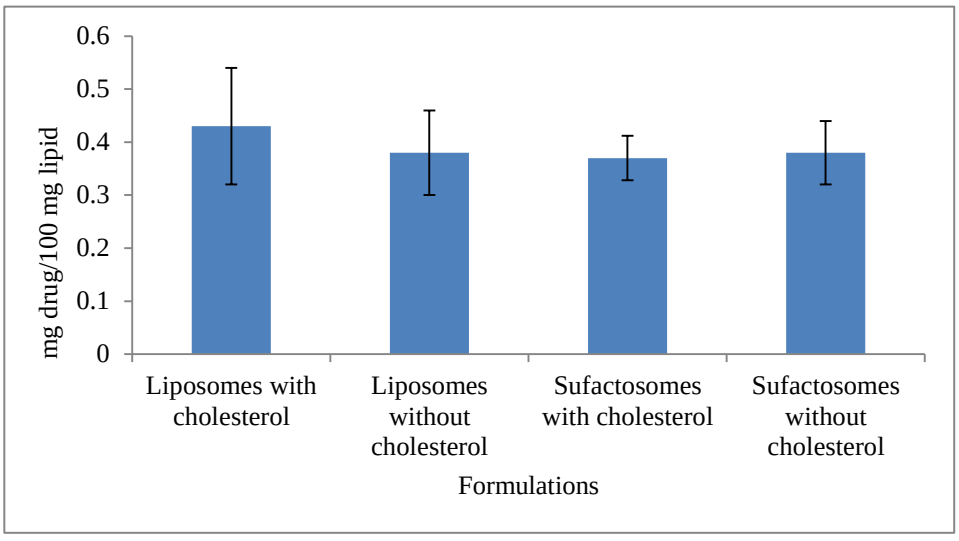

Figure 3.16 Drug loading of BDP in liposomes and surfactosomes. Data are mean ±SD, n=3.

3.3.13. Saturation solubility of BDP in deionised water and Tween 80 solution

Saturation solubility of BDP in water and Tween was studied. As shown in Table 3.2, solubility of BDP in water was 0.14µg/ml and in tween 80 solution (15:85 Tween 80: water v/v) was 12.67µg/ml. This shows that in presence of Tween 80 greater quantities of BDP became soluble. This means that formulations with surfactosomes had greater proportions of BDP solubilised in water and hence, less incorporation in the lipid bilayers was offered. This is thought to be responsible for the lower entrapment efficiency of BDP in surfactosomes compared to liposomes.

Table 3.2 Table showing the solubility of BDP in water and Tween 80

Solubility of BDP in water	0.14µg/ml
Solubility of BDP in water + Tween 80	12.67µg/ml

3.3.14. Effect of extrusion and cholesterol incorporation on drug entrapment in liposomes and surfactosomes

The liposomes and surfactosomes entrapping BDP were separated from the dispersed D_2O via centrifugation and were re-suspended in fresh D_2O. The dispersions were then extruded using polycarbonate membranes with pore size 5µm, 2µm, 1µm and 0.4µm. Their reactions to this external stress caused by extrusion and the resultant retained entrapment of the drug were presented in Figure 3.17. It was observed that without any extrusion the drug leaked slightly from both liposomes and surfactosomes (Figure 3.17). Without extrusion the drug entrapment in liposomes with cholesterol and without cholesterol was 92.3% and 88% respectively. Similarly for surfactosomes with cholesterol and without cholesterol it was 89.33% and 83% respectively. There was no significant difference in BDP retention between all four vesicles ($p>0.05$). For the non-extruded formulations it was expected that since no stress was applied, they separated vesicles should retain almost 100% of the originally entrapped BDP. However, this was not the case, possibly because the liposomes/surfactosomes have undergone physical changes during re-dispersion in D_2O, leading to alterations in the bilayer properties and subsequent drug leakage. It has been previously reported that physical alterations in the liposome bilayers may cause the entrapped drug to leak (Darwis and Kellaway, 2001). When extruded with 5µm polycarbonate membranes, the percentage entrapment

decreased further as a result of some vesicle disruption and forced drug leakage while "squeezing" the vesicles through the membrane filters. Liposomes with cholesterol and without cholesterol retained 88.33 % and 73.66 % BDP respectively with a significant difference between both the formulations ($p<0.05$); whereas surfactosomes with and without cholesterol retained 80.66% and 78.66% BDP respectively with no significant difference ($p>0.05$). When extruded through 2µm polycarbonate membranes, the entrapment efficiency decreased further. Liposome with cholesterol and without cholesterol retained 82.66% and 67.66% of BDP respectively with a significant difference between both the formulations ($p<0.05$); whereas surfactosomes with and without cholesterol retained 74.66% and 73.33 % respectively with no significant difference ($p>0.05$). When these vesicles were extruded through 1µm polycarbonate membranes, there was further loss of the drug. Liposomes with cholesterol and without cholesterol retained 75% and 60.6% of BDP respectively with a significant difference between both the formulations ($p<0.05$); whereas surfactosomes with and without cholesterol retained 71% and 69.3% respectively with no significant difference ($p>0.05$). Finally these vesicles were extruded through 0.4µm polycarbonate membrane. The entrapment of BDP in liposome and surfactosomes continued to decrease. Liposome with cholesterol and without cholesterol retained 68% and 56% with a significant difference between both the formulations ($p<0.05$); whereas surfactosomes with and without cholesterol retained 63.3 % and 62.66 % respectively with no significant difference ($p>0.05$).

In Figure 3.17 it can be observed that as the pore size of polycarbonate membranes used was smaller, the BDP retention decreased. Moreover after statistical analysis it can be observed that liposomes with cholesterol retained significant more drug than cholesterol-free liposomes after extrusion through all pore sizes ($p<0.05$). This, however, was not a trend for surfactosomes since cholesterol did not significantly enhance the retention of the drug, suggesting that the presence of the surfactant in surfactosomes was responsible for counteracting the drug retention effect caused by cholesterol.

The drug leakage in surfactosomes with cholesterol was slightly more compared to liposomes with cholesterol ($p>0.05$). This may be due to the reduced ability of surfactosomes to withstand the stress provided by the extruder or more likely the leakage rate of the drug from the bilayers was higher owing to the presence of surfactant in the surfactosomes. It was easier to extrude surfactosomes as compared to liposomes

because of their elasticity, but this, however, did not protect the drug from leakage. It is also possible that free Tween 80 molecules were present in the continuous phase of the surfactosomal formulation resulting in encouraging the steroid drug to partition between the bilayers and the continuous phase; this proposed behavior of the drug seemed to be further promoted by the stress exerted on the vesicles via extrusion. There is a significant decrease in the BDP entrapment before extrusion and after extrusion though smallest pore size membrane used i.e. 0.4µm in all formulations. This suggests that extrusion via small membrane leads to significant BDP leakage from vesicles.

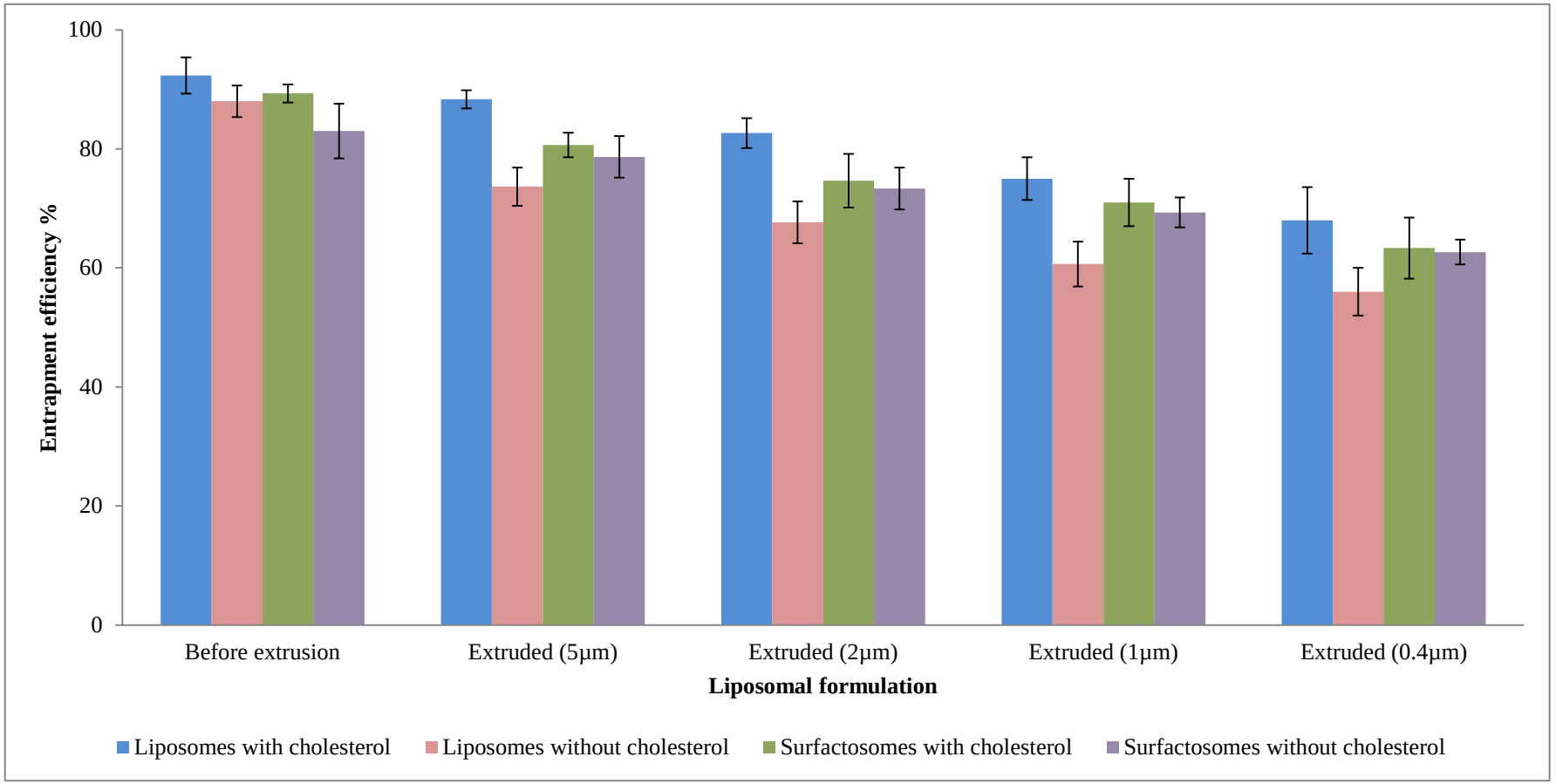

Figure 3.17 BDP retention in liposomes and surfactosomes with and without cholesterol before extrusion and after passing through various sized polycarbonate membranes like 5µm, 2µm, 1µm and 0.4µm. Here the originally entrapped liposomes and surfactosomes vesicles were re-suspended in fresh D_2O. Data are mean ± SD, n=3.

3.3.15. Stability of BDP entrapped in liposomes and surfactosomes using excessive extrusion

Here, the stability of liposomes and surfactosomes with or without cholesterol, using BDP as model drug was studied. Formulations were extruded 51 times using 1µm polycarbonate membranes. This was expected to replicate the environment in nebuliser chamber where the vesicles undergo stress multiple times and are subjected to excessive shearing. From Table 3.3 it can be seen that when the liposomes with and without cholesterol was extruded 51 times, liposomes incorporating cholesterol retained significantly greater BDP proportions than liposomes prepared without cholesterol ($p<0.05$). For surfactosomes, there was no significant difference between the BDP entrapment regardless of cholesterol incorporation in the formulation ($p>0.05$). It was observed earlier that liposomes retained significantly more SBS than surfactosomes, thus, liposomes are physically more stable candidate carriers compared to surfactosomes ($p<0.05$).

It can be observed that when the 1µm vesicles after separation of the unentrapped drug (i.e. with theoretically 100% drug entrapment efficiency) were again extruded using 1µm polycarbonate membrane, BDP retention was better than the extrusion of un-extruded sample. There was no significant difference in the drug retention in liposomes containing cholesterol compared to liposomes free of cholesterol ($p>0.05$). Surfactosomes with cholesterol retained significantly more BDP than the one without cholesterol ($p<0.05$). The drug retention by surfactosomes was significantly lower than retained entrapment of the drug in liposomes ($p<0.05$).

Thus, it can be concluded that vesicles with cholesterol are physically more stable than the ones without cholesterol. Liposomes retained significantly more BDP than surfactosomes. It is found similar to results with SBS as discussed in chapter 3 section 3.3.7. Hence, it is presumed that liposomes would be more capable of tolerating the shear force generated by nebulisers. This is investigated in further chapters of thesis using nebulisers instead of mini-extruder.

Table 3.3 The stability of liposomes and surfactosomes after excessive extrusion. Data are mean ±SD, n=3.

Formulations	BDP retention after 51 times extrusion through 1µm membrane	BDP retention after 51 times extrusion of previously extruded vesicles through 1µm membrane
Liposomes with cholesterol	65.8 ± 1.79	87.13 ± 1.8
Liposomes without cholesterol	55.27 ± 2.97	84.1 ± 1.1
Surfactosomes with cholesterol	55.3 ± 3.08	78.47 ± 1.45
Surfactosomes without cholesterol	49.5 ± 2.36	71.03± 2.15

3.3.16. Comparison between liposomes and surfactosomes for hydrophilic and lipophilic drug

As discussed in section 3.3.7 it can be concluded that liposomes are potentially better option than surfactosomes to deliver hydrophilic drugs like SBS to the lungs. Liposomes with cholesterol can help in maximising the retention of entrapped drug with minimum drug loss even if pressure is applied on the vesicles. As discussed in section 3.3.14, it can be concluded that liposomes are better than surfactosomes for retention of lipophilic drugs like BDP. For surfactosomes, the entrapment efficiency was much higher for the lipophilic drug compared to the hydrophilic drug. Moreover, drug leakage was much less under pressure in surfactosomes when using the lipophilic drug. This may be due to the encapsulation of the lipophilic (hydrophobic) drug in the liposomal bilayers instead of the aqueous core (Figure 3.18). Hydrophobic drugs are expected to be associated with the hydrocarbon chain region of the lipid molecule (Batavia et al., 2001). It is possible that the presence of Tween 80 in the surfactosomes has promoted the fluidity of the vesicle bilayers, resulting in enhanced localisation the steroid with the lipid composition of the vesicles. Hence, even after the fragmentation of surfactosomes/liposomes during extrusion, upon reassembly the drug can possibly associate itself within the bilayers of the downsized vesicles. However, in case of using the hydrophilic drug, fragmentation of the vesicles leads to the loss of drug which

cannot be again re-entrapped in the liposome aqueous spaces, rather the drug was present in the continuous aqueous phase outside the vesicles.

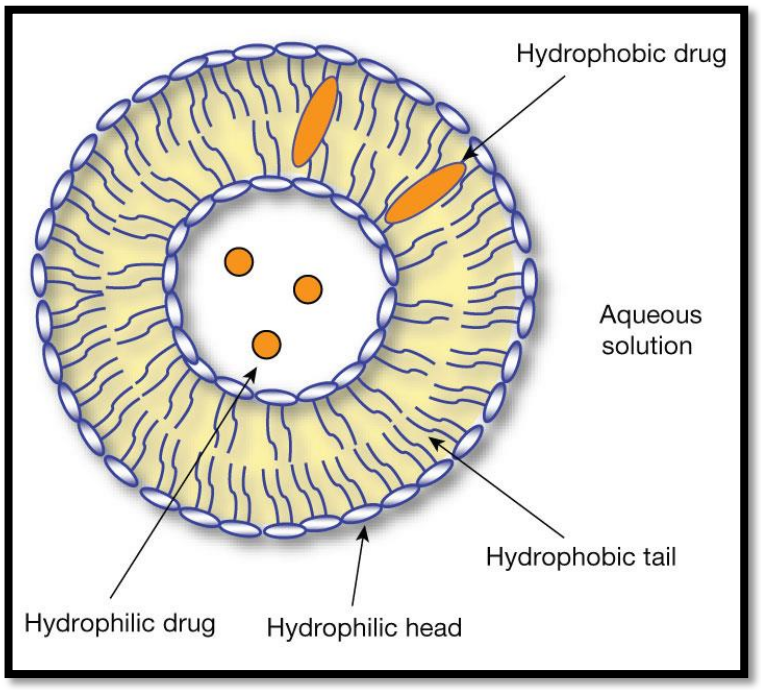

Figure 3.18 Structure of a liposome and schematic representation of drug incorporation (Lembo and Cavalli, 2010)

3.4. Conclusions

After observing the VMD and span of liposomes and surfactosomes, it was decided to carry out the subsequent studies using chloroform rather than ethanol. Evaporation of ethanol to dissolve the lipids resulted in formation of lipid films that were difficult to hydrate, leading to aggregation or fusion of the vesicles, which, due to their large measured size, are unlikely to be appropriate for pulmonary drug delivery. It was also observed that there was no significance difference in the size of liposomes with or without cholesterol. Surfactosomes with cholesterol were slightly but significantly smaller than surfactosomes without cholesterol.

After observing the behavior of liposomes and surfactosomes for drug entrapment, it was concluded that there was no significant difference in the SBS entrapment efficiencies of all four formulations. It can be concluded that liposomes with cholesterol are better for entrapping hydrophilic drugs as compared to surfactosomes. However, the use of surfactosomes resulted in excessive drug leakage when they were extruded with

5, 2, 1 and 0.4µm sized membrane in Avestin mini-extruder. The retention of SBS was better in liposomes than surfactosomes after passing through stress. After studying the loading efficiencies it was concluded that there was no significant difference between the loading efficiencies of all four formulations. On excessive extrusion (51 times) of all four vesicles through 1µm membrane, it was concluded that vesicles with cholesterol are more stable than the one without cholesterol for the retention of SBS. Liposomes are more stable than surfactosomes for SBS as sample drug. It can also be concluded that for hydrophilic drug like SBS smaller vesicles tend to retain more drug than larger vesicles after extensive extrusion.

For lipophilic drug BDP it was found that, there was no significant difference in the initial BDP entrapment efficiencies of all four formulations. Both surfactosomes and liposomes were appropriate as they both retained significant drug even when they were placed under the pressure of extrusion through 5, 2, 1 and 0.4µm sized membrane in Avestin mini-extruder. After studying the loading efficiencies it was seen that there was no significant difference between the loading efficiencies of all four formulations. On excessive extrusion (51 times) of all four vesicles through 1µm membrane it was concluded that vesicles with cholesterol are physically more stable than the ones without cholesterol. Liposomes retained significantly more BDP than surfactosomes. Hence, it is presumed that liposomes would be more capable of tolerating the shear force generated by nebulisers. It can also be concluded that smaller vesicles tend to retain more drug than larger vesicles after extensive extrusion.

Hence in this chapter it is concluded that chloroform is a better solvent for lipids than ethanol while preparing liposomes and surfactosomes. It can also be concluded that for hydrophilic drug like SBS, liposomes are better than surfactosomes for drug entrapment and retention. For hydrophobic like BDP both liposomes and surfactosomes are equally good for drug entrapment and retention. Moreover, cholesterol is an important component to be incorporated in both types of vesicles as they may increase the stability and decrease the drug losses during extrusion. Loading efficiencies of all four formulations are similar for SBS and BDP. Liposomes are better than surfactosomes for retaining more drugs (both SBS and BDP) after excessive extrusion. Smaller vesicles retain more drugs after undergoing extensive extrusion than larger vesicles

Chapter 4

Entrapment studies of SBS and BDP for proliposomes and prosurfactosomes using particulate based proliposome technology

4.1. Introduction

Liposomes made using thin film method were studied in the previous chapter. Thin film method is not appropriate for manufacturing liposomes on large scale. In addition to this, aqueous suspensions of liposomes may be subject to a variety of instability manifestations like aggregation, fusion and phospholipid hydrolysis which limit their stability. Hence an alternative approach to preparing liposomes was introduced by Payne et al in 1986; this was referred to as proliposome technology (Payne et al., 1986a). This approach has been suggested as a convenient alternative to freeze- drying and spray drying. Ethanol based (Perrett et al., 1991) and particulate based proliposomes are the two types of proliposomes (Payne et al., 1986a) described in literature. In particulate based proliposome technology a carbohydrate carrier like sucrose, mannitol or lactose was used as core carrier particles to be coated with phospholipid. Addition of aqueous phase and shaking causes instant dissolution of the carbohydrate carrier and generation of liposomes.

In this study, particulate based proliposomes were made using soya phosphotidylcholine (SPC), cholesterol (optional) and drug. Salbutamol sulphate (SBS) was used as a model hydrophilic drug and beclometasone dipropionate (BDP) was used as model hydrophobic drug. In this study a new type of vesicles called prosurfactosomes was prepared. In the preparation of prosurfactosomes, the surfactant Tween 80 was included within the lipid components. On hydration of prosurfactosomes, surfactosomes were formed which are presumed to be more elastic than conventional liposomes. Liposomes and surfactosomes prepared from proliposomes and prosurfactosomes respectively were studied for their VMD, size distribution (i.e. span), zeta potential (i.e. surface charge) and drug entrapment. Moreover, retention of the drug in liposomes after extrusion was investigated. Vesicles with different concentrations of cholesterol were considered. TEM was used to analyse the shape and lamellarity of liposomes and surfactosomes prepared from proliposomes and prosurfactosomes.

4.2. Methods

4.2.1. Preparation of proliposome

SPC and Cholesterol were used in 1:1 and 2:1 mole ratio. The lipid phase was dissolved in chloroform (20mg/ml) within a round bottom flask as described in Chapter 2 section 2.2.2. The carbohydrate carrier particles of mannitol were added to the lipid phase in 1:5 w/w and 1:10 w/w for SBS and BDP formulations respectively. The organic solvent was removed using a rotary evaporator under vacuum for 1h with the flask being partially immersed in a water bath (38ºC at maximum) and a rotation speed of 280rpm. After releasing the vacuum and detaching the flask from the rotary evaporator, the proliposomes were collected using a clean spatula and then stored in room temperature to be used on the same day. The proliposomes were hydrated by addition of isotonic NaCl solution (0.9%) for salbutamol sulphate (5mg/ml) and deuterium oxide (D_2O) for beclometasone dipropionate (BDP) (15mg/ml). Drug was incorporated into the formulations as described in chapter 2 section 2.2.5.1 and 2.2.5.2.

4.2.2. Preparation of prosurfactosomes

Lipid phase (SPC and cholesterol, 1:1 or 1:2 mole ratio) along with Tween 80 (15% w/w of the total lipid used) were loaded into a round bottom flask. These components were dissolved in chloroform (20 mg/ml) within the round bottom flask. Carbohydrate carrier particles of mannitol were added to the lipid phase in 1:5 and 1:10 w/w ratio for SBS and BDP formulations respectively. The organic solvent was evaporated and the resultant thin film was hydrated by addition of isotonic NaCl solution (0.9%) for salbutamol sulphate (5mg/ml) and deuterium oxide (D_2O) for beclometasone dipropionate (BDP) (15mg/ml). Drug was added as described in chapter 2 section 2.2.5.1 and 2.2.5.2.

4.2.3. Analysis of cholesterol in BDP spot

HPLC was used to analyse the concentration of cholesterol present in the BDP spot in the eppendorf after centrifugation. All samples for HPLC were diluted in a mixture of methanol before analysis. Methanol (HPLC-grade) was used as the mobile phase. HPLC instrument was set up using C18 column (HPLC column Eclipse XDB-C18, 4.6 x 50mm, Agilent, UK). The mobile flow rate was 1.5ml/min with a sample injection

volume of 20µl and UV detection at 207 nm. The assay was validated by using a calibration curve made by using solutions of different known concentrations of BDP.

4.2.4. Extrusion of formulations

Avestin Liposofast mini-extruder was used to extrude the liposome/ surfactosomes samples through polycarbonate membranes having pore sizes 5µm and 2µm. (Nucleopore Track-etched membranes). The sample was passed through 5µm and 2µm membrane 11 times. All samples were first passed through 5µm membrane and then they were passes through the 2µm membranes. This technique may minimise drug loss as well as reduce the physical labour-force needed for extrusion.

4.3. Results and discussion

4.3.1. VMD (size) and span (size distribution) of SBS entrapped liposomes and surfactosomes.

VMD of SBS entrapped liposomes and surfactosomes prior to extrusion were analysed using Malvern Mastersizer as shown in Figure 4.1. Formulations with cholesterol had 1:1 molar ratio of cholesterol and SPC. Size of liposomes and surfactosomes with cholesterol was around 5.92µm and 5.93µm respectively. By contrast, VMD of liposomes and surfactosomes without cholesterol was around 6.32µm and 6.05µm respectively. It was observed that there was no significant difference between the VMD of vesicles ($p>0.05$).

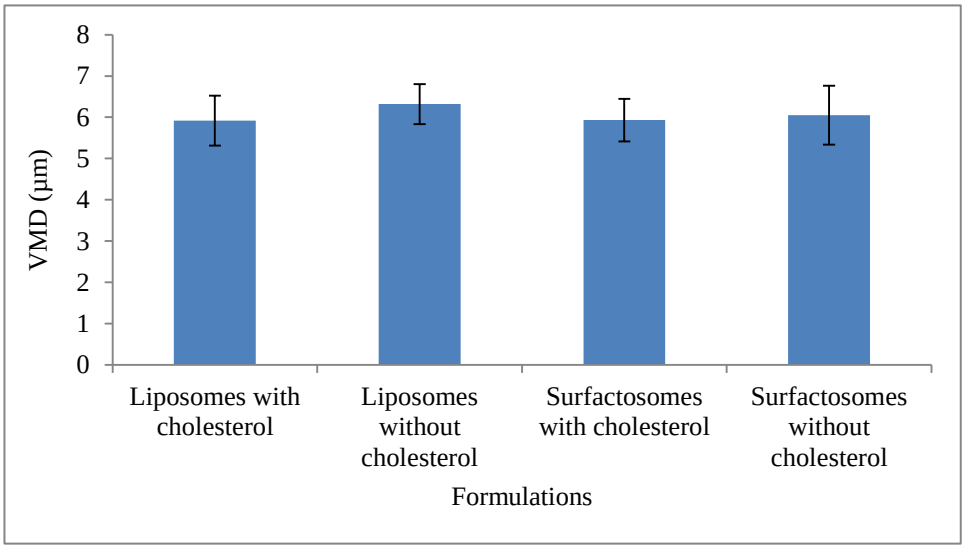

Figure 4.1 VMD of SBS entrapped liposomes and surfactosomes with (1:1 molar ratio) and without cholesterol. Data are mean ±SD, n=3.

Span of SBS entrapped liposomes and surfactosomes prior to extrusion were analysed using Malvern Mastersizer as shown in Figure 4.2. Formulations with cholesterol had 1:1 molar ratio of cholesterol and SPC Span of liposomes and surfactosomes with cholesterol was around 2.03 and 1.5 respectively. By contrast, span of liposomes and surfactosomes without cholesterol was around 1.96 and 1.94 respectively. It was observed that there was no significant difference between the span of vesicles for formulations used in this study ($p>0.05$).

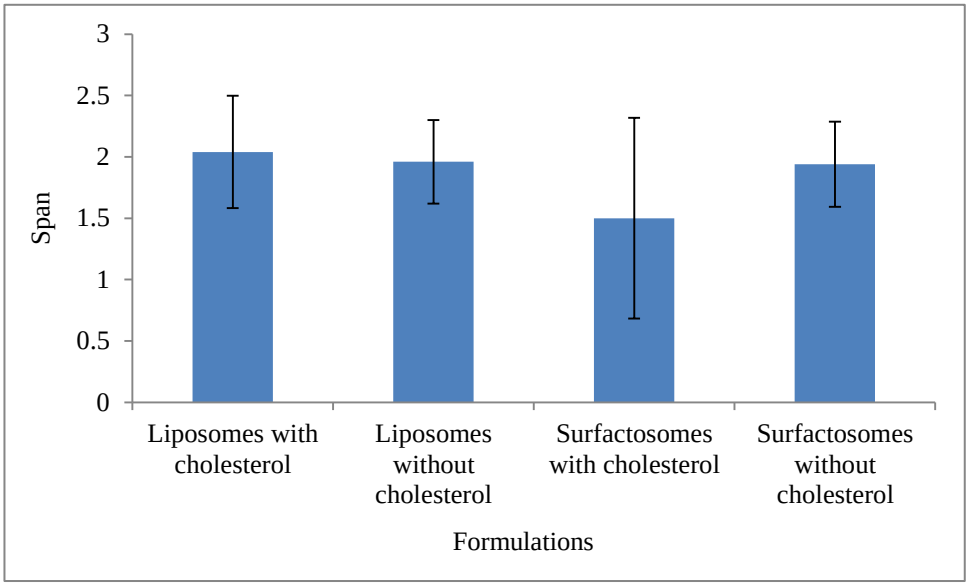

Figure 4.2 Span of SBS entrapped liposomes and surfactosomes with (1:1 molar ratio) and without cholesterol. Data are mean ±SD, n=3.

4.3.2. Zeta potential of SBS liposomes and surfactosomes.

Zeta potential (i.e. surface charge) of SBS liposomes and surfactosomes prior to extrusion were analysed using Malvern Zetasizer as shown in Figure 4.3. The zeta potential values of liposomes and surfactosomes with cholesterol was around -1.77mV and -3.25mV respectively. It can be observed that surface charge of surfactosomes were significantly more negative than that of liposomes ($p < 0.05$) (Figure 4.3). Charge of liposomes and surfactosomes without cholesterol were around -1.85mV and -2.28mV respectively, with no significant difference between the two formulations ($p>0.05$). Surfactosome with coleaterol appeared to have more negative zeta potential than liposomes. The presence of Tween 80 seems to increase the negative surface charge of vesicle. Sorbitan esters, polyoxyethelene delivatives, are fatty acid esters of sorbitol and its anhydrides copolymerised with a varying number of moles of ethylene oxide.

Polysorbate 80 (Tween 80) is an oleate ester (Remington et al., 2006, Rowe et al., 2009). There is a possibility of by products like free fatty acids like linoleic acid, palmitic acid and stearic acid to be present as impurities as verified from Sigma Aldrich, UK. It is possible that these acids on dissociation on the surface of a particle gave rise to a negatively charged surface as discussed in section 1.7. Similar observation of increase in negative charge of solid lipid nanoparticle on addition of Tween 80 in the formulation was found by Prabhakar *et al.* in 2013 (Prabhakar et al., 2013). Hence, surfactosomes are significantly more negative than liposomes.

Zeta potentials of both liposomes and surfactosomes are tending towards zero with very minor differences in the negativity of formulations although statistic disagrees with it. It is stated that a higher absolute value of zeta potential indicates a more stable suspension and lower value indicates colloid instability, which could lead to aggregation of nanoparticles.

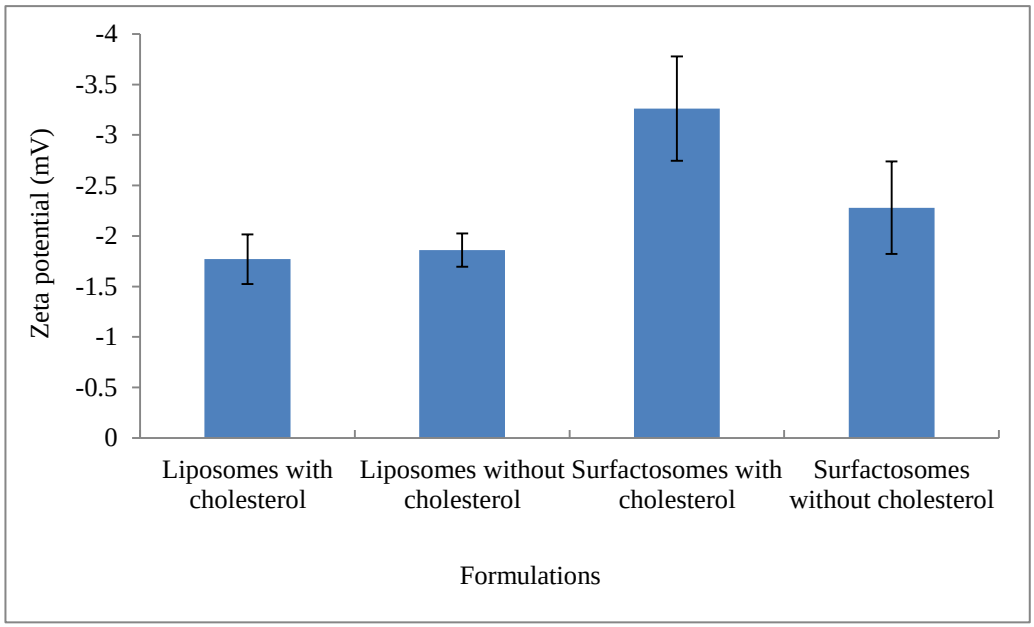

Figure 4.3 Zeta potential of SBS entrapped liposomes and surfactosomes with and without cholesterol. Data are mean ±SD, n=3.

4.3.3. Entrapment of SBS in proliposomes and prosurfactosomes with and without cholesterol

Entrapment of SBS in liposomes and surfactosomes prepared from proliposomes and prosurfactosomes were studied as shown in Figure 4.4. It was observed that liposomes

with and without cholesterol entrapped 8.5% and 4.03% respectively while surfactosomes with and without cholesterol entrapped 11.7% and 4.27% respectively. It can be observed that vesicles without cholesterol entrapped significantly lower amount of SBS than vesicles made with cholesterol ($p<0.05$). Hence, inclusion of cholesterol in formulation is desirable. It can also be observed that there is no significant difference in the entrapment efficiency of both liposomes and surfactosomes ($p>0.05$). Hence both the vesicles with cholesterol can be considered desirable for the entrapment of SBS. It is, however, important to note that entrapment of hydrophilic drugs in liposomes is known to be generally low since the aqueous spaces within liposome structures are limited compared to the aqueous phase outside the liposomes (Taylor et al., 1990, Elhissi et al., 2006).

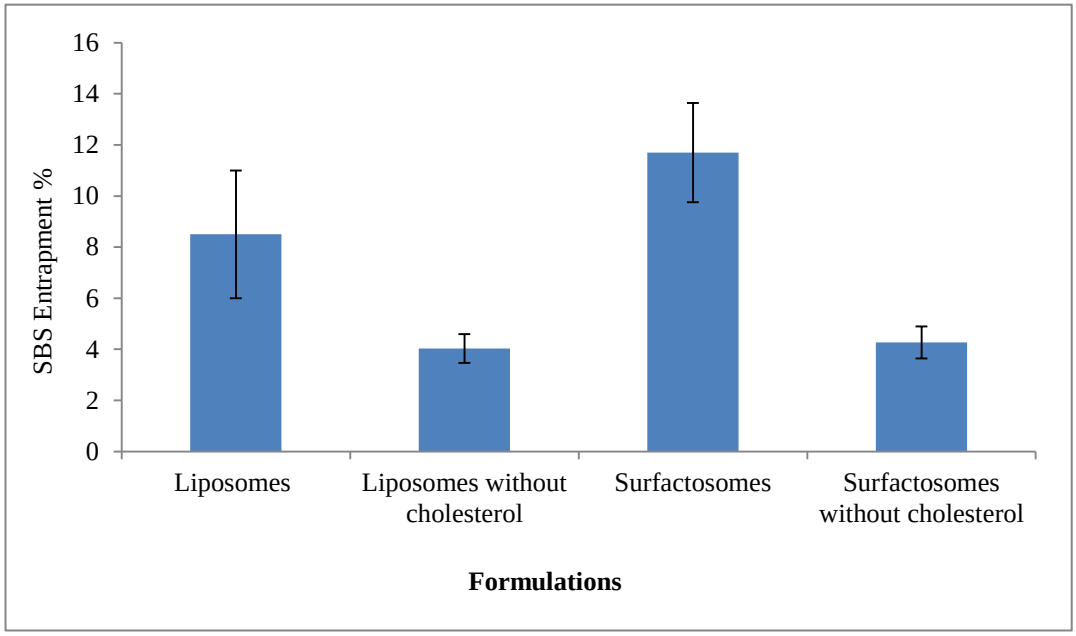

Figure 4.4 Initial entrapment of SBS in liposomes and surfactosomes with and without cholesterol. Data are mean ±SD, n=3.

The entrapment of SBS in proliposomes and prosurfactosomes as compared to the vesicles prepared by thin film method in chapter 3 section 3.3.4 is very low. Maximum entrapment achieved was only 11.7% by prosurfactosomes with cholesterol. Hence, no further extrusions of these vesicles were carried out.

4.3.4. Drug loading of SBS in proliposomes and prosurfactosomes with and without cholesterol

The drug loading of SBS in proliposomes and prosurfactosomes were calculated. This was calculated to give the quantity of drug entrapped by 100mg of lipid. This study will help to analyse if formulation is economically feasible for drug entrapment. As shown in Figure 4.5, vesicles with cholesterol loads significantly more SBS that the vesicle without cholesterol ($p<0.05$). Hence, inclusion of cholesterol is desirable. However, there is no difference between the drug loading of liposomes and surfactosomes ($p>0.05$).

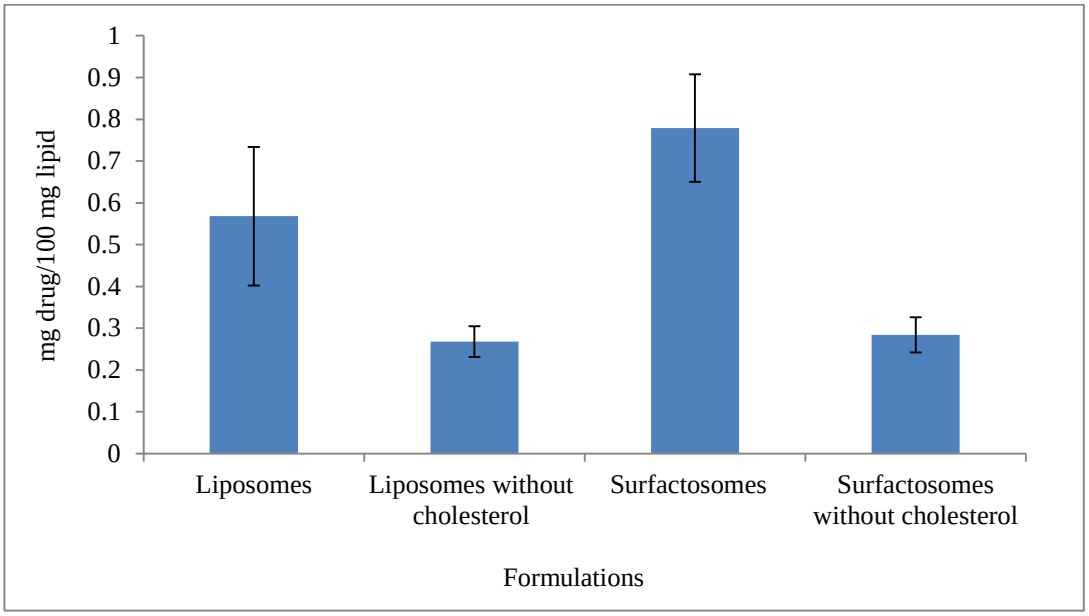

Figure 4.5 Drug loading of SBS in liposomes and surfactosomes with and without cholesterol. Data are mean ±SD, n=3.

4.3.5. VMD (size) and span (size distribution) of BDP liposomes and surfactosomes.

VMD of BDP liposomes and surfactosomes prior to extrusion were analysed using the Malvern Mastersizer as shown in Figure 4.6. Formulations with cholesterol had 1:1 molar ratio of cholesterol and SPC. VMD of liposomes and surfactosomes with cholesterol was around 5.17µm and 7.46µm respectively. By contrast, when no cholesterol was included the VMD measurements of liposomes and surfactosomes were around 4.4µm and 5.53µm respectively. It was observed that, regardless of cholesterol inclusion, liposomes were significantly smaller than surfactosomes ($p<0.05$). The larger

VMD of surfactosomes is may be due to the increase in fluidity of bilayer in the presence of Tween 80. This increases the gaps in bilayers, thus, making them bigger than liposomes (Young et al., 1983, Tasi et al., 2003). It was also observed that addition of cholesterol had slight but significant effect on the VMD of vesicles ($p<0.05$) (i.e. cholesterol increased the size of vesicles). Similar findings were proved by Tseng *et al.* where increase in cholesterol concentration increased the vesicular size (Tseng et al., 2007a).

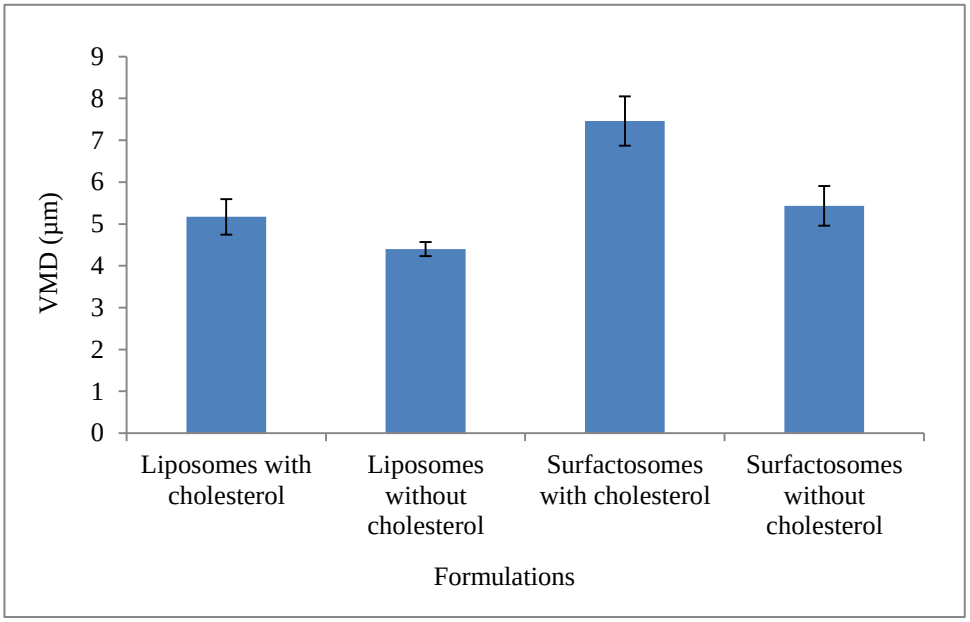

Figure 4.6 VMD of BDP entrapped liposomes and surfactosomes with (1:1 molar ratio) and without cholesterol. Data are mean ±SD, n=3.

Span of BDP entrapped liposomes and surfactosomes prior to extrusion were analysed using Malvern Mastersizer as shown in Figure 4.7. Span of liposomes and surfactosomes with cholesterol was around 1.73 and 5.2 respectively. Span of liposomes and surfactosomes without cholesterol was around 1.66 and 6.8 respectively. It was observed that the span values of liposomes were significantly lower than those of surfactosomes. It was also shown that inclusion of cholesterol had no significant effect on the span of the vesicles ($p>0.05$). The large span values of surfactosomes indicate that they have wide size distribution (i.e. low uniformity in size). Aggregation of vesicles was possibly the reason for this wide size distribution, which may indicate that Tween 80 has promoted the aggregation of vesicles. Overall, liposomes had narrower size distribution compared to surfactosomes, and although inclusion of Tween 80 was

though to enhance formulation stability, it actually reduced stability by promoting the interaction between the adjacent vesicles.

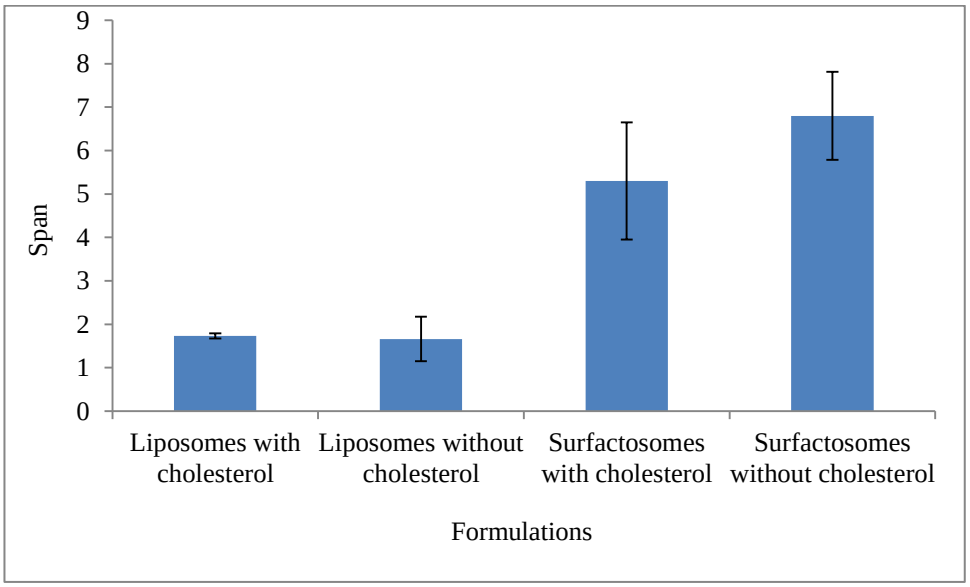

Figure 4.7 Span of BDP entrapped liposomes and surfactosomes with and without cholesterol. Data are mean ±SD, n=3.

4.3.6. Zeta potential of BDP liposomes and surfactosomes

Zeta potential of BDP liposomes and surfactosomes prior to extrusion were analysed using Malvern Mastersizer as shown in Figure 4.8. The surface charge of liposomes and surfactosomes with cholesterol were around -4.15mV and -6.74mV respectively. Size of liposomes and surfactosomes without cholesterol was -2.3mV and -8.4mV respectively. It was demonstrated that the surface charge of liposomes were significantly less negative than that of surfactosomes ($p<0.05$). The surfactosomes were slightly more negative than liposomes may be due to the presence of Tween 80. Sorbitan esters, polyoxyethelene delivatives, are fatty acid esters of sorbitol and its anhydrides copolymerised with a varying number of moles of ethylene oxide. Polysorbate 80 (Tween 80) is an oleate ester (Remington et al., 2006, Rowe et al., 2009). There is a possibility of by products like free fatty acids and their dissociation as discussed in section 1.7 and section 4.3.2. It was also seen that inclusion of cholesterol had no significant effect on the charge of the vesicles ($p>0.05$).

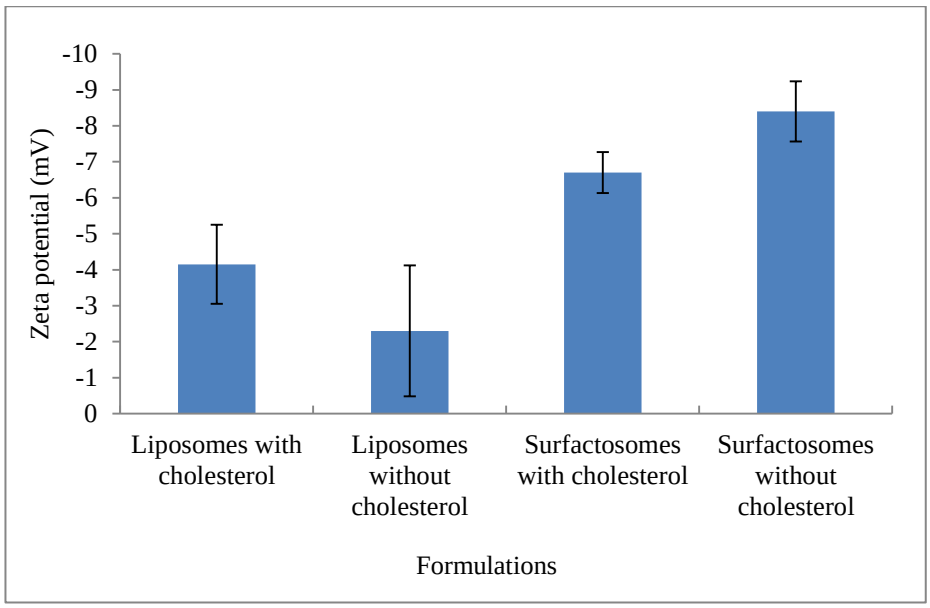

Figure 4.8 Zeta potential of BDP entrapped liposomes and surfactosomes with and without cholesterol. Data are mean ±SD, n=3.

4.3.7. Evaluation of mannitol behaviour in aqueous environment

In this study mannitol was used as the carbohydrate carriers for the manufacture of proliposomes. Mannitol has been previously proved to be the most promising candidate for pulmonary drug delivery (Steckel and Bolzen, 2004). Mannitol is a sugar alcohol which acts as an osmotic agent by improving the hydration of airways. This leads to the easy clearance of sputum by coughing and ciliary action (Jaques et al., 2008). Mannitol inhalation leads to long term improvement to lung function (Chan et al., 2011). E. Daviskas had made several studies on the inhalation of mannitol. In one of the study, inhalation of mannitol for 2 weeks improved the lung function in patients with cystic fibrosis. The change in sputum's physical property in favour of patients after treatment with mannitol was also observed. Mannitol increased the hydration of the airway surface by creating an osmotic gradient for water efflux into the airway lumen, thus, improving the mucus clearance (Daviskas et al., 2010). In another study, mannitol improved the health status of the patient with bronchiectasis by improving the hydration of mucus, thus, improving the clearance of mucus (Daviskas et al., 2005). Mannitol also has a positive effect on asthmatic patients (Daviskas et al., 2007). Mannitol inhalation reduced the viscoelasticity, surface tension, contact angle and the solids content of sputum, thus, improving the hydration in airway lumen. Mannitol is proved to be safe

and efficient in asthmatic and non-asthmatic patients (Brannan et al., 2005). Mannitol along with other sugar alcohols exhibit reduced caloric value. They are metabolised independently of insulin, thus, can be safely used by diabetic people (Schiweck et al., 2000). Hence, Mannitol was selected as carbohydrate carrier for liposomes and surfactosomes in this study.

However, during the studies, it was observed that mannitol formed needle-like filaments/crystals when stored in water for more than one hour, forming a gel-like product. The crystallization of mannitol in water was monitored using light microscopy as shown in Figure 4.9. The resultant samples became no longer suitable for HPLC analysis. Thus, immediate usage of these mannitol-based proliposomes in HPLC was recommended. Moreover, if this formulation will have a future clinical application, the proliposomes should be used immediately after hydration with aqueous phase.

Mannitol is a naturally occurring sugar alcohol commonly used as a pharmaceutical excipient due to its compatibility with drugs and safety. It has different polymorphic forms like alpha (α), beta (β) and delta (δ) forms (Yoshinari et al., 2002, Raut et al., 2011). It has been previously reported that mannitol has a strong tendency to crystallise (Yu et al., 1998). Thus, when mannitol is exposed to high relative humidity this usually results in moisture-induced polymorphic transition followed by changes in morphology of the particles. It is also observed that transition from δ form to β form is manifested by formation of filament-like crystals. It has also been demonstrated that prolonged contact with moisture can decrease the surface area and increase the size of mannitol particles. With the increase in the amount of water added, the SEM images have revealed the greater percentage of needle like β form (Yoshinari et al., 2003). This transformation of an amorphous material to become crystalline is referred to as glass transition (Yu et al., 1998). These crystals cannot revert back to their original form at room temperature (Raut et al., 2011). This polymorphic property of mannitol had adverse effects on outcomes of freeze drying products and shelf stability of drugs having mannitol (Beattie et al., 2007).

Figure 4.9 Light microscopy image showing the formation of mannitol crystals on hydration of mannitol based proliposomes. Magnification used is 400x. This is a typical of three images

4.3.8. Initial entrapment of BDP in proliposomes and prosurfactosomes

Initial entrapment of BDP in liposomes and surfactosomes were analysed. For this purpose vesicle with SPC only (no cholesterol), SPC with cholesterol (1:1) or SPC with cholesterol (2:1) were studied. As shown in Figure 4.10 it can be observed that in the formulation without cholesterol, BDP entrapment was 95.7% and decreased significantly to 40.3% and 48.3% on addition of cholesterol in the ratio 1:1 and 1:2 ratio respectively ($p<0.05$). Moreover, entrapment in liposomes made from SPC and cholesterol (1:1) was slightly but significantly lower than the entrapment in liposomes made from SPC and cholesterol (2:1). Entrapment significantly reduced from 48.3% to 40.3% ($p<0.05$). This shows that addition of cholesterol decreases the entrapped percentage of BDP in liposomes. This is due to the similarity between the structures of cholesterol and BDP leading to a competition for entrapment. This competition leads to decrease in BDP entrapment. Hence, increase in cholesterol tends to decrease the BDP entrapment in the bilayers (Tsotas et al., 2007, Ali et al., 2010).

For surfactosomes, the formulation without cholesterol entrapped 86.33% of BDP while the formulation with SPC and cholesterol (2:1) entrapped 92% of the drug with no significant difference in BDP entrapment between the two formulations ($p>0.05$). For the formulation made from SPC and cholesterol (1:1), BDP entrapment was reduced significantly to 25.67% ($p<0.05$). This shows that surfactosomal formulations with no

cholesterol or low cholesterol content (i.e. 2:1 SPC to cholesterol ratio) are superior than corresponding formulations made with greater cholesterol proportion (i.e 1:1 SPC to cholesterol). This may be due to the similarity in structures of cholesterol and BDP which increases the competition to be incorporated in the vesicular bilayer.

It was also observed that there is no significant difference in the BDP entrapment by liposome and surfactosome with only SPC ($p>0.05$). Similarly there was no significant difference between liposomes and surfactosome with 1:1 cholesterol to SPC ratio ($p>0.05$). However, surfactosomes entrapped significantly more BDP than liposomes in 2:1 SPC to cholesterol ratio ($p<0.05$).

Hence, surfactosome has good entrapment of BDP in no or low cholesterol concentration, but liposome has good entrapment only in formulation with no cholesterol.

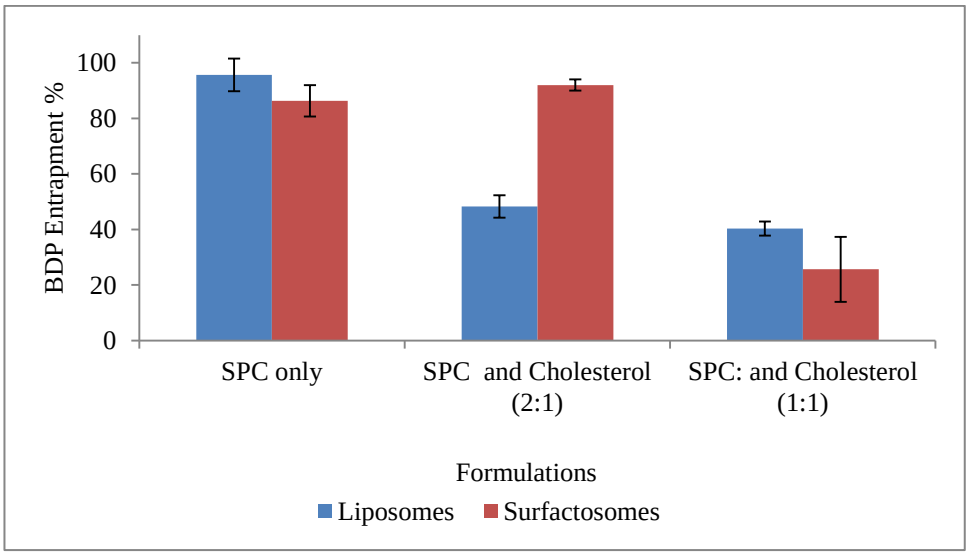

Figure 4.10 Initial entrapment of BDP in liposomes and surfactosomes with different concentrations of cholesterol. Data are mean ±SD, n=3.

4.3.9. Initial drug loading of BDP in proliposomes and prosurfactosomes

The drug loading of BDP in proliposomes and prosurfactosomes were calculated. This was calculated to give the quantity of drug entrapped by 100mg of lipid. This study will help to analyse if formulation is economically feasible for drug entrapment. As shown in Figure 4.11, BDP loading in liposome without cholesterol was significantly more that in liposomes with cholesterol ($p<0.05$). Moreover, loading of BDP in liposomes made from SPC and cholesterol (1:1) was slightly but significantly lower than in liposomes

made from SPC and cholesterol (2:1) ($p<0.05$). This shows that the addition of cholesterol decreases the loading of BDP in liposomes

In surfactosomes, there was no significant difference in the BDP drug loading of formulation with only SPC and with SPC and cholesterol (2:1) ($p>0.05$). However, the BDP loading decreased significantly in surfactosomes with SPC and cholesterol (1:1) ($p<0.05$). This shows that as discussed in section 4.3.8, the surfactosomal formulations with no cholesterol or low cholesterol content (i.e. 2:1 SPC to cholesterol ratio) are superior than corresponding formulations made with greater cholesterol proportion (i.e 1:1 SPC to cholesterol).

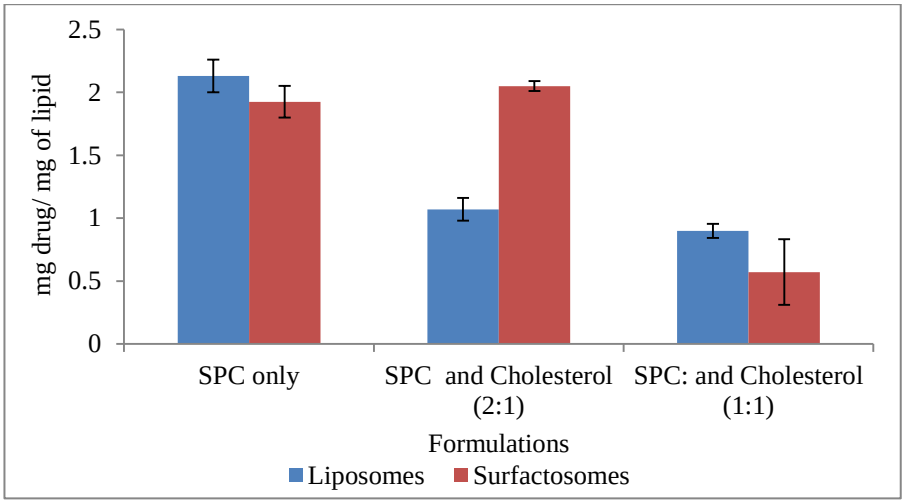

Figure 4.11 Drug loading of BDP in liposomes and surfactosomes with different concentrations of cholesterol. Data are mean ±SD, n=3.

4.3.10. Entrapment of BDP in vesicles (i.e. top layer) upon centrifugation using cholesterol-free or cholesterol-enriched formulations

The entrapment of BDP in liposomes and surfactosomes was evaluated with a focus on formulations that are free from cholesterol. When no cholesterol was incorporated in both formulations and when low cholesterol content was used in surfactosomes formulation, no BDP sediment was formed upon centrifugation using D_2O as dispersion medium (described in chapter 3 sections 3.3.8 and 3.3.9). Hence, the entire amount of the drug was suspended in the aqueous phase or incorporated into the vesicle structures. The top liposomal layer was studies under light microscope to detect the presence of BDP crystals, if any. In Figure 4.12 (a) top suspended liposome layer of formulation with no drug added was observed as a reference sample for comparison with samples

enriched with BDP. When no drug was included, the liposome layer was devoid of any crystals, indicating that crystals that might be seen in drug-containing formulations are BDP crystals. In Figure 4.12 (b) top layer of liposomal formulation without cholesterol was observed. This layer had a few scattered BDP crystals floating amidst liposomes; these crystals did not form a spot on the bottom of the eppendorf and their appearance in the liposome layer possibly indicates they were either adsorbed onto vesicle surfaces or leaked from the bilayers under the experimental conditions. In Figure 4.12 (c) top layer of liposomes with cholesterol (1:1 molar ratio) was observed. Here there no BDP crystals were seen, thus confirming their sedimentation in the bottom of the eppendorf or the ability of cholesterol to prevent leakage of the drug under experimental conditions.

In liposomal formulations without cholesterol, the entrapment of BDP was more as compared to the ones with cholesterol. This is due to the similarity in structure of cholesterol and BDP, resulting in competition for entrapment in the liposomal bilayers. Thus, displacement of the lipophilic drug from the liposomal membranes that cholesterol has occupied is likely, resulting in lower entrapment efficiency of the drug in the liposomal bilayers. Similar effect of cholesterol was observed for the steroid drug dexamethasone due to its similarity in structure to cholesterol (Tsotas et al., 2007). In other studies, loading of drugs such as diazepam, ibuprofen, midazolam and propofon in liposome bilayers was shown to be influenced by the cholesterol content in the formulations; increasing cholesterol content was shown to reduce drug incorporation into liposomes. Another report confirmed the presence of a trend of decreasing drug loading with increasing cholesterol content in liposomes (Ali et al., 2010). It has also been reported that high levels of cholesterol interferes with the close packing of lipids in vesicles by modifying the membrane fluidity. This increases the distribution of aqueous phase within the liposomal vesicles, hence, reducing the entrapment of hydrophobic drug (Ramana et al., 2010).

On placement of formulation in a release medium, an initial large bolus of drug is released before achieving a stable controlled release profile. This phenomenon is termed as “burst effect” (Chandy and Sharma, 1996). Such burst happens in a very short time compared to the entire release process. Burst release is also unpredictable and the amount of drug leakage cannot be robustly controlled (Huang and Brazel, 2001). The appearance of floating BDP crystals in the vesicle samples (Figure 4.12 b) is possibly due to the burst release phenomenon. Hence it can be likely that the entrapment of BDP

in vesicles without cholesterol is overestimated since rapid drug leakage will not count for the controlled release effect.

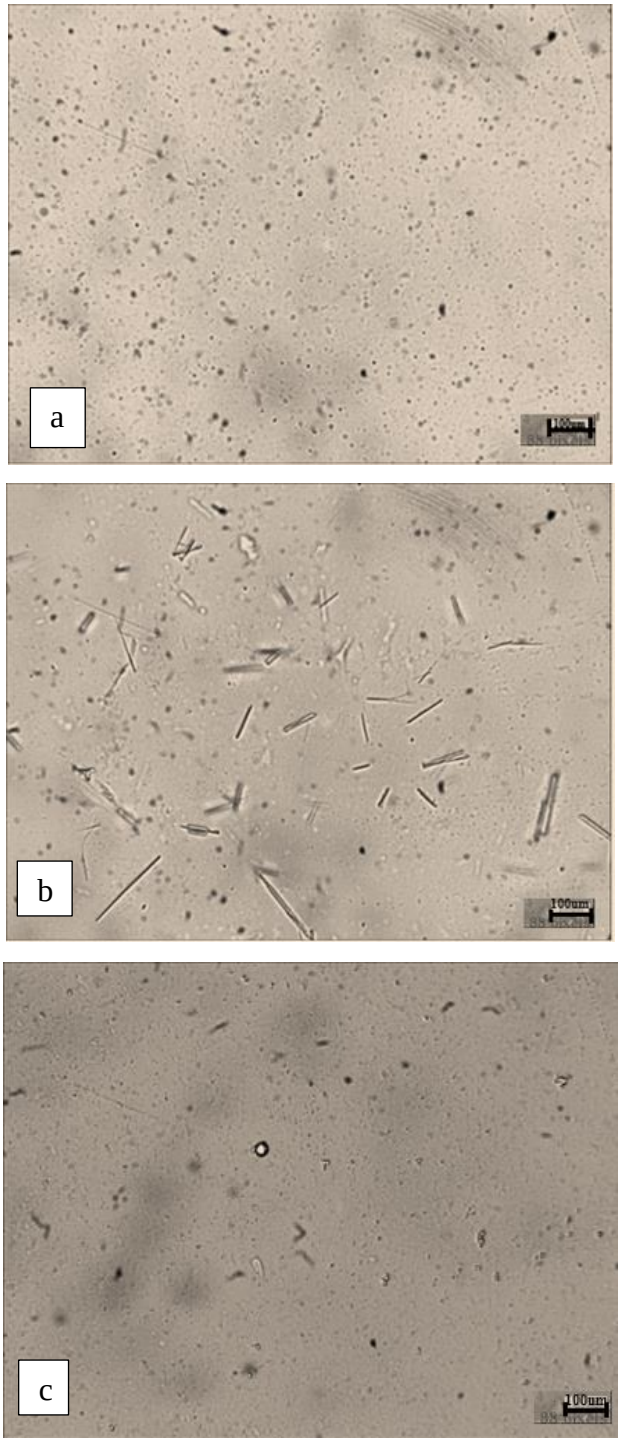

Figure 4.12 Light microscopy picture showing the top liposomal layer in formulations with (a) no drug. There are no crystals present and only vesicles floating (40x magnification). (b) No cholesterol. There are BDP crystals present (40x magnification). (c) With cholesterol. There are no BDP crystals present (40x magnification). This is a typical of three such images

4.3.11. Cholesterol assay for analysis of cholesterol in BDP sediment spot

Cholesterol assay was performed to study if there is any cholesterol present in the BDP spot sedimented at the bottom of eppendorf tube upon centrifugation. This experiment was conducted only for cholesterol-containing formulations because BDP spot was formed only when cholesterol was included in the formulation. HPLC analysis has revealed that the BDP spot had only 5% cholesterol whilst the remaining 90-95 % was BDP crystals. Hence, lipid bilayers had greater affinity to incorporate cholesterol which displaced BDP from the bilayers, causing it to sediment as a spot, resulting in reduced drug entrapment. Formulations without cholesterol had more BDP entrapment and thus, no BDP sediment spot was observed. This study has confirmed the "competitional" affinity of cholesterol towards liposome bilayers.

4.3.12. Effect of extrusion on BDP entrapment

Effect of extrusion on the retention of BDP was studied using a mini-extruder with polycarbonate membranes having pore sizes of 5µm and 2µm with 11 cycles of each and HPLC for BDP retention studies as shown in Figure 4.13. Vesicles with SPC, SPC and cholesterol (2:1) and SPC and cholesterol (1:1) were studied after they were re-suspended in fresh drug-free D2O. It was seen that liposome with SPC retained 90% BDP, vesicle with SPC and cholesterol (2:1) retained 94.3% and vesicle with SPC and cholesterol (1:1) retained 93.5% of BDP It was observed that without extrusion there was no significant difference in the entrapment of BDP ($p>0.05$), regardless of formulation. When 5µm polycarbonate membrane was used for extrusion, the drug started to leak and entrapment decreased. There was no significant difference among the formulation with regard to drug retention by the liposomes ($p>0.05$). This indicates that when stress was applied using 5µm membranes, all formulations behaved similarly and were unaffected by the shearing effect, indicating that the stress exerted on the vesicle was insufficient to cause marked leakage of BDP from liposomes. On further extrusion of 5µm with 2µm polycarbonate membranes, it was observed that liposomes made from SPC and cholesterol (1:1) retained significantly lower proportion of BDP than the other two liposomal formulations ($p<0.05$).

Similar observation was found for surfactosomes. Vesicles with only SPC and vesicles with SPC and cholesterol (2:1) had no significant difference in terms of drug retention in the vesicles ($p>0.05$).

It can be concluded from this study that inclusion of more cholesterol leads to excessive leakage of drug and decreases the ability of vesicles to tolerate stress of extrusion. Thus, with less or no cholesterol the vesicles were more stable. Cholesterol has been proved to give stability to the liposomes but in this study it was observed that high cholesterol levels may displace the BDP from vesicular bilayer. Hence, it is possible that stress has encouraged this displacement.

It can also be observed that there is no significant difference between the retention of BDP by liposomes and surfactosomes without extrusion in all three formulations ($p>0.05$). Similar observations with no significant difference were found for retention of BDP by liposomes and surfactosomes on extrusion with 5µm and 2µm in all three formulations ($p>.05$). Hence, it can be concluded that liposomes and surfactosomes prepared from proliposomes and prosurfactosomes behaved similarly

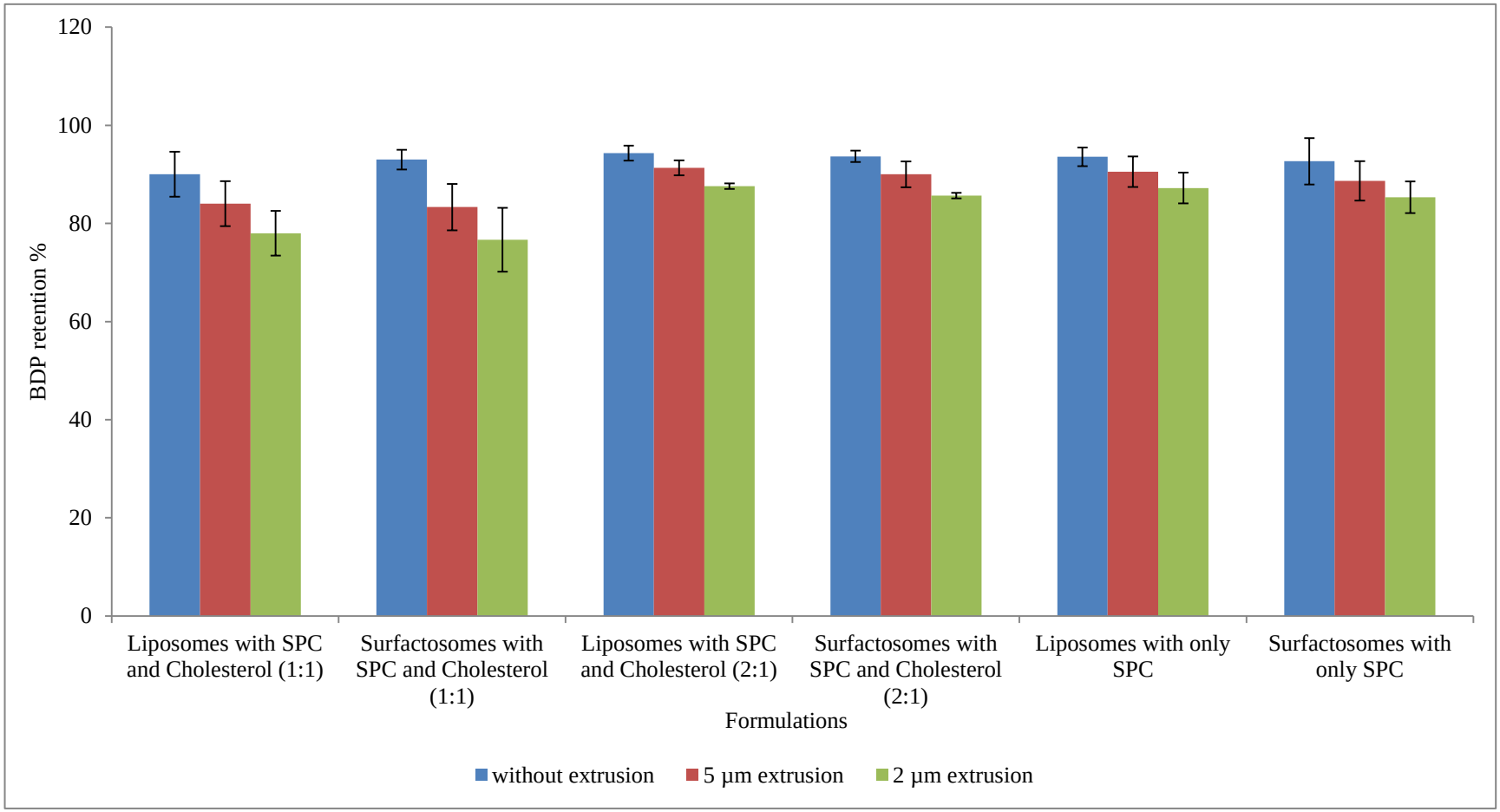

Figure 4.13 Retention of BDP in liposomes and surfactosomes with different concentrations of cholesterol after extrusion with 5µm and 2µm polycarbonate membrane. Data are mean ±SD, n=3.

4.3.13. TEM analysis of liposomes and surfactosomes

TEM analysis of liposomes and surfactosomes prepared from proliposomes and prosurfactosomes respectively were studied in Figure 4.14 (a) and (b). It is observed that the vesicles were unilamellar.

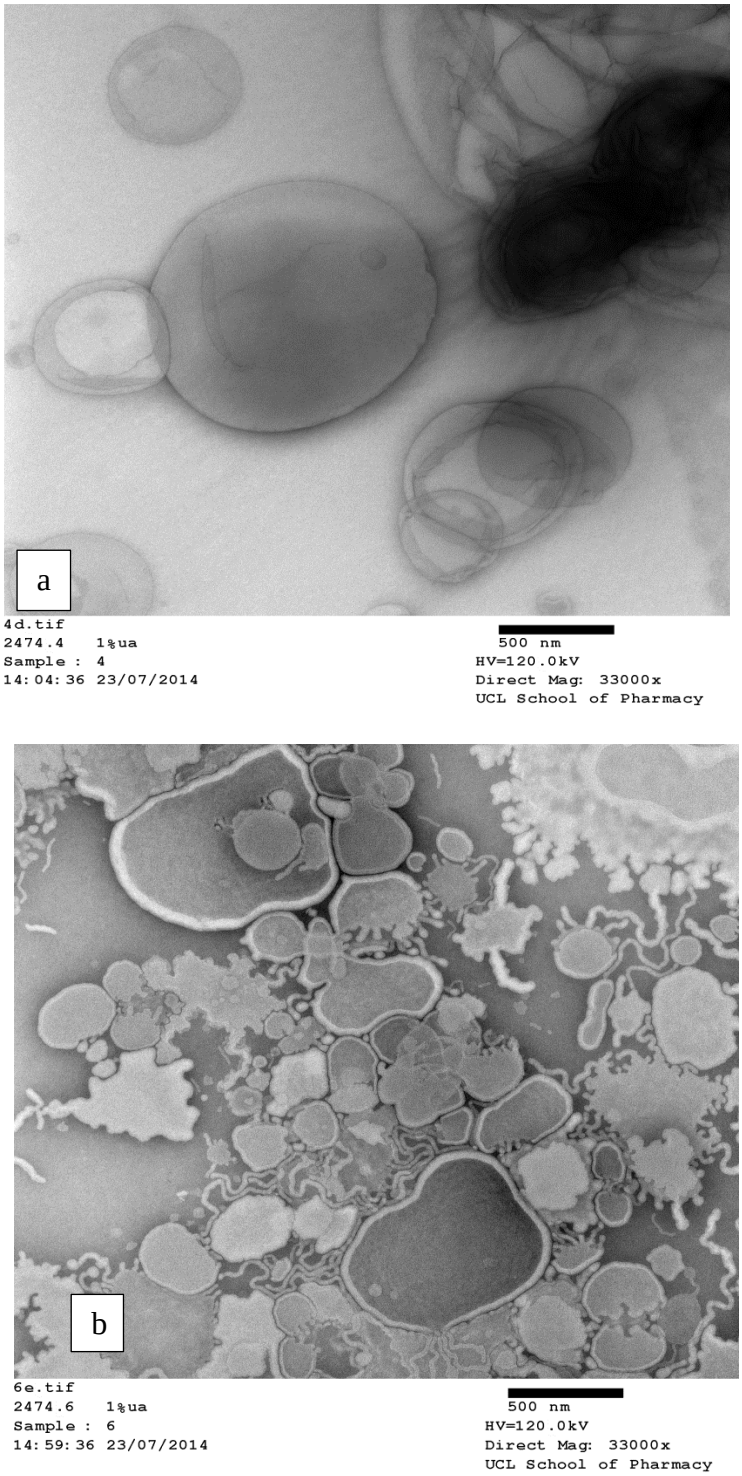

Figure 4.14 (a): TEM image of liposomes prepared from hydration of proliposomes (b): TEM image of surfactosomes prepared from hydration of prosurfactosomes

4.4. Conclusion

From this study it was concluded that for hydrophilic drug like SBS or hydrophobic agent like BDP, liposomes and surfactosomes had similar VMD and size distribution (span). It was observed that for both of the drugs the surface charge of surfactosomes was more negative than liposomes. This was attributed to the presence of surfactant Tween 80 in the formulation along with the impurities like linoleic, palmitic and stearic acids which has possibly affected the surface charge intensity on the vesicle surfaces.

In the entrapment studies it was shown that liposomes and surfactosomes retained very low proportions of SBS. Vesicles without cholesterol entrapped significantly lower proportions of this drug as compared to vesicles with cholesterol. After studying chapter 3 and chapter 4, it was found that the drug entrapment by proliposome technology was significantly low as compared the entrapment by thin film method. Therefore, this technology is proved to be inappropriate for hydrophilic drug like SBS. For this reason, no extrusion was carried out using SBS formulations.

For BDP, three formulations: vesicles with only SPC, vesicles with SPC and cholesterol (2:1) and SPC and cholesterol (1:1) were studied. For liposomes, formulation with no cholesterol was proved to be the best because it provided the highest drug entrapment. In other formulations with cholesterol it was demonstrated that drug entrapment was lower, suggesting that cholesterol has displaced the drug from the lipid bilayer due to the similar chemical structure of BDP and cholesterol, resulting in low drug entrapment in cholesterol-enriched formulations. For surfactosomes, formulations with no cholesterol and those with low cholesterol concentration (i.e. SPC and cholesterol; 2:1) proved to provide higher entrapment than formulation with high cholesterol concentration (i.e. SPC and cholesterol; 1:1). This is possibly due to displacement of BDP by cholesterol. In the presence of low concentration of cholesterol in the formulation BDP was not displaced in surfactosomes. Formulation with SPC only was analysed under light microscope BDP crystals were found floating on the top layer along with the vesicles. This was attributed to the “burst effect” shown by these vesicles.

It can also be concluded that there was no significant difference between the entrapment of BDP by liposomes and surfactosomes prepared by proliposome technology in formulations with only SPC and those with 1:1 SPC to cholesterol ratio. However,

surfactosomes entrapped significantly more than liposomes in formulations with 2:1 SPC to cholesterol ratio.

When extruded with 5µm and 2µm polycarbonate membranes both liposomes and surfactosomes behaved similarly in terms of BDP retention in the bilayers. Vesicles with more cholesterol (i.e. SPC and cholesterol 1:1) retained lower proportions of BDP than the vesicles with no cholesterol or vesicle with less cholesterol (SPC and cholesterol 2:1) in both liposomes and surfactosomes. This is due to the excess rigidity provided by the cholesterol; hence it was difficult for BDP to locate within the bilayers. However, there was no significant difference between the BDP retention by liposomes and surfactosomes prepared by proliposome technology in all three formulations without extrusion and with extrusion through 5µm and 2µm polycarbonate membrane.

TEM studies revealed that both proliposomes and prosurfactosomes form unilamellar vesicles on hydration.

Hence, for proliposome formulation with SPC is more preferable over other formulations studied and for prosurfactosome formulation with SPC and cholesterol (2:1) is more preferable over other studied formulations due to their high BDP entrapment/ retention and low standard deviation

Chapter 5

Characterisation of particulate based proliposomes and prosurfactosome with and without cholesterol using medical nebulisers

5.1. Introduction

Liposomes and surfactosomes on nebulisation delivers drug to the respiratory tract. The liposomes and surfactosomes ensure a prolonged and localised drug delivery to the lungs (Huang et al., 2010a). These vesicles have the tendency of fragmentation on being nebulised due to the shear force generated by the nebuliser. Freeze drying and spray drying of liposomes using the cryoprotectant before nebulisation has shown an improved drug delivery (Lo et al., 2004, Lu and Hickey, 2005). Payne *et al* in 1986 introduced proliposome technology which was more economical and easier to manufacture. These proliposomes on hydration with water above T_m of the phospholipid used produced liposomes (Payne et al., 1986b). The carrier particle dissolves in water and the lipids generate vesicles.

In this chapter, four vesicular formulations were investigated for their drug delivering capacity using Aeroneb Pro, Beurer iH50 vibrating nebulisers and the standard air jet PARI LC sprint nebuliser. Proliposomes and prosurfactosome with or without cholesterol were investigated. On hydration they produced liposomes and surfactosomes. The VMD, span, zeta potential, initial entrapment, BDP delivered to both stages of twin impinger, BDP retained by vesicles after nebulisation and aerosol droplet sizes were all investigated. The aim of this study was to investigate the four formulations (Table 5.1) for its efficiency and stability on being nebulised by three nebulisers in both the stage of impinger.

Table 5.1 List of formulations used for nebulisation

Proliposomes with cholesterol (1:1 molar ratio SPC to cholesterol)
Proliposomes without cholesterol
Prosurfactosomes with cholesterol (1:1 molar ratio SPC to cholesterol)
Prosurfactosomes without cholesterol

5.2. Methods

5.2.1. Preparation of proliposomes for nebulisation

For proliposomes without cholesterol, only soya phosphotidylcholine (SPC) was used. For proliposomes with cholesterol, SPC and cholesterol were used in 1:1 molar ratio. The lipid phase was dissolved in chloroform (20mg/ml) within a round bottom flask. Carbohydrate based carrier particle mannitol was added to this lipid phase in 1:5 lipid to mannitol ratio for proliposomes with BDP formulations. BDP was added in 2.5 Mole % to lipid phase. The organic solvents were removed using a rotary evaporator as described in section 2.2.1. After detaching the flask, the proliposomes were collected using a clean spatula. The formulations were stored in room temperature and were used on the same day for nebulisation.

5.2.2. Preparation of prosurfactosome for nebulisation

For prosurfactosome without cholesterol, SPC and Tween 80 were used in the ratio 85:15. For prosurfactosome with cholesterol, SPC and cholesterol were used in 1:1 molar ratio with Tween 80 (15% w/w of the total lipid) in a round bottom flask. These were dissolved in chloroform (20 mg/ml) in a round bottom flask. Carbohydrate based carrier particle mannitol was added to this lipid phase in 1:5 lipid to mannitol ratio for prosurfactosome with BDP. BDP was added in 2.5 Mole % to lipid phase. The organic solvent was evaporated as described in section 2.2.1. After evaporation of the solvent the flask was detached and the proliposomes were collected using a clean spatula. It was stored in room temperature and was used on the same day for nebulisation.

5.2.3. Hydration of vesicles for nebulisation

Proliposomes and prosurfactosome were hydrated to form liposomal and surfactosomal dispersions. They were hydrated with 75% of HPLC water and 25% of NaCl isotonic water to a concentration 10mg/ml. This formed an isotonic vesicular dispersion ready for nebulisation. The isotonicity was contributed by mannitol and NaCl.

5.2.4. Assembly of twin impinger

Twin impinger, also called the two stage impinger, was assembled and isotonic NaCl solution was used as a collection medium in both stages of the impinger. Thus, 7ml and 30 ml solution was placed in upper stage and lower stage of impinger respectively. Flow

rate through the impinger was set at 60 L/min for collecting the aerosols generated from nebulisers. Figure 5.1 shows the assembly of the twin impinger.

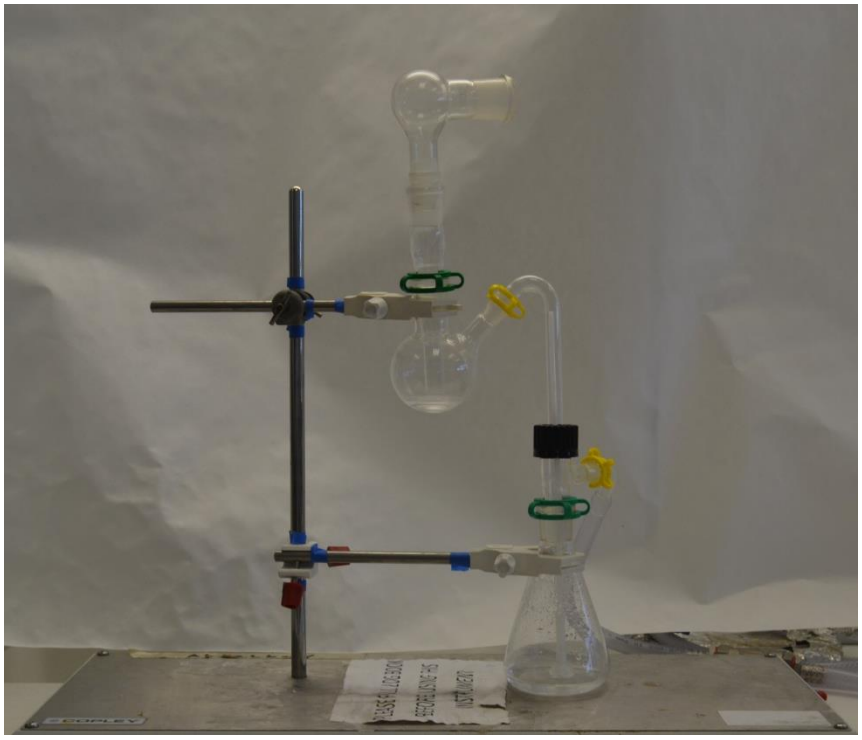

Figure 5.1 Assembly of twin impinge

5.2.5. Delivery of drug in vesicles via nebulisation

Vesicular dispersion (liposomes/surfactosomes) of 20ml was placed in Aeroneb pro, Beurer iH50 and PARI LC sprint nebuliser. The mouthpiece of the nebuliser was directed towards the throat of twin impinger as shown in Figure 5.2. The vesicular dispersion was nebulised till it reached “dryness”. The liposomal and surfactosomal samples delivered were collected from upper stage and lower stage of twin impinger for further analysis. The residual concentration of BDP was also calculated by washing the nebuliser chamber and running HPLC.

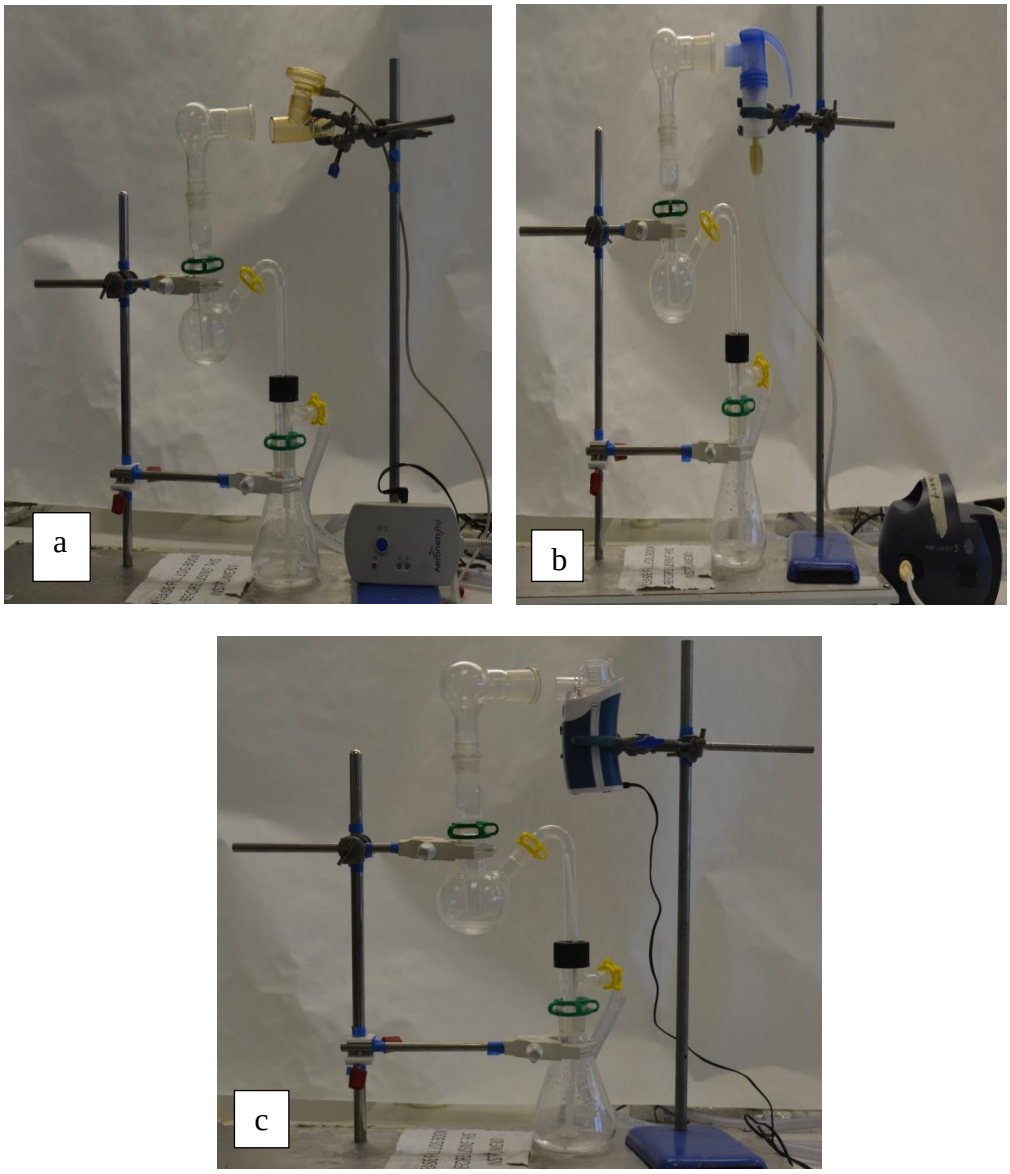

Figure.5.2 Assembly of nebuliser with twin impinger (a) Aeroneb Pro vibrating mesh nebuliser (b) PARI LC sprint air jet nebuliser and (c) Beurer iH50 vibrating mesh nebuliser.

5.2.6. Retention of BDP in vesicles after nebulisation.

To analyse the BDP retention in vesicles in both the stages, HPLC was carried out after centrifuging the samples collected from both stages. The BDP entrapped layer was separated from the unentrapped suspension as described in chapter 2 section 2.2.8(b) and HPLC analysis was carried out as explained in chapter 2 (section 2.2.9(b)).

5.2.7. Spraytech experiments

To detect the aerosol droplet VMD, Malvern spray tech laser diffraction size analyser was used. The aerosols generated from the nebuliser were directed perpendicularly to the laser beam and vacuum was applied from the other side of the laser to draw the

aerosols across it. The nebuliser was held 2.5cm away from the laser and VMD and span of aerosols were recorded.

5.3. Results and discussion

5.3.1. Initial BDP entrapment

Entrapment of BDP in liposomes and surfactosomes was analysed as shown in Fig 5.3. It was observed that there was no significant difference in the entrapment of BDP by both liposomes and surfactosomes with cholesterol ($p>0.05$). Similarly, there was no significant difference in BDP entrapment by both the vesicles without cholesterol ($p>0.05$). However, vesicles with cholesterol entrapped significantly lower amount of BDP than vesicles without cholesterol ($p<0.05$). This high entrapment in vesicles without cholesterol is due the excess entrapment of BDP in liposomal bilayer. This is due to the absence of competition from cholesterol which has a similar structure to BDP (Tsotas et al., 2007). This can lead to the displacement of the lipophilic drug from positions in the membrane that cholesterol may have occupied as explained in Chapter 4 (section 4.3.10). However, some BDP crystals are trapped in the top vesicular layer in the eppendorf tube after centrifugation. This is possibly due to the "burst effect" as described in chapter 4 section 4.3.10.

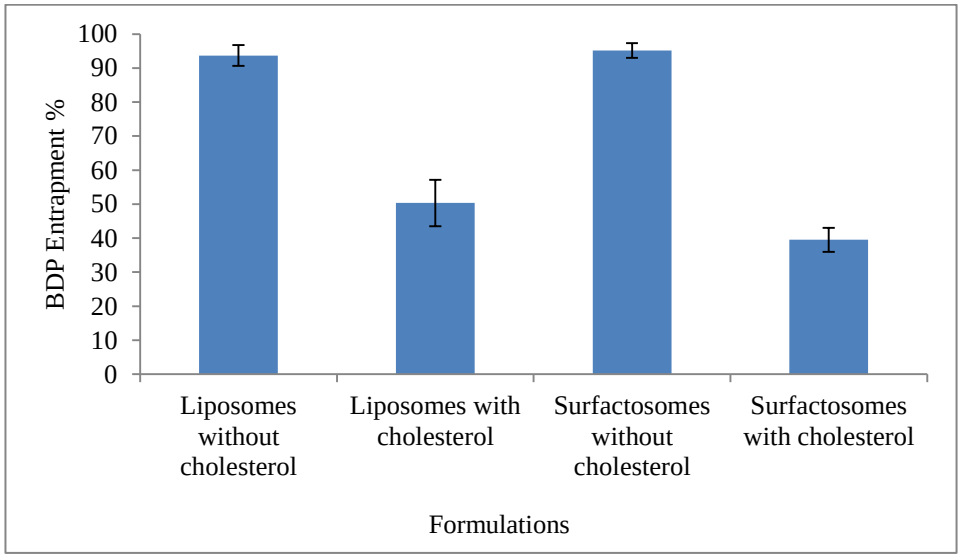

Figure 5.3 Entrapment of BDP in liposomes and surfactosomes with and without cholesterol before nebulisation. Data are mean ±SD, n=3.

5.3.2. Initial BDP drug loading

The drug loading of BDP in proliposomes and prosurfactosomes were calculated. This was calculated to give the quantity of drug entrapped by 100mg of lipid. This study will help to analyse if formulation is economically feasible for drug entrapment. As shown in Figure 5.4, it can be seen that there is no significant difference between the drug loading of liposomes and surfactosomes with and without cholesterol ($p>0.05$). However, significantly more drug is loaded in vesicles without cholesterol ($p<0.05$). Hence, exclusion of cholesterol from formulation is beneficial for better drug loading in proliposomes and prosurfactosomes due to lack of competition from cholesterol in incorporation of BDP in bilayers.

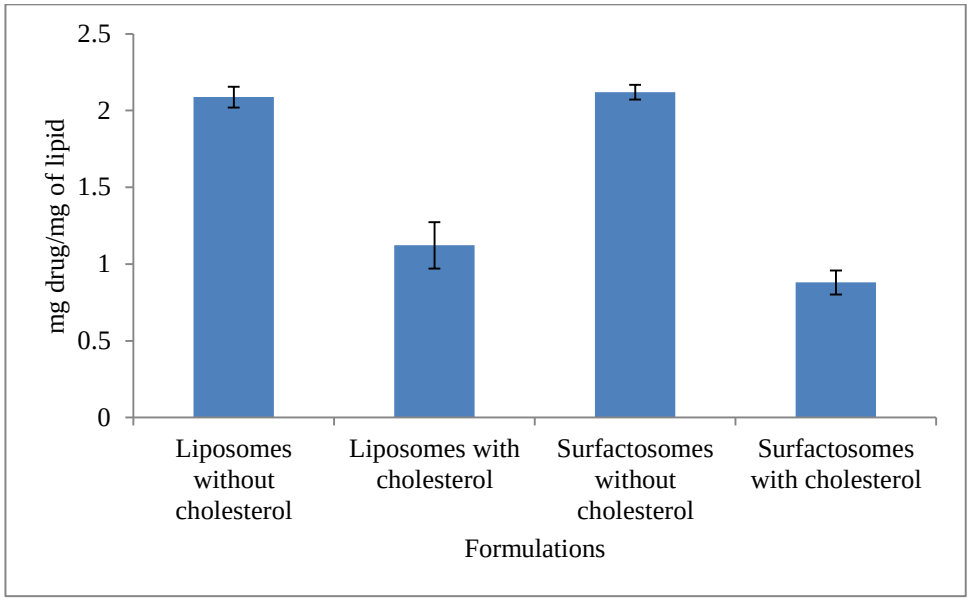

Figure 5.4 BDP drug loading in liposomes and surfactosomes with and without cholesterol before nebulisation. Data are mean ±SD, n=3.

5.3.3. VMD (size) and span (size distribution) of conventional liposomes and surfactosomes before nebulisation

The VMD of liposomes and surfactosomes with and without cholesterol before nebulisation were analysed as shown in Table 5.2. It was observed that liposomes without cholesterol were significantly larger than the vesicles with cholesterol ($p<0.05$). Vesicles with cholesterol were slightly smaller than the ones without cholesterol. This concludes that inclusion of cholesterol decreases the VMD of liposomes and surfactosomes possibly by changing the bilayer packing of the vesicles. Cholesterol

increases the attraction between bilayer lipids, thus, decreasing the size (Wang et al., 2006)

The span of liposomes and surfactosomes with and without cholesterol before nebulisation was analysed. It was observed that there was no significant difference between the span of all vesicles. The size distribution of all vesicles was similar.

Table 5.2 VMD and span of BDP entrapped liposomes and surfactosomes with and without cholesterol. Data are mean ±SD, n=3.

Formulations	**VMD (µm)**	**Span**
Liposomes	6.376 ± 0.45	2.047 ± 0.13
Liposomes with cholesterol (1:1)	5.38 ± 0.41	1.64 ± 0.48
Surfactosomes	5.53 ± 0.2	1.47 ± 0.42
Surfactosomes with cholesterol (1:1)	4.79 ± 0.22	1.7 ± 0.24

5.3.4. VMD (size) and span (size distribution) of conventional liposomes and surfactosomes delivered to twin impinger using Aeroneb pro nebuliser

Liposomes and surfactosomes with and without cholesterol were nebulised using Aeroneb Pro vibrating mesh nebuliser. The VMD and span of the vesicles collected in the upper and lower stage of twin impinger were analysed for their VMD and span as shown in Table 5.3. It was observed that there was no significant difference in the VMD of vesicles collected in the upper and lower stage of impinger ($p > 0.05$) except for surfactosomes without cholesterol. In surfactosomes without cholesterol, VMD of vesicles accumulated in upper stage was significantly larger than those in lower stage ($p<0.05$). The VMD of all the vesicles was less than 4µm and thus, can be easily incorporated into aerosol droplets with less leakage. As studied in chapter 3 sections 3.3.7 and 3.3.15, small vesicles leak fewer drugs as compared to large vesicles when under stress of excessive extrusion. Vesicles can, thus, possibly be incorporated into respirable aerosol droplets that may reach the lung with maximum drug retention on nebulisation.

It was observed that VMD of all the vesicles after nebulisation decreased significantly ($p<0.05$). This size reduction was due to the vibrating mesh which acts as an extruder.

Span of all the vesicles deposited in both the stages of twin impinger were studied and compared with the initial span. It was observed that in upper stage span of liposomes were significantly lower than the span of surfactosomes ($p<0.05$). Size distribution of surfactosomes was more possibly due to its aggregation after nebulisation. In lower stage of twin impinger it was observed that the span of surfactosomes without cholesterol were significantly higher than that of liposomes without cholesterol ($p<0.05$). There was no significant difference in the span of liposomes and surfactosomes with cholesterol ($p >0.05$). There was no significant difference also between the span of vesicles deposited in upper stage and lower stage of twin impinger ($p>0.05$) with an exception of surfactosomes with cholesterol where there was a significant difference ($p<0.05$). Here the span of surfactosomes in lower stage was slightly but significantly higher than that in upper stage. In the previous studies, inclusion of tween surfactant in liposomal bilayers has resulted in increase in liposome interaction, resulting in aggregation. It was also studied by Tasi *et al.* that inclusion of Tween 80 into liposomes decreases the liposomal energy barrier of aggregation or fusion from the interaction potential. This aggregation is due to the increase in the hydrophobic interaction between the (CH_2-CH_2-O) of the tween surfactant headgroup and the liposomal bilayers. (Tasi et al., 2003, Elhissi et al., 2012). It has also been studied that nebulisation may increase aggregation by increasing the fluid concentration during nebulization due to solvent evaporation (Muppidi et al., 2012). It is also studied that the air flow through the impinger leads to evaporation of water, thus, promoting to surfactosomal aggregation and fusion (Dua et al., 2012)

Thus, it can be concluded that the overall span of surfactosomes is higher than liposomes which may be due to the tendency of surfactosomes to aggregate on nebulisation

It was also observed that the span of liposomes remain unchanged after nebulisation but span of surfactosomes increased significantly after nebulisation in both the stages ($P<0.05$). This concludes that on nebulisation with Aeroneb pro vibrating mesh nebuliser with a mesh pore size 3µm, the size distribution of surfactosomes has increased. This may again be due to the aggregation of these vesicles during nebulisation.

Table 5.3 VMD and span of BDP entrapped liposomes and surfactosomes with and without cholesterol after nebulisation through Aeroneb Pro nebuliser. Data are mean ±SD, n=3.

Formulations	**VMD (µm)**		**Span**	
	Upper stage	**Lower stage**	**Upper stage**	**Lower stage**
Liposomes	2.98±0.75	1.93± 0.07	1.15±0.29	1.47 ± 0.78
Liposomes with cholesterol (1:1)	2.71±0.45	1.59± 0.27	0.87 ± 0.34	1.1 ± 0.82
Surfactosomes	3.76 ± 0.29	1.72± 0.21	2.67 ± 0.46	3.3± 0.75
Surfactosomes with cholesterol (1:1)	3.18 ± 0.58	1.41± 0.26	2.1± 0.18	2.97± 0.07

5.3.5. VMD (size) and span (size distribution) of conventional liposomes and surfactosomes delivered to twin impinger using Beurer nebuliser

Liposomes and surfactosomes with or without cholesterol were nebulised using Beurer vibrating mesh nebuliser. The VMD and span of the vesicles collected in the upper and lower stage of twin impinger were collected and were analysed for their VMD and span as shown in Table 5.4. It was seen that in upper stage liposome without cholesterol VMD was significantly larger than the vesicles with cholesterol ($p<0.05$). In lower stage it was observed that liposomes without cholesterol were significantly larger than the surfactosomes with and without cholesterol ($p<0.05$). It was also observed that liposome with cholesterol was significantly larger than the surfactosomes with cholesterol. Here it can be concluded that liposomes without cholesterol are larger than the other vesicles. Inclusion of cholesterol decreases the VMD of the vesicles. This may be due to the close packing of phospholipids due to the presence of cholesterol (Wang et al., 2006).

VMD of vesicles in upper stage and lower stage of twin impinger were compared with the VMD before nebulisation with Beurer iH50. It was observed that the VMD of vesicles significantly decreased after nebulisation ($p<0.05$) with an exception of surfactosomes with cholesterol in upper stage. This shows that on nebulising with Beurer iH50 vibrating mesh nebuliser the vesicle size decreases and the mesh of the device has acted as an extruder. It was also observed that there was a significant difference in the size of vesicles without cholesterol in upper stage and lower stage of

twin impinger ($p<0.05$). VMD of vesicles without cholesterol in lower stage was significantly smaller than those in upper stage. Vesicles with cholesterol didn't have much size difference between both stages. This may be due to the absence of cholesterol makes the vesicle less stable and more prone to fragmentation, thus, making it smaller in size. The VMD of all the vesicles was less than 4µm and as studied in chapter 3 sections 3.3.7 and 3.3.15, small vesicles leak fewer drugs as compared to large vesicles when under stress of excessive extrusion. Hence, these vesicles can be incorporated in aerosols when nebulised with maximum drug retention.

Span of all the vesicles deposited in both the stages of twin impinger was studied and compared with the initial span. It was observed that in upper stage of twin impinger liposomes with and without cholesterol was significantly smaller than the surfactosomes with and without cholesterol ($p<0.05$). There was no significant difference in the vesicles with and without cholesterol. Similar finding was found in vesicles collected in lower stage of twin impinger. This concludes that the span of surfactosomes was significantly higher than liposomes. This is due to the tendency of surfactosomes to aggregate and decrease the uniformity of vesicles after nebulisation as discussed in section 5.3.4.

It was also observed that for liposomes without cholesterol, after nebulisation the span decreased significantly ($p <0.05$). For other vesicles span deceased significantly only in upper stage of twin impinger. In the lower stage the decrease in span was not significant. This decrease in span after nebulisation with Beurer iH50 suggests that after nebulisation the size distribution of vesicles decreases and they become more uniform in size. This vibrating mesh nebuliser acts as an extruder to vesicles via forcing the vesicles through apertures with defined pore dimensions. Pore dimension of Beurer iH50 was between 3.8µm and 4.8µm as confirmed by the company's technical team. Except for liposomes without cholesterol, for all other vesicles there was no significant difference between the span of upper and lower stage of twin impinger ($p>0.5$). This suggests that the uniformity of all vesicles collected in both the stages of impinger was similar.

Table 5.4 VMD and span of BDP entrapped liposomes and surfactosomes with and without cholesterol after nebulisation through Beurer iH50 nebuliser. Data are mean ±SD, n=3.

Formulations	**VMD in µm**		**Span**	
	Upper stage	**Lower stage**	**Upper stage**	**Lower stage**
Liposomes	4.07±0.27	3.33 ± 0.28	0.84 ± 0.22	0.68 ± 0.2
Liposomes with cholesterol (1:1)	3.03 ± 0.17	2.52 ± 0.11	0.75 ± 0.22	0.92 ± 0.14
Surfactosomes	3.72 ±0.3	2.33 ± 0.3	2.69 ± 0.59	1.80 ± 0.25
Surfactosomes with cholesterol (1:1)	3.18 ± 0.48	1.52 ± 0.47	2.16 ± 0.17	2.24 ± 0.52

5.3.6. VMD (size) and span (size distribution) of conventional liposomes and surfactosomes delivered to twin impinger using PARI LC sprint nebuliser

Liposomes and surfactosomes with or without cholesterol were nebulised using PARI LC sprint air jet nebuliser. The VMD and span of the vesicles collected in the upper and lower stage of twin impinger were analysed for their VMD and span as shown in Table 5.5. It was observed that in upper stage of twin impinger there was significant difference between the VMD of liposomes and surfactosomes ($p<0.05$). Liposomes were significantly smaller than surfactosomes with and without cholesterol. This may be due to the loose packing of phospholipids in the presence of Tween 80. However, there was no significant difference in the vesicular VMD with and without cholesterol. This shows that cholesterol does not significantly affect the VMD of liposomes and surfactosomes deposited following aerosolisation with the PARI LC sprint nebuliser. However in the presence of cholesterol the VMD of the vesicle was slightly decreased. In lower stage there was no significant difference between VMD of all vesicles ($p>0.05$) with an exception of surfactosomes without cholesterol being significantly larger than liposomes with cholesterol. Here it can be concluded that cholesterol decreases the size of the vesicles, possibly because of the change of the bilayer packing patterns.

VMD of vesicles in upper stage and lower stage of twin impinger were compared with the VMD before nebulisation with the PARI LC sprint nebuliser. Here it was observed that the VMD of all the vesicles significantly decreased after the nebulisation ($p<0.05$)

with an exception of surfactosomes without cholesterol in upper stage of the twin impinger. It was also observed that there was no significant difference in VMD between the vesicles in upper stage and lower stage ($p>0.05$). Similar finding were observed by Saari *et al.*in 1999 where nebulisation using air jet nebuliser reduced the VMD of liposomes due to shear force provided by continuous recycling of liposomes during nebulisation (Saari et al., 1999). Liposomal VMD reduction during jet nebulisation was also observed by Bridges and Taylor and Elhissi *et al.* due to shear force generated by the jet nebuliser during nebulisation. This leads to fragmentation of vesicle (Bridges and Taylor, 1998, Elhissi et al., 2007).

This concludes that vesicles on being nebulised by PARI LC sprint nebuliser decreases in size due to its shear forces, leading to vesicle fragmentation. The VMD of all the vesicles was less than 5µm and as studied in chapter 3 sections 3.3.7 and 3.3.15, small vesicles leak fewer drug as compared to large vesicles when under stress of excessive extrusion. Hence, these vesicles can be incorporated in aerosol droplet on being nebulised with maximum drug retentions to be deposited in the lungs.

Span of all vesicles deposited in upper stage and lower stage of twin impinger were analysed after nebulisation with PARI LC sprint nebuliser. It was observed that in upper stage there was no significant difference in the span of liposomes and surfactosomes with or without cholesterol ($p>0.05$). The uniformity of all the deposited vesicles was similar. Similar results were observed in the lower stage of twin impinger with an exception of surfactosomes without cholesterol which was significantly larger than the liposomes regardless of cholesterol incorporation within formulation ($p<0.05$). This was possibly due to the tendency of surfactosomes without cholesterol to aggregate on nebulisation as they lack the stability in the absence of cholesterol.

It was also observed that the span of the vesicles changed significantly after nebulisation with PARI LC sprint nebuliser. After nebulisation the span of vesicles significantly decreased ($p<0.05$) with an exception of surfactosomes without cholesterol in lower stage where the there was a slight decrease in the size. This decrease in vesicular size distribution after nebulisation was due to the shear forces offered by the nebuliser which makes them more uniform in size. It was also observed that there was no significant difference between the span of vesicles in upper stage and lower stage of twin impinger.

Table 5.5 VMD and span of BDP entrapped liposomes and surfactosomes with and without cholesterol after nebulisation through AirJet nebuliser. Data are mean ±SD, n=3.

Formulations	**VMD in µm**		**Span**	
	Upper stage	**Lower stage**	**Upper stage**	**Lower stage**
Liposomes	3.07± 0.24	1.74±0.35	2.59±0.35	2.76±0.22
Liposomes with cholesterol (1:1)	2.83±0.33	1.53± 0.1	2.27± 0.32	1.82±0.11
Surfactosomes	4.32±0.39	2.29± 0.26	2.83±0.32	4.08±0.96
Surfactosomes with cholesterol (1:1)	3.8± 0.24	1.79± 0.36	2.73±0.17	2.95± 0.1

5.3.7. Zeta potential of conventional liposomes and surfactosomes before nebulisation

Zeta potential of liposomes and surfactosomes with and without cholesterol before nebulisation was studied as shown in Table 5.6. Surfactosomes appeared to have more negative zeta potential than liposomes. The presence of Tween 80 seems to increase the negative surface charge of vesicle. Tween 80 is a fatty acid ester made from fatty acid and sorbitol.This may be attributed by the presence of impurities/free fatty acids like linoleic acid, palmitic acid and stearic acid as discussed in section 4.3.2. It is possible that these acids on dissociation on the surface of a particle gave rise to a negatively charged surface as discussed in sections 4.3.2 and 1.7. Similar observation of increase in negative charge of solid lipid nanoparticle on addition of Tween 80 in the formulation was found by Prabhakar *et al.* in 2013 (Prabhakar et al., 2013). Hence, surfactosomes are significantly more negative than liposomes.

It is stated that that a higher absolute value of zeta potential indicates a more stable suspension and lower value indicates colloid instability, which could lead to aggregation of nanoparticles.

Table 5.6 Zeta potential of BDP entrapping liposomes and surfactosomes with or without cholesterol before nebulisation. Data are mean ±SD, n=3.

Formulation	Zeta potential (mV)
Liposomes	-2.85± 0.17
Liposomes with cholesterol (1:1)	-2.82 ± 0.19
Surfactosomes	-3.44 ± 0.25
Surfactosomes with cholesterol (1:1)	-3.70 ± 0.24

5.3.8. Zeta potential of conventional liposomes and surfactosomes delivered to twin impinger using Aeroneb pro, Beurer and PARI LC sprint nebulisers

Following nebulisation directed towards the twin impinger impinger, zeta potential of liposomes and surfactosomes with or without cholesterol was determined using Aeroneb Pro, Beurer and PARI LC sprint nebulisers (Table 5.7).

For Aeroneb pro it was observed that there was no significant difference between the zeta potentials of all vesicles in upper stage and in lower stage ($p>0.05$). Moreover, no significant difference between the zeta potential measurements of vesicles collected from upper stage and lower stage of the twin impinger. This indicates that the surface morphology of vesicles remains same in both stages of impinger.

For Beurer iH50 similar findings as Aeroneb pro were observed. There was no significant difference in the zeta potential of vesicles in both upper and lower stage of twin impinger ($p>0.05$). There was also no significant difference between the zeta potentials of vesicles collected in upper stage and lower stage of twin impinger. Beurer iH50 changes the surface morphology of all the vesicles similarly irrespective of the vesicles and site of delivery.

For PARI LC sprint nebuliser similar findings as Aeroneb pro and Beurer iH 50 were observed. There was no difference in the zeta potential of vesicles in both upper and lower stage of twin impinger ($p>0.05$). There was also no significant difference between the zeta potentials of vesicles collected in upper stage and lower stage of twin impinger.

These findings indicate that the zeta potential of all vesicles is similar irrespective of the nebuliser and formulation used and regardless of the site of deposition within the twin impinger (i.e. upper stage or lower). Similar zeta potential of vesicles indicate that the

vesicle uptake by the cells will be similar irrespective of the site of delivery. Similar zeta potential also indicates similar pharmacokinetics of the vesicles after being delivered by nebuliser (Henriksen et al., 1994).

However, the zeta potential of the vesicles significantly increased after nebulisation. This is possibly due to the reduction in VMD of vesicles after nebulisation. Reduction of size increased the surface area which in turn may increase the zeta potential. Similar trend was observed where the increase in liposomal VMD decreased the zeta potential when stored for 24 hours (Tseng et al., 2007a). Schubert *et al* in 2005 observed that when the particle size of solid-lipid nanoparticles (SLN) decreased with increase in emulsifier-lipid ratio, thus increasing zeta potential (Grahame, 1947). The negatively charged particles binds to the cationic sites of the cell in the form of cluster for adsorption (Henriksen et al., 1994).

Table 5.7 Zeta potential of BDP entrapped liposomes and surfactosomes with and without cholesterol after nebulisation through all three nebulisers nebuliser. Data are mean ±SD, n=3.

Formulations	**Aeroneb Pro**		**Beurer**		**Air jet**	
	Upper stage	**Lower stage**	**Upper stage**	**Lower stage**	**Upper stage**	**Lower stage**
Liposomes	-19.43± 2.3	-20.31± 2.22	-21.03 ± 1.04	-19.93 ± 1.09	-15.43 ± 0.63	-15.06± 0.22
Liposomes with cholesterol (1:1)	-21.30± 0.69	-20.39± 0.5	-19.16 ± 1.38	-20.3 ± 2.25	-15.45 ± 0.49	-15.37± 0.42
Surfactosomes	-20.10± 1.91	-21.42± 1.9	-21.53 ± 2.2	-22.26 ± 2.3	-16.88 ± 0.09	-17.01± 0.2
Surfactosomes with cholesterol (1:1)	-20.05± 1.94	-19.51± 1.15	-20.52 ±2.06	-21.16 ± 1.5	-17.93 ± 0.55	-17.43± 0.45

The efficiency of Aeroneb Pro, Beurer iH50 and PARI LC sprint nebuliser to deliver liposomes and surfactosomes with or without cholesterol to twin impinger was studied. This study may help at providing indications on which nebuliser and formulation are most desirable in terms of deposition in lower respiratory airways, if further in vivo studies are to be conducted in the future.

5.3.9. Delivery of BDP using conventional liposomes and surfactosomes to twin impinger using Aeroneb Pro nebuliser

Aeroneb pro was used to study the efficiency of nebulisation using different vesicular formulations for pulmonary delivery as shown in Figure 5.5. Surfactosomes without cholesterol were delivered higher proportions compared to the other vesicles as measured from both the stages. In upper stage, 22% of surfactosomes without cholesterol was delivered which was significantly higher than liposomes and surfactosomes with cholesterol ($p<0.05$). Cholesterol-free surfactosomes were delivered slightly more than cholesterol-free liposomes ($p>0.05$). In lower stage, 38.5% of surfactosomes without cholesterol was delivered which was significantly more than the other formulations investigated ($p<0.05$). Liposome without cholesterol was also delivered in significantly higher proportions than liposome with cholesterol ($p<0.05$). These findings suggest that surfactosomes without cholesterol may offer the greatest potential in terms of pulmonary deposition profile using the Aeroneb Pro nebuliser. This is due to the high flexibility and lack of rigidity of surfactosomes when cholesterol is excluded (Benson, 2010, Samad et al., 2007). This helped the vesicle to squeeze through the mesh of the vibrating nebuliser without fragmentation. This may lead to less leakage of drug and more delivery. In vesicles with cholesterol, due to presence of cholesterol they become rigid. Hence, they are more liable to be broken/ to be fragmented when under stress during nebulisation due to excess rigidity. Vesicles were more ultradeformable and elastic in the absence of cholesterol. Similar findings were concluded by Zaru *et al.* where liposomes rich in cholesterol and with rigid liposome membranes were unable to efficiently retain amphiphilic drug Rifampicin during nebulization processes. Liposomes rich in cholesterol demonstrated very low nebulisation efficiency. It was also observed that addition of cholesterol at different concentrations had an impact on bilayer rigidity as well as on the interactions between the drug and the membrane components, influencing the stability of drug incorporation in the lipid membrane. This also affected the retention of the drug in the vesicles during nebulization (Prabhakar et al., 2013). Cholesterol can also lead to burst effect and excessive displacement of BDP from bilayer, thus, leading to excessive BDP leakage.

It was also observed that BDP was delivered in significantly greater proportions to lower stage of twin impinger than upper stage ($p<0.05$) except with surfactosomes with cholesterol. Hence, it can be concluded that more BDP is likely to be delivered to the lower respiratory tract than upper tract using the Aeroneb Pro nebuliser. Lower

respitatory tract is the desired region for BDP delivery as corticosteroid (BDP) inhalation has adverse effects on upper respiratory tract. They include dysphonia, hoarseness of voice, cough and oral candiditis. Oral candiditis is rare and dose related and can be prevented by gargling, washing and spitting after taking inhaler or with local anti-fungal treatment. They are more common with patients sensitive to inhaled steroids. Hoarseness in voice is caused by the deposition of corticosteroid on the vocal cord. These can be avoided to some extent by gargling and washing after inhalation (Barnes, 2007). Hence, delivery of maximum BDP directly to the lower respiratory system via vesicles is more desirable.

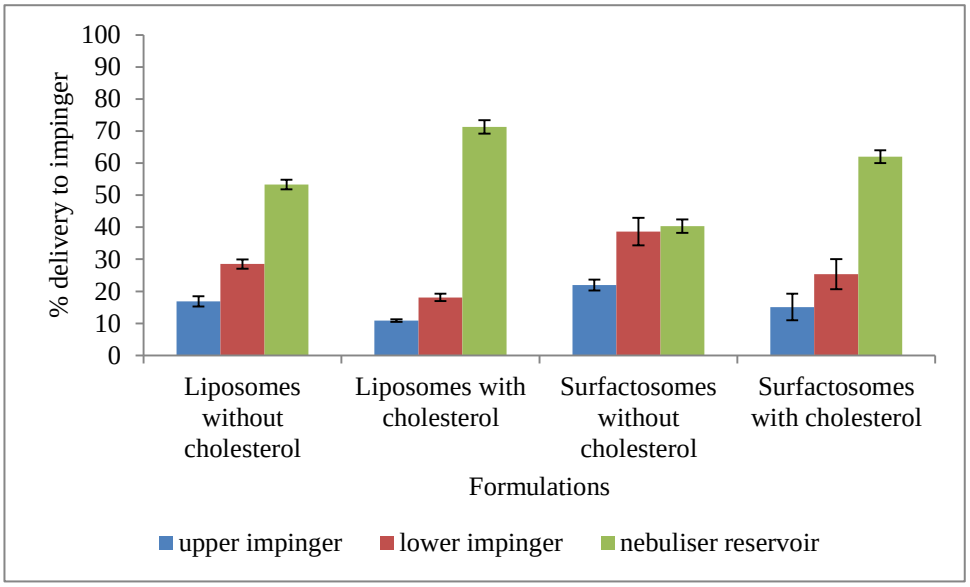

Figure 5.5 The delivery of BDP entrapped liposomes and surfactosomes with and without cholesterol after nebulisation through Aeroneb Pro nebuliser to both stages of impinger. Data are mean ±SD, n=3.

5.3.10. BDP retained in conventional liposomes and surfactosomes in twin impinger following nebulization with the Aeroneb Pro Nebuliser

BDP retained in vesicles after nebulisation was studied as shown in Figure 5.6. It was found that in upper stage vesicles with cholesterol retained significantly lower drug proportions than vesicles without cholesterol after nebulisation. Similar results were observed in lower stage. However, as studied and discussed in Chapter 4 (section 4.3.10) there is competition between cholesterol and BDP for the entrapment in the bilayers, hence, in vesicles without cholesterol higher drug proportions were retained.

However, as discussed in chapter 4 section 4.3.10 burst release effect may lead to an over estimation of drug entrapped in vesicles without cholesterol. Overall in liposomes and surfactosomes with cholesterol the drug entrapped was 75.2% and 78.2% respectively in upper stage and 79.3% and 84.6% respectively in lower stage. In liposomes and surfactosomes without cholesterol the drug entrapped was 97.1% and 96.2% respectively in upper stage and 98% and 98.1% respectively in lower stage.

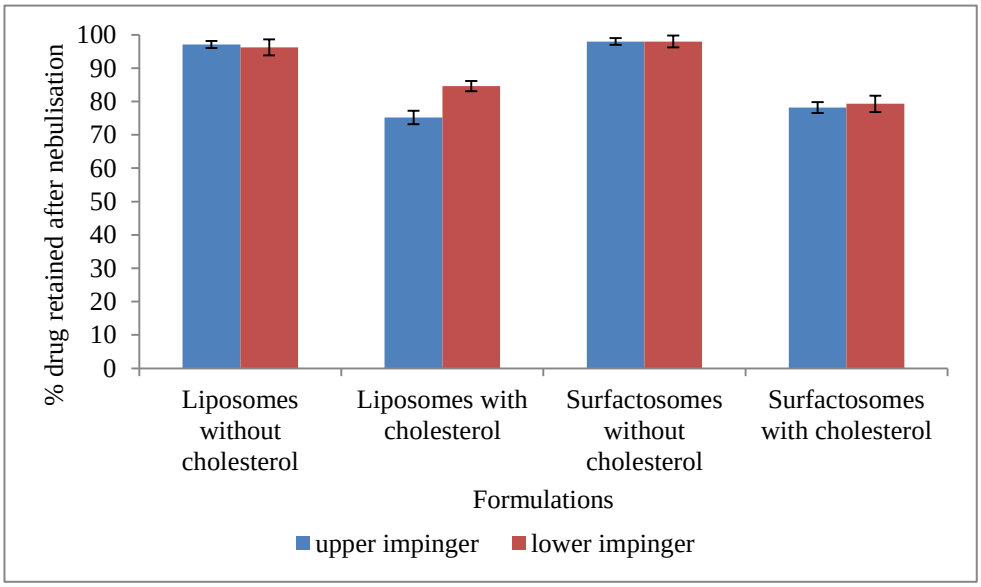

Figure 5.6 The retention of BDP in liposomes and surfactosomes with and without cholesterol after nebulisation through Aeroneb Pro nebuliser in both stages of impinger. Data are mean ±SD, n=3.

5.3.11. Nebulisation of BDP in conventional liposomes and surfactosomes to twin impinger using Beurer nebuliser

Beurer iH50 was used to study the efficiency of nebulisation using different vesicular formulations for pulmonary delivery as shown in Figure 5.7. It was observed that in upper stage, 23.2% of BDP in surfactosomes without cholesterol was delivered which was significantly higher than other vesicles ($p<0.05$). It was also observed that surfactosomes with cholesterol were the least delivered vesicle with only 10.7% BDP being delivered. In lower stage, there was significant difference between the delivery of all the vesicles ($p<0.05$). Surfactosomes without cholesterol delivered more BDP than the other formulations which was 44%. Hence, it was concluded that surfactosomes without cholesterol was the best formulation to deliver BDP using Beurer iH50 nebuliser to both stages of the twin impinger. This is possibly due to the high flexibility

and low rigidity of surfactosomes without cholesterol (Samad et al., 2007). This helped the vesicle to squeeze through the mesh of vibrating nebuliser without fragmentation. In vesicles with cholesterol, they tend to fragment more due to the rigidity provided by cholesterol. The vesicles are more elastic when they are cholesterol-free. Similar findings were found with Aeroneb Pro nebuliser in section 5.3.9. Similar findings were found in another study by Zaru *et al.* where presence of cholesterol in the formulation increased the drug leakage (Prabhakar et al., 2013).

It was also found that the vesicles delivered BDP significantly more in lower stage than in upper stage. Hence, it can be concluded that more BDP is likely to be delivered to lower respiratory tract than to the upper respiratory tract using Beurer iH50 vibrating mesh nebuliser. This is more desirable as BDP has many side effects in upper respiratory tract as discussed in section 5.3.9.

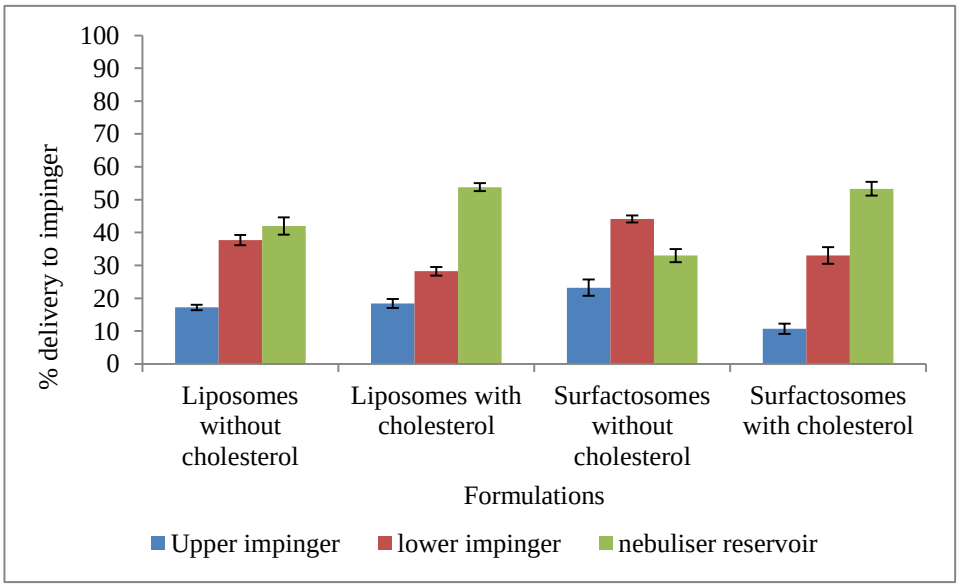

Figure 5.7 The delivery of BDP entrapped liposomes and surfactosomes with and without cholesterol after nebulisation through Beurer iH50 nebuliser to both stages of impinger. Data are mean ±SD, n=3.

5.3.12. BDP retained in conventional liposomes and surfactosomes following nebulization into twin impinger using Beurer nebuliser

Drug retained in vesicles after nebulisation using Beurer iH50 vibrating mesh nebuliser was studied as shown in Figure 5.8. It was observed that in upper stage liposome without cholesterol retained significantly more drug than other vesicles ($p<0.05$). There

was no significant difference between other vesicle's drug retention capacity ($p>0.05$). In lower stage, vesicles without cholesterol retained significantly more drug than vesicles with cholesterol ($p<0.05$). As studied and discussed in Chapter 4 section 4.3.10 there is competition between cholesterol and BDP for the entrapment in the bilayers, hence, in vesicles without cholesterol higher drug retention was found. However, as discussed in chapter 4 section 4.3.10 burst release effect may lead to an over estimation of drug entrapped in vesicles without cholesterol. Similar results were observed while using Aeroneb pro as discussed in section 5.3.10. Overall in liposomes and surfactosomes with cholesterol the drug entrapped was 89.3% and 85.4% respectively in upper stage and 86% and 89% respectively in lower stage. In liposomes and surfactosomes without cholesterol the drug entrapped was 97.7% and 91.3% respectively in upper stage and 95.1% and 94% respectively in lower stage.

It was also observed that there was significant difference in the drug retention by vesicles without cholesterol between upper stage and lower stage ($p<0.05$).Liposomes are delivered more to upper stage whereas surfactosomes are delivered more to lower impinger stage. This may be due to the elasticity of surfactosomes which make the aerosols reach to the lower stage more than upper stage. The mechanism of operation of Beurer iH50 is more specific to this characterisation. However, the percentage of BDP delivery is more than 85% for both the formulation in spite of the significant difference.

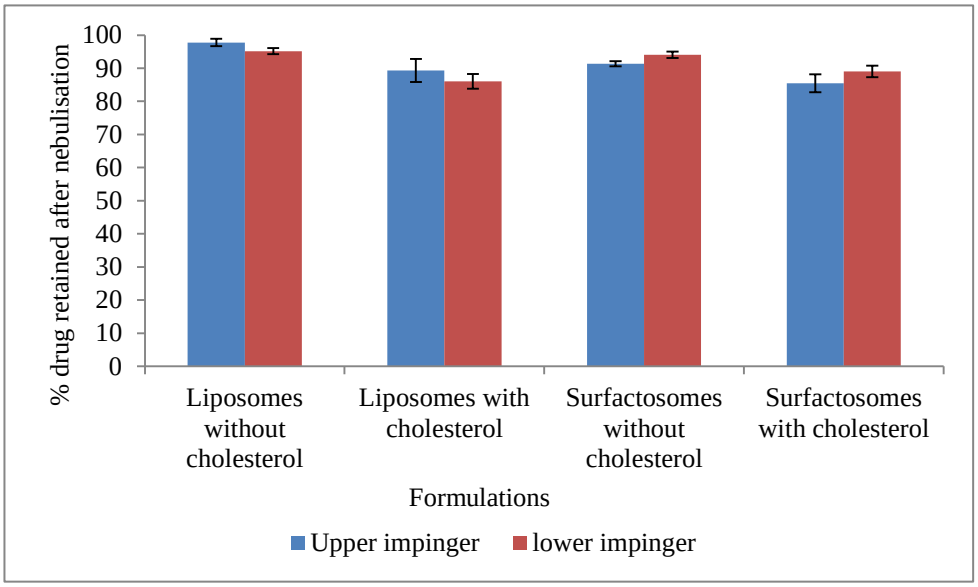

Figure 5.8 The retention of BDP in liposomes and surfactosomes with and without cholesterol after nebulisation through Beurer iH50 nebuliser in both stages of impinger. Data are mean ±SD, n=3.

5.3.13. Delivery of BDP using conventional liposomes and surfactosomes to twin impinger using PARI LC sprint nebuliser.

PARI LC sprint air Jet nebuliser was used to study the potential of different vesicle-based formulations for pulmonary delivery as shown in Figure 5.9. It was observed that in upper stage, 17.8% of BDP was delivered by surfactosomes with cholesterol which was significantly more than other vesicles ($p<0.05$). In lower stage there was significant difference in the drug delivery between all the vesicles ($p<0.05$). Surfactosomes without cholesterol delivered maximum BDP i.e. 62.8%. It was concluded that surfactosomes with cholesterol was the best formulation to deliver BDP using PARI LC sprint nebuliser to upper impinger while surfactosomes without cholesterol was the best formulation for lower impinger. This is due to the high flexibility of surfactosomes (El Maghraby et al., 2004). This helped the vesicle to squeeze through the mesh of vibrating nebuliser without fragmentation. In liposomes due to the absence of surfactant Tween 80 they become rigid, hence, more fragmentation.

It was also observed that there was significantly more BDP delivery in lower stage than in upper stage of twin impinger ($p<0.05$). Hence, it can be concluded that more BDP is likely to be delivered to lower tract than upper pulmonary tract using Air Jet nebuliser. This is more desirable as BDP has many side effects in upper respiratory tract as discussed in section 5.3.9.

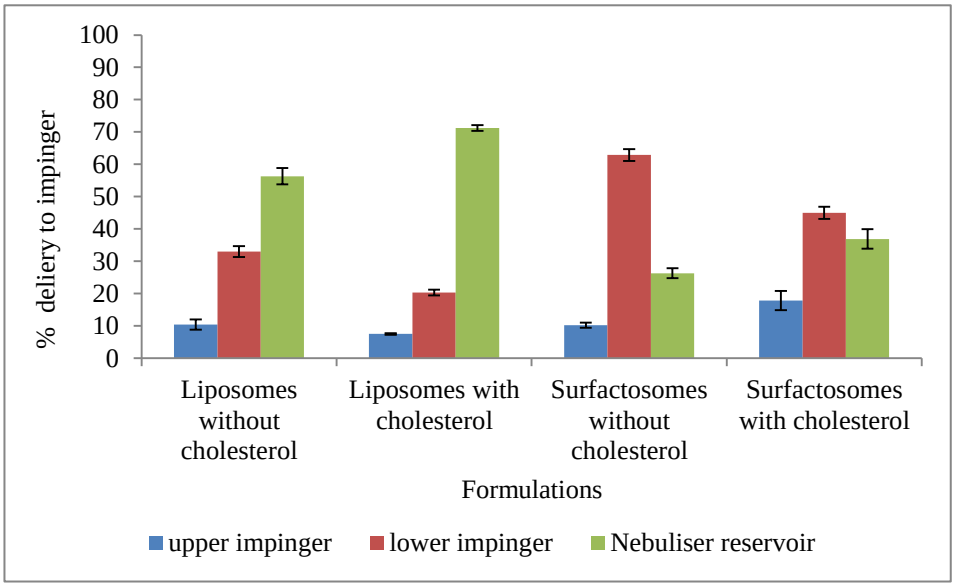

Figure 5.9 The delivery of BDP entrapped liposomes and surfactosomes with and without cholesterol after nebulisation through PARI LC sprint air jet nebuliser to both stages of impinger. Data are mean ±SD, n=3.

5.3.14. BDP retained in conventional liposomes and surfactosomes following nebulisation to the twin impinger using PARI LC sprint air Jet nebuliser

BDP retained in vesicles after nebulisation was studied as shown in Figure 5.10. It was observed that in upper stage, surfactosomes without cholesterol retained significantly less BDP than other vesicles ($p<0.05$). Other vesicle retained similar amount of drug. In lower stage similar results were obtained where only 88.2% of BDP was retained by surfactosomes without cholesterol ($p<0.05$). Other vesicles retained similar amount of BDP. Overall in liposomes and surfactosomes with cholesterol the drug entrapped was 98.6% and 98.1% respectively in upper stage and 98.7% and 97.4% respectively in lower stage. In liposomes and surfactosomes without cholesterol the drug entrapped was 98.7% and 88.1% respectively in upper stage and 98.9% and 88.2% respectively in lower stage. This shows that surfactosomes without cholesterol retains least BDP when nebulised via air jet nebuliser. This may be due to the loose packing of lipids in surfactosomes (Young et al., 1983, Tasi et al., 2003). Mechanism of operation of this nebuliser may also have a huge impact.

It was also observed that there was no significance difference between delivery of vesicles containing BDP in upper stage and lower stage of impinger ($p>0.05$).

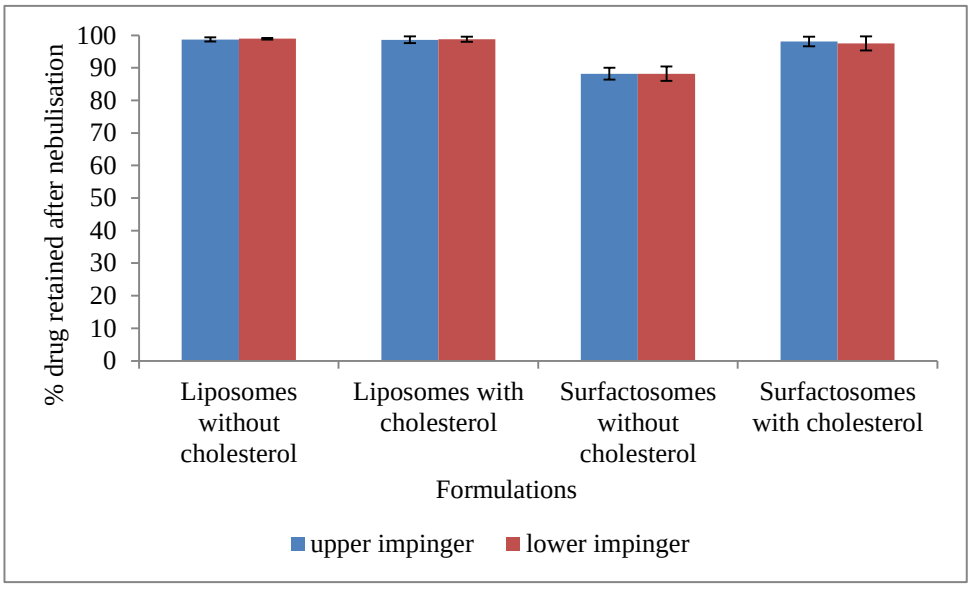

Figure 5.10 The retention of BDP in liposomes and surfactosomes with and without cholesterol after nebulisation through PARI LC sprint air jet nebuliser in both stages of impinger. Data are mean ±SD, n=3.

Hence, the best formulation for all three nebuliser was analysed. It is expected that the delivery of BDP to the lung can be maximised by using the formulation delivering the maximum drug proportion through the selected nebuliser.

Surfactosomes without cholesterol can be concluded as the best formulation after analysis of all the formulation. This was due to the elastic nature of these vesicles offered by the presence of Tween 80 and absence of cholesterol. Hence, the vesicles can easily squeeze through the meshed apertures of Aeroneb pro and Beurer iH50 and also sustain themselves without fragmentation upon shearing within the PARI LC sprint nebuliser. It can also be concluded that surfactosomes are better than liposomes for carrying higher BDP proportions to the twin impinger.

The nebuliser suitable for each formulation was also analysed. It was observed that for liposomes with and without cholesterol, Beurer iH50 vibrating mesh nebuliser was the best among the three nebulisers studied. It was suitable for liposomal formulation for both upper and lower stages of the impinger. For surfactosomes without cholesterol Beurer and Aeroneb Pro both were suitable for BDP delivery to upper impinger while the PARI LC sprint nebuliser was better for BDP delivery to the lower impinger. PARI LC sprint nebuliser delivers significantly higher drug proportions to lower impinger ($p<0.05$). For surfactosomes with cholesterol, all three nebulisers were equally good for upper stage impinger, while for lower stage impinger air jet nebuliser is significantly preferable over other nebulisers ($p<0.05$).

The aerosols generated for all there nebulisers were investigated for their VMD and span using laser diffraction. All four formulations were analysed for their stability and efficiency in their aerosol form.

5.3.15. VMD (size) and span (size distribution) of aerosols generated from conventional liposomes and surfactosomes using Aeroneb Pro nebuliser

VMD and span of aerosols generated from Aeroneb Pro vibrating mesh nebuliser were analysed as shown in Figure 5.11 and 5.12.

The experiment showed that there was no significant difference in VMD of aerosols generated from all four formulations ($p>0.05$). Similar results were observed for span. All four formulation using Aeroneb pro nebuliser produces aerosols with similar VMD and size distribution. This concludes that Aeroneb pro generates similar aerosols

irrespective of the formulations. This proves that the physiochemical characteristics of liposomes and surfactosomes with and without cholesterol did not affect the size of aerosol produced by Aeroneb Pro.

Hence, it can be concluded that the aerosol VMD and span are similar for all formulations generated from Aeroneb Pro nebuliser for all four formulations used in this study.

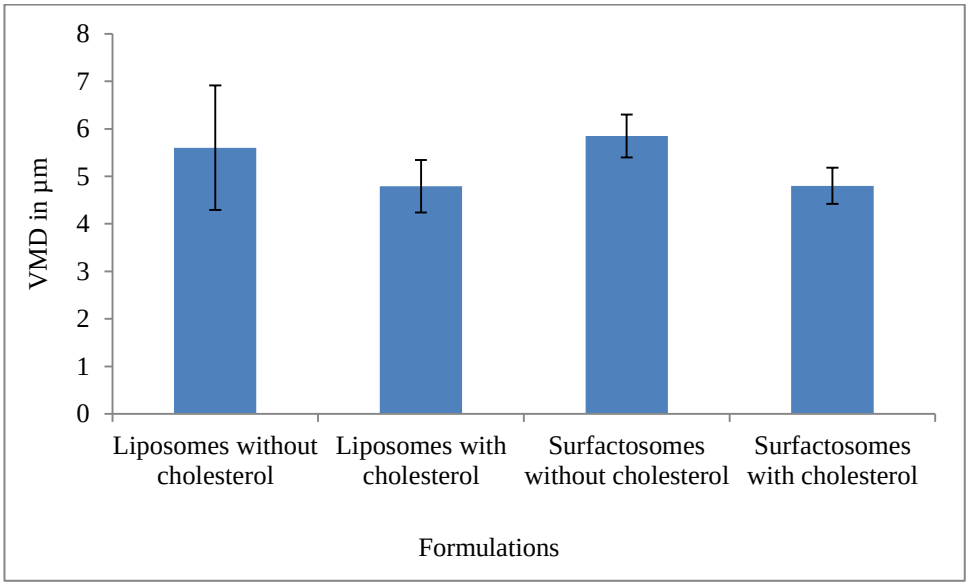

Figure 5.11 The VMD of aerosols of liposomes and surfactosomes with and without cholesterol after nebulisation through Aeroneb Pro nebuliser. Data are mean ±SD, n=3.

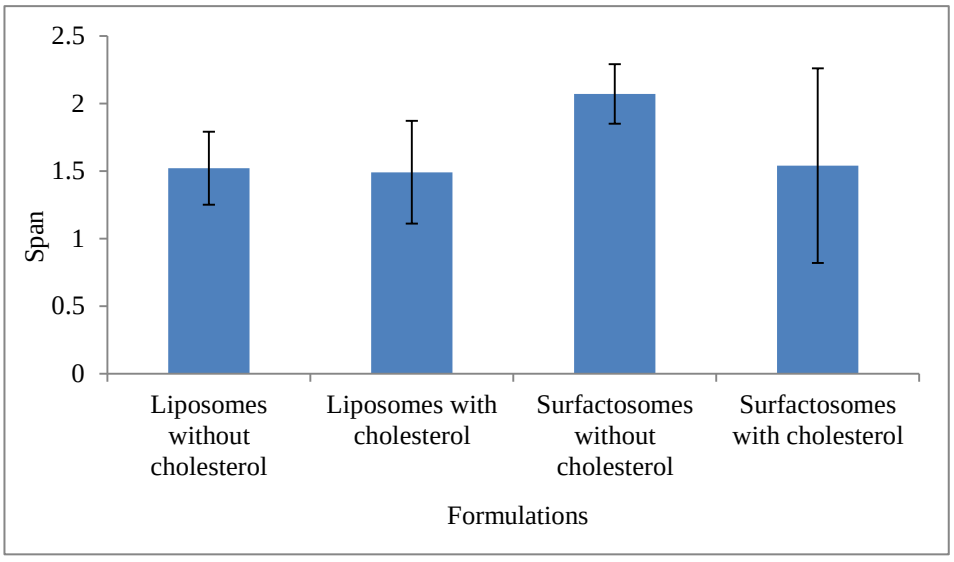

Figure 5.12 The span of aerosols of liposomes and surfactosomes with and without cholesterol after nebulisation through Aeroneb Pro nebuliser. Data are mean ±SD, n=3.

5.3.16. VMD (size) and span (size distribution) of aerosols generated from conventional liposomes and surfactosomes using Beurer iH50 nebuliser

VMD and span of aerosols generated from Beurer iH50 vibrating mesh nebuliser were analysed as shown in Figure 5.13 and 5.14.

It was observed that there was no significant difference in VMD of aerosols generated from all four formulations ($p>0.05$). Similar results were observed for span. All four formulation using Beurer iH50 nebuliser produces aerosols with similar VMD and size distribution. Similar to Aeroneb Pro, Beurer iH50 is a vibrating mesh nebuliser. As discussed in previous section 5.3.15, the properties of the aerosol generated from vibrating mesh nebuliser are independent of the physiochemical properties of the fluid nebulised. Hence, there is no difference in the VMD and size distribution of aerosols.

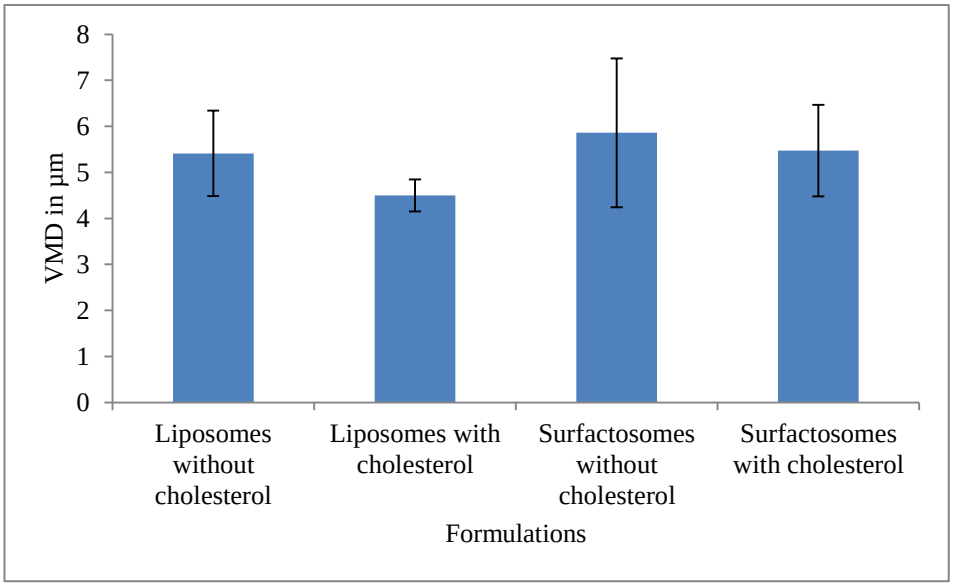

Figure 5.13 The VMD of aerosols of liposomes and surfactosomes with and without cholesterol after nebulisation through Beurer iH50 nebuliser. Data are mean ±SD, n=3.

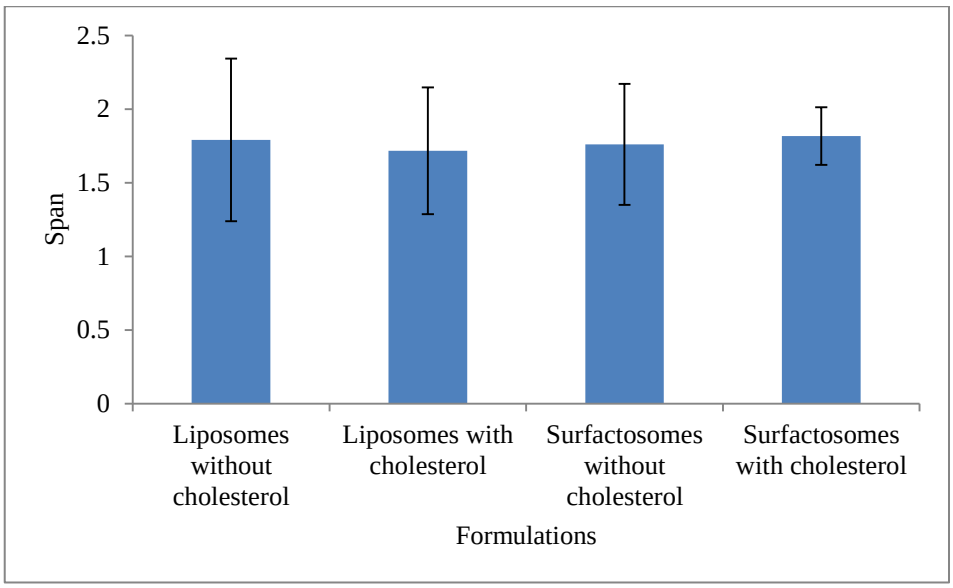

Figure 5.14 The span of aerosols of liposomes and surfactosomes with and without cholesterol after nebulisation through Beurer iH50 nebuliser. Data are mean ±SD, n=3.

5.3.17. VMD (size) and span (size distribution) of aerosols generated from conventional liposomes and surfactosomes using PARI LC sprint nebuliser

VMD and span of aerosols generated from PARI LC sprint air jet nebuliser were analysed as shown in Figure 5.15 and 5.16.

It was observed that the VMD of aerosols generated from surfactosomes with cholesterol was significantly more than liposome with cholesterol and surfactosomes without cholesterol ($p<0.05$) and slightly more than liposomes without cholesterol ($p>0.05$). Surfactosomes with cholesterol had the largest aerosol size. This may be due to the physiochemical properties of surfactosomes which is particularly affected in Jet nebulisers (Bridges and Taylor, 1998).

It was also observed that the span of liposomes without cholesterol was slightly but significantly greater than surfactosomes with and without cholesterol ($p<0.05$) and slightly more than liposome with cholesterol ($p>0.05$). This may be due the mechanism of operation of air jet nebuliser.

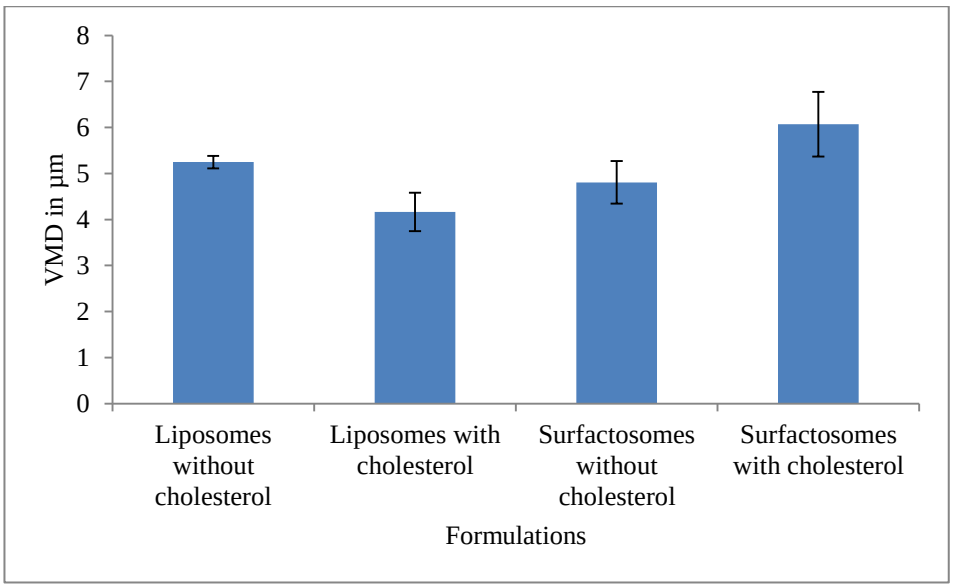

Figure 5.15 The VMD of aerosols of liposomes and surfactosomes with and without cholesterol after nebulisation through PARI LC sprint nebuliser. Data are mean ±SD, n=3.

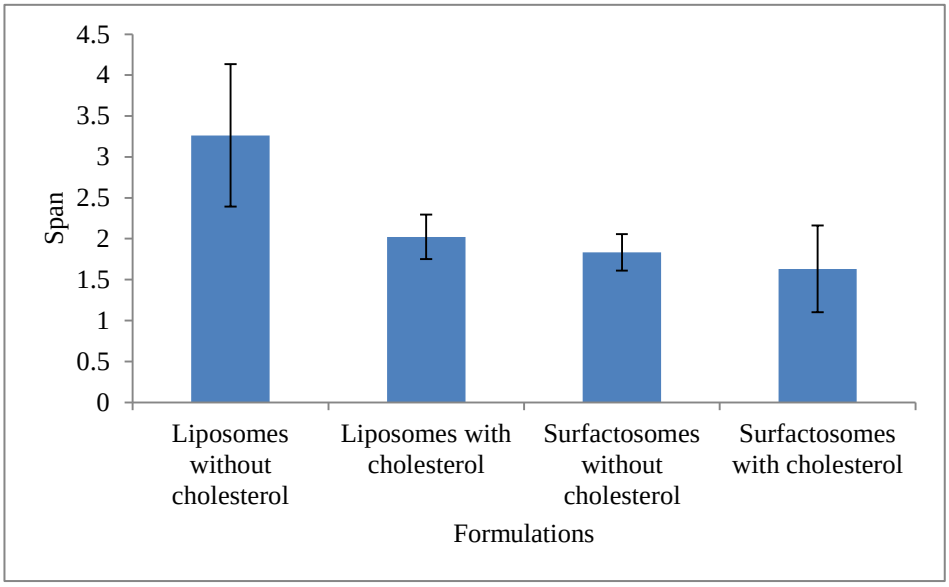

Figure 5.16 The span of aerosols of liposomes and surfactosomes with and without cholesterol after nebulisation through PARI LC sprint nebuliser. Data are mean ±SD, n=3.

It was observed that there was no significant difference in the VMD and span of aerosols generated using all the four formulations with Aeroneb pro, Beurer iH50 and PARI LC sprint nebuliser ($p>0.05$). All four formulations can be used with any of the three nebuliser as they produce similar sized and uniform aerosols.

It was also observed that the VMD of aerosols from all four formulations with all three nebulisers were larger than the VMD of liposomes and surfactosomes after nebulisation. Hence, the vesicle can easily be accumulated in the aerosol without being fragmented. This may minimise the drug leakage from vesicles during nebulisation. As all the vesicles are below 10µm in diameter they have the potential of being biologically active in susceptible individuals (Heyder, 2004, Hussain et al., 2011).

5.4. Conclusion

From this study it can be concluded that VMD and span of liposomes and surfactosomes with and without cholesterol decreases after nebulisation. This is due to the extrusion offered by meshes of Aeroneb Pro and Beurer iH50 nebuliser and sheer vibration by the PARI LC sprint nebuliser. The decrease in span also concludes that the uniformity of vesicles increase after nebulisation. It was found that zeta potential of all the vesicles after nebulisation were similar irrespective of the formulations, nebuliser used and the stage of impinger. This indicates that the vesicle may have similar pharmacokinetics after being delivered by nebulisation. However, zeta potential of the vesicles increased significantly after being delivered to impinger. This is may be due to the decrease in VMD of vesicles.

On studying the initial entrapment of BDP on all four formulations it was observed that vesicles without cholesterol entrapped significantly more BDP than vesicles with cholesterol. This was due to the completion between BDP and cholesterol to be incorporated into the vesicular bilayer due to their similar structure. It was also concluded that surfactosomes without cholesterol is the best formulation to deliver BDP via nebulisers. Due to its high elasticity and flexibility it can easy squeeze through the meshes of vibrating nebulisers without being fragmented. Hence, maximum hydrophobic drug BDP was delivered to both the stages of impinger using surfactosomes as a carrier.

All three nebulisers were analysed and compared for delivery of BDP using liposomes and surfactosomes with and without cholesterol. It was found that liposomes with and without cholesterol, Beurer iH50 was the better nebuliser for maximum delivery of BDP via liposomes to both lower and upper impinger when compared to Aeroneb pro and PARI LC sprint nebuliser. Hence, Beurer iH50 can be concluded as the better nebuliser

to deliver liposomes. Similarly, for surfactosomes with cholesterol PARI LC nebuliser was better than other two to deliver BDP to lower impinger.

It was also studied that using all three nebulisers maximum BDP was delivered to the lower impinger stage as compared to upper impinger stage. This also concludes that for hydrophobic drug delivery, vibrating mesh nebulisers are better for upper stage whereas jet nebulisers are better for lower stage. Delivery of BDP is not much desirable for upper respiratory system as it has many side effects in upper respiratory tract as discussed in section 5.3.9. This study will, thus, help to choose the perfect nebuliser and vesicle for hydrophobic drug delivery.

On studying the BDP retention in vesicles after nebulisation, it was concluded that using both vibrating mesh nebulisers, vesicles with cholesterol retained less BDP that those without cholesterol. This is due to the competition between cholesterol and BDP which displaces the BDP. In Air jet nebuliser, surfactosomes without cholesterol retained the least drug after nebulisation compared to other formulations. This may be due to the inability of surfactosomes to retain its stability in the presence of sheer force generated by the jet nebuliser.

It can also be found that VMD of aerosols were bigger than the VMD of liposomes and surfactosomes after nebulisation. Hence, the vesicle can easily be accumulated in the aerosol without being fragmented. This may minimise the drug leakage from vesicles during nebulisation.

Hence, from this study it can be concluded that an ultradeformable vesicle, surfactosome, is formulated and delivers more BDP to lower impinger than conventional liposomes due to improved elasticity. These surfactosomes can withstand the stress provided by nebuliser during nebulisation better than liposomes.

Chapter 6

Characterisation of vesicles and stability studies

6.1. Introduction

Various processes are available to make a dry powder formulation of vesicles to increase their stability. Freeze drying and spray drying are common techniques used for this purpose.

Spray drying is a one-step process of drying that can have applications in designing dry powders of vesicle formulations. This process has the ability to produce spherical micro-particle powders with good flow properties, high porosity and low density. Thus, "respirable" dry powdered liposomes have been manufactured using the spray drying technology (Lo et al., 2004). Typical spray drying sequences that occur within fractions of a second are atomization of feed into a spray, spray-air contact, moisture evaporation of the sprayed droplets, and separation of the dried particles from the air (Lo et al., 2004, Charnvanich et al., 2010). High temperature in spray drying may lead to thermal degradation of protein which is a major concern. This activity loss of proteins occurs due to protein's sensitive structural alteration due to heat (Lo et al., 2004).

Freeze drying is commonly known as "lyophilisation" and is considered a promising means of extending the shelf-life of vesicles. As freeze drying is a low temperature process it is unlikely to cause thermal degradation of liposomes and surfactosomes (Lu and Hickey, 2005, Pikal, 2006). Freeze drying can be viewed as a three-step process consisting of freezing, primary drying and secondary drying. Both freezing and drying may cause instability problems and stress to liposomes and surfactosomes as a result of the induced structural or functional damage to the vesicles during freezing and drying. This in turn leads to leakage of the encapsulated drug on rehydration, thus compromising integrity of vesicular formulations (Bridges and Taylor, 2001). Even the addition of lycoprotectants like mannitol may not prevent the destruction of vesicles.

In this chapter, the stability of liposomes and proliposomes are analysed. Spray drying and freeze drying was performed on liposomal and surfactosomal dispersion to analyse their stability due to heat, freezing and drying. Stability of proliposomes and prosurfactosomes on being stored for a long time in different environments like room temperature, 2-8°C and 40°C were also analysed. The appearance of proliposomes and prosurfactosomes were analysed using Scanning electron microscopy (SEM) after and before spray drying and freeze drying. X-ray diffraction was performed for further analysis of the samples after freeze drying and spray drying. Transmission electron

microscopy was also used in this chapter to visualise liposomes and surfactosomes with SBS and BDP.

6.2. Methods

6.2.1. Preparation of proliposomes

For proliposomes with cholesterol, SPC and cholesterol were used in 1:1 molar ratio. The lipid phase was dissolved in chloroform (20mg/ml) within a round bottom flask. Carbohydrate based carrier particle mannitol was added to this lipid phase in 1:5 lipid to mannitol ratio for proliposomes with BDP formulations. BDP was added in 2.5 Mole % to lipid phase. The organic solvents were removed using a rotary evaporator as described in section 2.2.1. After detaching the flask, the proliposomes were collected using a clean spatula. They were stored in room temperature and used immediately.

6.2.2. Preparation of prosurfactosomes

For prosurfactosomes with cholesterol, SPC and cholesterol were used in 1:1 molar ratio with Tween 80 (15% w/w of the total lipid) in a round bottom flask. These were dissolved in chloroform (20 mg/ml) in a round bottom flask. Carbohydrate based carrier particles were made by using mannitol which was incorporated into the lipid phase in 1:5 lipid to mannitol ratio for prosurfactosomes with BDP. BDP was added in 2.5 mole% to the lipid phase. The organic solvent was evaporated as described in section 2.2.1. After the evaporation of the solvent the flask was detached and the proliposomes were collected using a clean spatula. It was stored in room temperature and was used on the same day.

6.2.3. Hydration of vesicles

The proliposomes and prosurfactosomes were hydrated to form liposomal and surfactosomal dispersions. They were hydrated with HPLC water in the concentration 10mg/ml. For entrapment studies formulations were hydrated with D_2O in to make dispersions with lipid concentration of 10mg/ml.

6.2.4. Spray drying

Spray dryer was used to convert the liposomes and surfactosomes prepared by proliposome technology to dry powder form. BUCHI mini spray dryer B-290,

Switzerland, was used for this purpose. All parts of the spray dryer were assembled to ensure it was air tight. The temperature was set to 120ºC, Aspiration was 100% and pump was 15%. After the final assembly, aspirator and temperature were turned on. After reaching a stable input and output temperature, nitrogen inlet was turned on. The pressure of 8-9 bars was attained, and the pump was also turned on. Initially distilled water was spray dried to clean the instrument and tubes. Liposomal and surfactosomal dispersions (30 ml) were passed through the nozzle to be spry dried. The vesicular dry powder was collected from the collecting chamber after it was allowed to cool down and the powder was used for further characterisation.

The yield of dry proliposomes and prosurfactosomes was calculated. 30ml of liposomal and surfactosomal formulation contained 1.8g of dry ingredients (lipid, BDP and mannitol). Hence, yield was the weight of dried liposomes and surfactosomes collected after spray drying in the collecting chamber.

Percentage yield was calculated by comparing the collected weight of proliposomes/prosurfactosomes after spray drying and the original weight of proliposomes/prosurfactosomes used.

$$\%\ yield = \frac{Amount\ of\ dry\ vesicles\ \ collected\ (g)}{Amount\ of\ dry\ ingredients\ originally\ used\ (g)} \times 100$$

6.2.5. Freeze drying

As an alternative to spray drying, freeze drying was used to convert the liposomes and surfactosomes prepared by proliposome technology to dry powder. The freeze dryer Scanvac Coolsafe 110-4, UK was used for this purpose. The liposomes and surfactosomes were initially kept in wide mouthed 30ml glass vials and frozen overnight to get a frozen sample. The vials were covered with parafilm having small holes to enable the frozen water to sublime during freeze drying. The freeze dryer was turned on and the temperature was set at -110ºC. Once this temperature was attained, the frozen samples were kept in the freeze dryer and the vacuum was turned on using a vacuum pump. This sample was kept in the freeze dryer for 24 hours till water sublimed leaving a dry sample. The vacuum was released and the samples were taken out. The condensed water was removed from the instrument

6.2.6. X-ray diffraction

X-ray diffraction was performed to check the crystallinity of proliposomes and prosurfactosomes before and after the process of spray drying and freeze drying. X-ray diffraction instrument (D2 Phaser, Bruker, UK) was used for this purpose. The powdered sample was placed on the dry shallow well of the sample holder having the diameter of 25mm. A densely packed flat and smooth surface was attained to get accurate results. A flat surface was used to press down the powder packing into the well. The sample holder was placed in the X-ray diffraction instrument. A range of 5-50 theta was selected and the instrument was set for 30 min.

6.2.7. Stability studies

For the stability studies, proliposomes and prosurfactosomes were stored in different environmental conditions like room temperature, 40°C and in 5-6ºC. For room temperature, the samples were kept on the bench, whilst for 40°C the samples were kept in an incubator (Binder, USA), and for 2-8°C they were stored in the refrigerator (Labcold, Sparkfree Fridge Freezer RLFF13246, UK). Proliposomes/prosurfactosomes (600 mg) was hydrated to form 10ml of liposomal and surfactosomal dispersion once every 2 weeks for a period of 3 months. They were analysed for VMD, span, surface charge (i.e. zeta potential), pH and BDP entrapment. Only 1 repetition of experiment was considered in this particular study.

6.2.8. pH test

PH meter was used to test the acidity/basicity of the liposomal and surfactosomal dispersions. Hanna Instruments 2221 pH meter, UK was used for this purpose. Initially the instrument was calibrated using the standard pH buffers with pH values of 4 and then with pH value of 7. The desired samples were then tested for their respective pH values. The pH meter was calibrated after every 2 readings for accuracy.

6.3. Results and discussions

6.3.1. Initial VMD, span and zeta potential of liposomes and surfactosomes

VMD, span and zeta potential of liposomes and surfactosomes before spray drying and freeze drying were recorded as shown in Table 6.1. As discussed in chapter 5, there was no significant difference between liposomes and surfactosomes with regard to VMD, span ad zeta potential ($p>0.05$). The VMD, span and zeta potential after applying the drying procedures were compared with those before drying.

Table 6.1 VMD, span and zeta potential of liposomes and surfactosomes before spray drying and freeze drying. Data are mean ±SD, n=3.

Formulations	VMD (µm)	Span	Zeta potential (mV)
Liposomes	5.38 ± 0.41	1.64 ±0.49	-2.8± 0.19
Surfactosomes	4.79 ± 0.22	1.7 ± 0.24	-3.7 ±0.24

6.3.2. Spray drying of liposomes and surfactosomes

6.3.2.1. VMD, span and zeta potential after spray drying

The VMD, span and zeta potential (surface charge) of liposomes and surfactosomes after spray drying were analysed in shown in Table 6.2. It was observed that after spray drying there was no significant difference in the VMD of liposomes and surfactosomes ($p>0.05$). Similar results were found for span of liposomes and surfactosomes after spray drying ($p>0.05$). However, it was observed that there is significant difference between the surface charge of vesicles after spray drying ($p<0.05$). Surfactosomes appeared to have more negative zeta potential than liposomes ($p<0.05$). The presence of Tween 80 seems to increase the negative surface charge of vesicle. Sorbitan esters, polyoxyethelene delivatives, are fatty acid esters of sorbitol and its anhydrides copolymerised with a varying number of moles of ethylene oxide. Polysorbate 80 (Tween 80) is an oleate ester (Remington et al., 2006, Rowe et al., 2009). There is a possibility of by products like free fatty acids like linoleic acid, palmitic acid and stearic acid to be present as impurities as verified from Sigma Aldrich, UK. It is possible that these acids on dissociation on the surface of a particle gave rise to a negatively charged surface as discussed in section 1.7 and 4.3.2. Similar observation of increase in negative

charge of solid lipid nanoparticle on addition of Tween 80 in the formulation was found by Prabhakar *et al.* in 2013 (Prabhakar et al., 2013). Hence, surfactosomes are significantly more negative than liposomes.

It can also be observed that there was no significant difference between the VMD and span of vesicles before and after spray drying as shown in Table 6.1 ($p>0.05$). However there was a significant difference between the charge of vesicles before and after spray drying ($p<0.05$). This leads to the conclusion that heat and stress generated during spray drying did not compromise the stability of liposomes and surfactosomes in terms of VMD and span.

Table 6.2 Table showing the VMD, span and charge of liposomes and surfactosomes after spray drying. Data are mean ±SD, n=3.

Formulations	**VMD (µm)**	**Span**	**Zeta potential (mV)**
Liposomes	4.42 ± 0.39	1.42 ± 0.08	-5.8 ±1.83
Surfactosomes	6.36 ± 1.02	3.76 ± 2.27	-10.1 ± 1

6.3.2.2. Retained entrapment of BDP after spray drying

Entrapment of liposomes and surfactosomes before and after spray drying was analysed as shown in Figure 6.1. It was observed that there was no significant difference in the entrapment of BDP in both the vesicles before and after spray drying (Figure 6.1). Liposomes and surfactosomes retained 92.8% and 90.84% of BDP respectively after spray drying. Thus, surfactosomes and liposomes were stable after facing the heat and stress generated during spray drying.

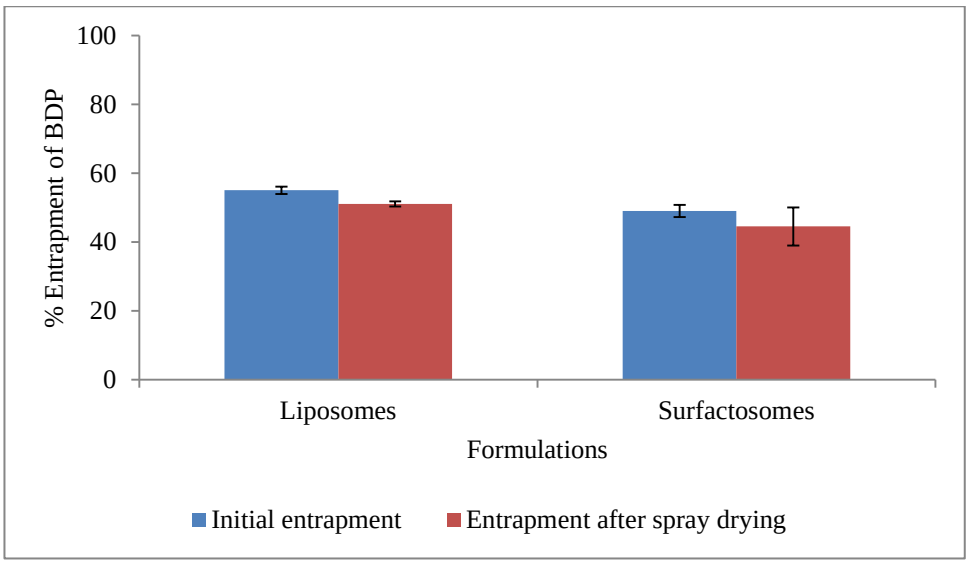

Figure 6.1 The entrapment of BDP before and after spray drying in liposomes and surfactosomes. Data are mean ±SD, n=3.

6.3.2.3. Drug loading of BDP in liposomes and surfactosomes before and after spray drying

The drug loading of BDP in proliposomes and prosurfactosomes before and after spray drying were calculated. This was calculated to give the quantity of drug loaded in 100mg of lipid. This study will help to analyse if formulation is economically feasible for drug entrapment. As shown in Figure 6.2, there is no difference in the loaded BDP before and after spray drying in both vesicles ($p>0.05$). This shows that both surfactosomes and liposomes were stable after facing the heat and stress generated during spray drying.

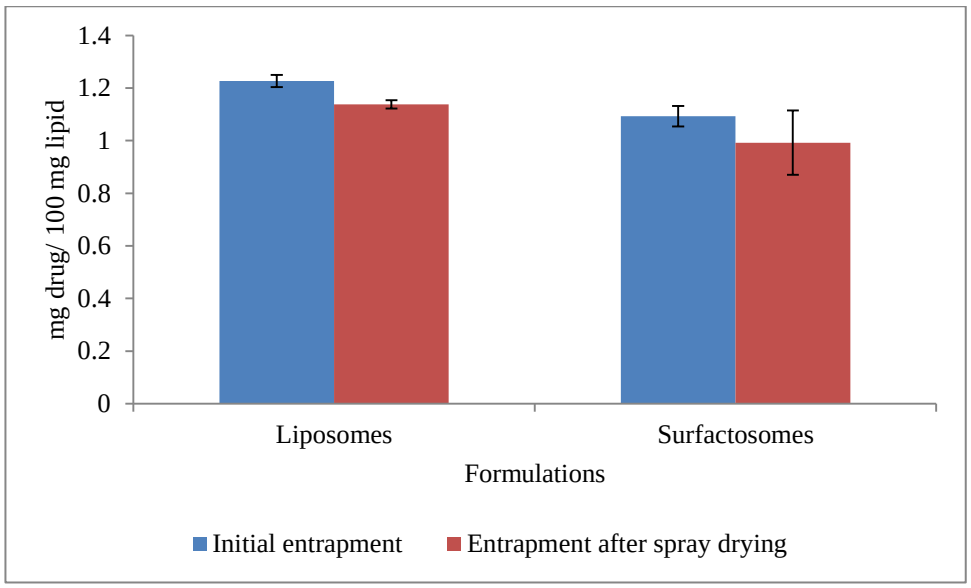

Figure 6.2 The drug loading of BDP before and after spray drying in liposomes and surfactosomes. Data are mean ±SD, n=3.

6.3.2.4. Yield after spray drying

Amount of proliposomes and prosurfactosomes collected in the collecting chamber after spray drying was recorded as shown in Table 6.3. It was observed that there was no significant difference in the percentage yield of proliposomes and prosurfactosomes after spray drying ($p>0.05$). 73% of proliposomes was collected while 82% of prosurfactosomes were collected compared to the original amount of dry ingredients used in the vesicular dispersion. After spray drying 30ml (1.8g) of vesicular dispersion (lipid with mannitol), 1.3g of spray dried proliposomes and 1.4g of spray dried prosurfactosomes were collected.

Hence, it can be concluded that surfactosomes have a trend for slightly higher yield than liposomes.

After analysing the VMD and span, zeta potential, BDP retention and yield of liposomes and surfactosomes it was found that both liposomes and surfactosomes are equally stable to spray drying. They are not much affected by the heat and stress generated during the process. This may be due to the absence of heat sensitive materials in the vesicles. Spray drying can, hence, be used to increase the stability of surfactosomes.

Table 6.3 Yield and percentage yield of spray dried proliposomes and prosurfactosomes after spray drying. Data are mean ±SD, n=3.

	Proliposomes	**Prosurfactosomes**
Yield	1.314g ± 0.46	1.480 g ± 0.08
% yield	73%	82%

6.3.3. Freeze drying of liposomes and surfactosomes

6.3.3.1. VMD, span and zeta potential after freeze drying

VMD, span and zeta potential of liposomes and surfactosomes after freeze drying was analysed as shown in Table 6.4. It was observed that there was significant difference between the VMD and span of both vesicles after freeze drying ($p<0.05$). Surfactosomes had significantly large VMD and span than liposomes. However, there was no significant difference between the zeta potential values of both formulations ($p>0.05$).It was also observed that there was no significant difference for VMD and span of liposomes after freeze drying when compared to the measured values before freeze drying (Figure 6.1) ($p>0.05$). However, VMD and span of surfactosomes increased significantly after freeze drying ($p<0.05$). This may indicate that liposomes are more stable to freeze drying than surfactosomes. Surfactosomes had physical instability like aggregation and fusion after freeze drying. Surfactosomes may be more fragile than liposomes due to its elastiticity. This may make it less resistant and more vulnerable during freeze drying. Concentration of cryoprotectant is an important factor determining its efficiency in protecting the nanoparticle (Abdelwahed et al., 2006). As discussed in section 1.14.1.1, it is possible that the concentration of cryoprotectant mannitol used in formulation was not optimum for surfactosomes as the integrity of vesicle was not protected by mannitol. The used quantity of mannitol in the formulation did not help in vitrification and replacing water efficiently. This was observed by increased size and span after freeze drying. Further studies are required to optimise it. This may also be due to the presence of surfactant which increases the tendency of surfactant to aggregate (Tasi et al., 2003).

Table 6.4 VMD, span and zeta potential of liposomes and surfactosomes after freeze drying. Data are mean ±SD, n=3.

Formulations	VMD (µm)	Span	Zeta potential (mV)
Liposomes	4.95 ± 0.3	1.91 ± 0.36	-10.7 ± 1.05
Surfactosomes	7.88 ± 0.93	7.11 ± 1.59	-12.76 ± 0.4

6.3.3.2. Retained entrapment of BDP after freeze drying

BDP entrapment of liposomes and surfactosomes before and after freeze drying was analysed as shown in Figure 6.3. It was observed that there was no significant difference in the initial entrapment of BDP in proliposomes and prosurfactosomes. However, It was observed that there was significant loss of BDP in prosurfactosomes as compared to proliposomes after freeze drying ($p<0.05$). Liposomes and surfactosomes retained 91.3% ± 2.1 and 86.3% ±1.2 of the originally entrapped BDP respectively after freeze drying. Hence, surfactosomes are possibly less stable than liposomes, owing to the greater leakage of the originally entrapped steroid. It is likely that freezing followed by drying have caused structural damage to the surfactosomes, causing the bilayers to leak greater proportions of the originally entrapped BDP. Freezing may cause phase transition changes, osmotic stress and expansion of the bilayers due to ice formation (Bridges and Taylor, 2001). The stability of bilayers depends on hydrogen bonding between water molecules and the polar head groups of the phospholipid molecules in the bilayers. The process of drying may lead to loss of water which may lead to changes in the bilayer behavior and loss of vesicle integrity. like bilayer damage, or vesicle fusion or aggregation, ultimately causing loss of the originally entrapped material (Bridges and Taylor, 2001). Lyoprotectants like mannitol helps in stabilisation of nanoparticles by water replacement hypothesis where there is a formation of hydrogen bond between the lyoprotectant and the polar groups on the surface of nanoparticles. It also helps in vitrification as discussed in 1.14.1.1 (Grahame, 1947, Olton, 2008). Surfactosomes are possibly more fragile than liposomes due to presence of surfactant, Tween 80, and its elasticity. Concentration of mannitol in surfactosomal formulation used in this study also may not be optimum enough to preserve its native structure. Further studies are required to optimise it.

Hence, surfactosomes were more unstable than liposome and less able to resist the stress provided by freeze drying.

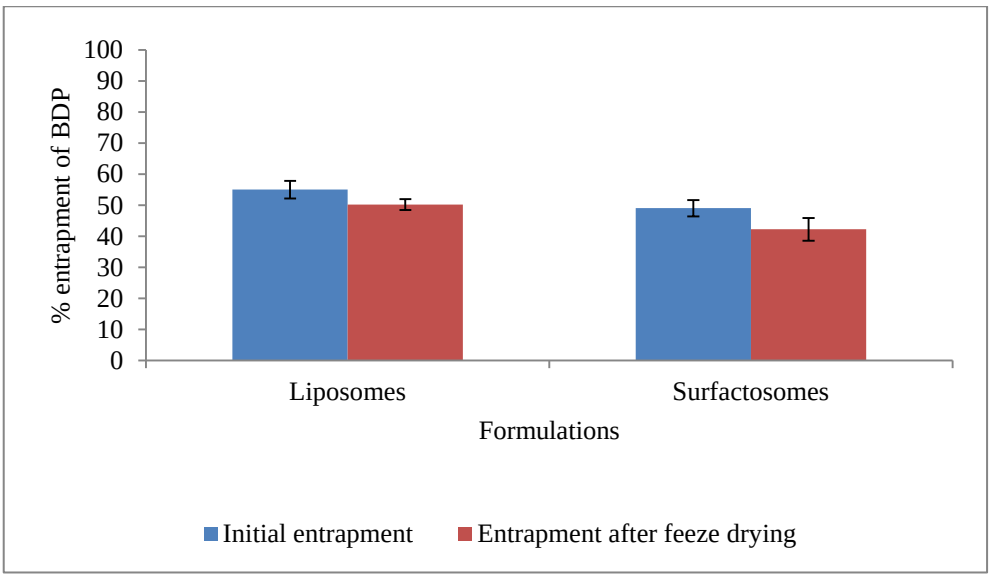

Figure 6.3 Entrapment of BDP before and after freeze drying in liposomes and surfactosomes. Data are mean ±SD, n=3.

6.3.3.3. Drug loading of BDP in liposomes and surfactosomes before and after freeze drying

The drug loading of BDP in proliposomes and prosurfactosomes before and after freeze drying were calculated. This was calculated to give the quantity of drug entrapped by 100mg of lipid. This study will help to analyse if formulation is economically feasible for drug entrapment. As shown in Figure 6.4, there is no significant difference in the BDP loading in liposomes and surfactosomes ($p>0.05$). However, after freeze drying there is significant loss of BDP from surfactosomes as compared to liposomes. This shows that surfactosomes are less stable that liposomes after freeze drying owing to the greater leakage of the originally entrapped drug.

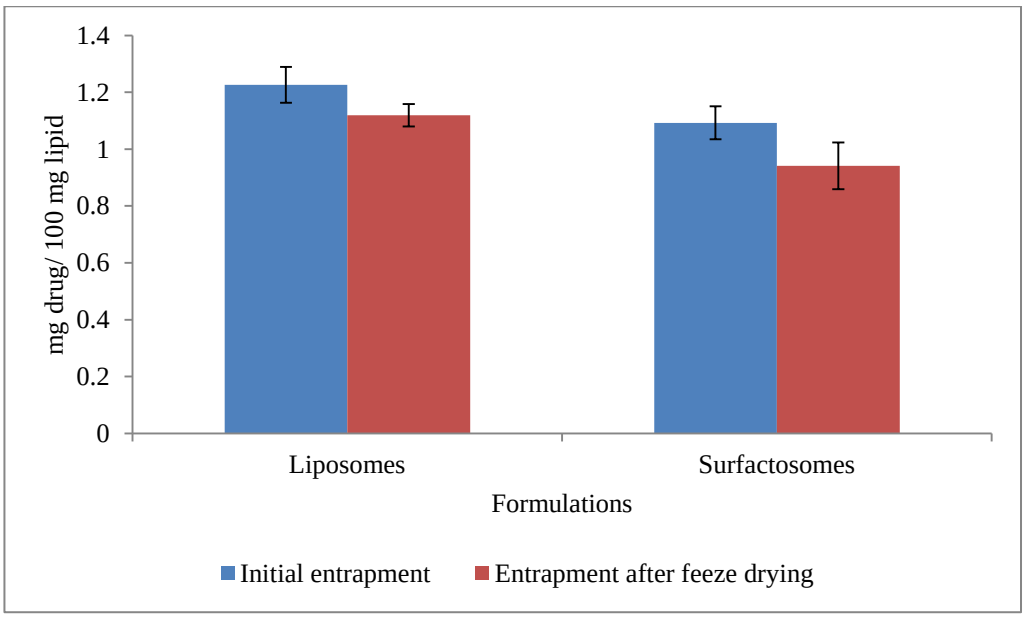

Figure 6.4 Dug loading of BDP before and after freeze drying in liposomes and surfactosomes. Data are mean ±SD, n=3.

6.3.4. SEM analysis of proliposomes and prosurfactosomes before and after spray drying ad freeze drying.

6.3.4.1. SEM studies of mannitol, proliposomes and prosurfactosomes

Morphology of mannitol, proliposomes and prosurfactosomes were studied in Figure 6.5 (a) and (b) and Figure 6.6(a)-(d) using SEM analysis. It was observed that the surface of mannitol became smoother after coating with phospholipid and cholesterol for both proliposome and formulations. The porosity of mannitol was decreased because of coating with lipids on the outer surfaces of the mannitol carrier particles. According to the SEM images, prosurfactosomes appeared to be slightly smoother than proliposomes, which may be due to the presence of Tween 80 in prosurfactosomes. The VMD of proliposomes and prosurfactosomes ranged between approximately 100-300µm while size of mannitol particles was less than 200µm. Thus, coating the carrier particles with phospholipid and cholesterol was responsible for size increments.

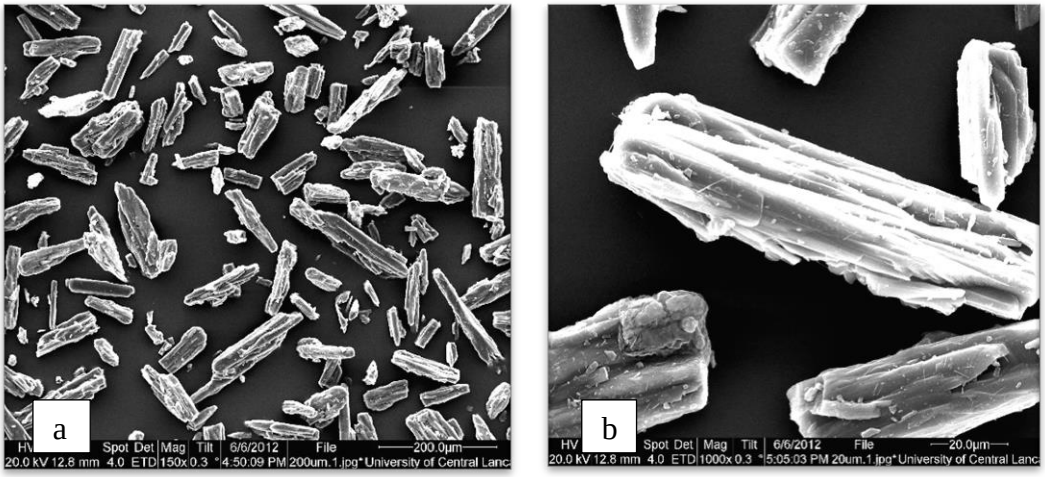

Figure 6.5 (a) and (b). Structure of mannitol under SEM

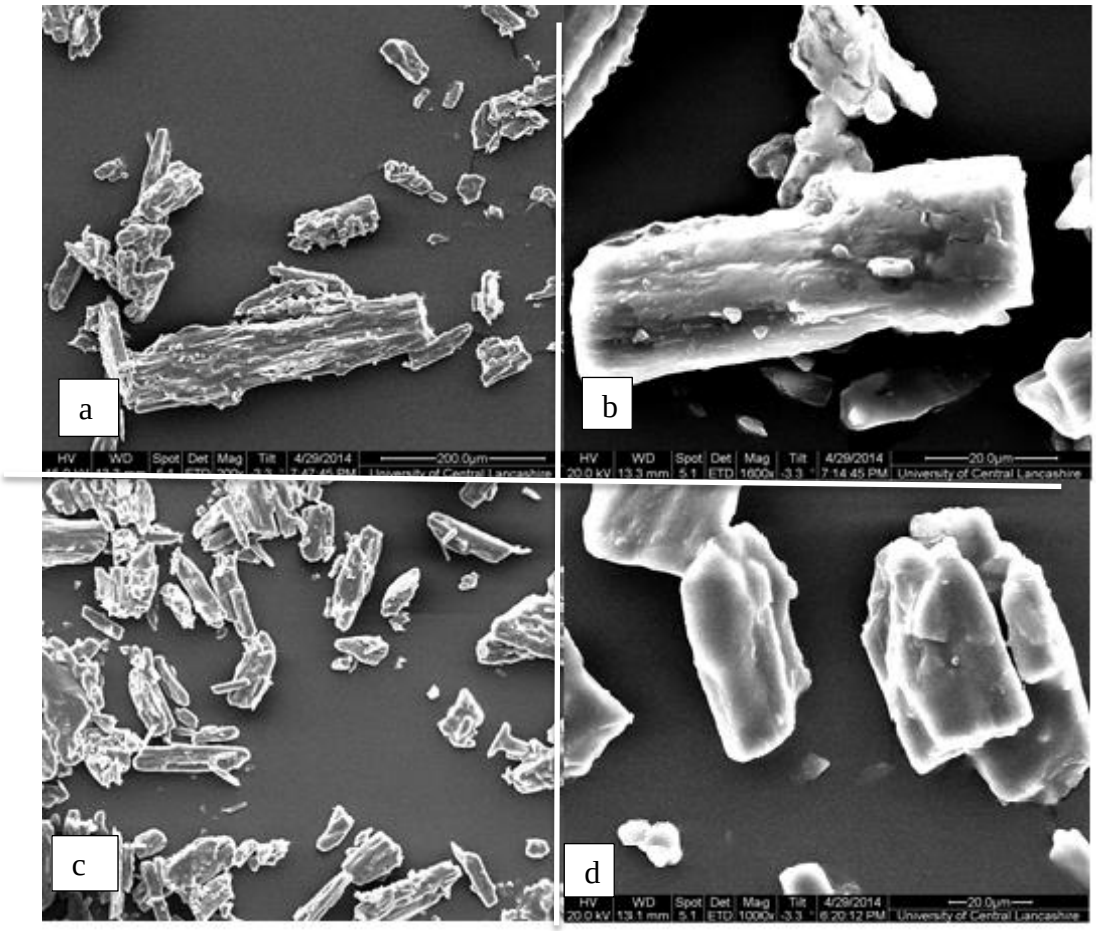

Figure 6.6 SEM of (a) proliposomes (200µm scale) (b) Proliposomes (20µm scale) (c) Prosurfactosomes (200µm scale) and (d) Prosurfactosomes (20µm scale)

6.3.4.2. SEM analysis of proliposomes and prosurfactosomes with cholesterol after spray drying

Morphology of proliposomes and prosurfactosomes were analysed after spray drying in Figure 6.7 (a)-(d). It was observed that after spray drying proliposomes and prosurfactosomes became small in size and spherical in shape. They formed microspheres and their size was reduced significantly to less than 5µm. The particle size distribution was homogenous. There was also no apparent difference in the morphology when proliposomes were compared to prosurfactosomes.

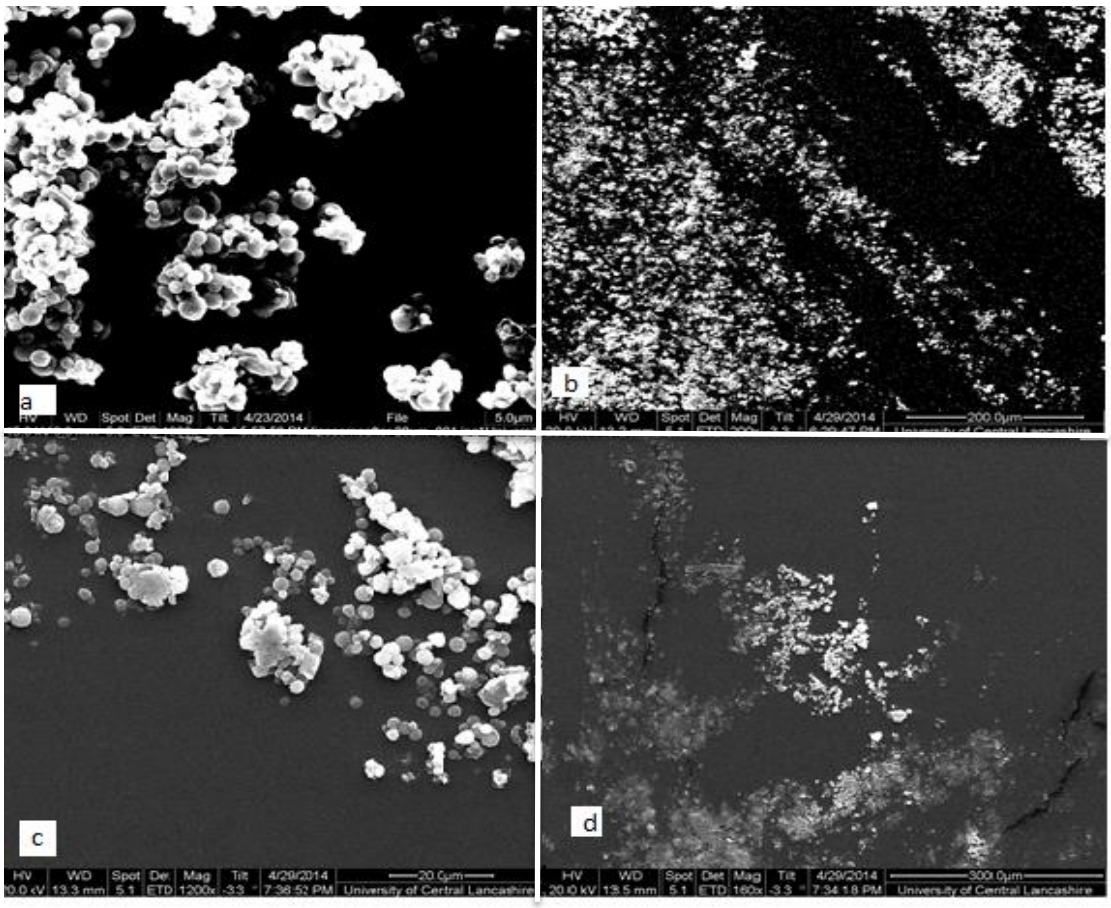

Figure 6.7 SEM of (a) proliposomes after spray drying (20µm scale) (b) Proliposomes after spray drying (200µm scale) (c) Prosurfactosomes after spray drying (20µm scale) and (d) Prosurfactosomes after spray drying (200µm scale)

6.3.4.3. SEM analysis of proliposomes and prosurfactosomes after freeze drying

Morphology of proliposomes and prosurfactosomes were analysed after freeze drying in Figure 6.8 (a)-(d). It was observed that after freeze drying, proliposomes and prosurfactosomes underwent a change in their smooth morphology to become needle

shaped and porous. The needle shaped structure may be due to the crystallisation of mannitol as discussed in chapter 4 (section 4.3.6). Yoshinari *et al* (2003) have shown that SEM images of mannitol had needle shaped structures due to the presence of β form of mannitol (Yoshinari et al., 2003). They had flake like structure. Porous structures can lead to fast reconstitution (Lee et al., 2007). The pores formed may be due to the vestige of sublimation of ice in the drying stage, leaving sharpness to the structure. They became sharper and smaller than those before freeze drying. The size decreased to approximately 20-50µm. It was observed that the morphology of prosurfactosomes after freeze drying was less porous than that of proliposomes. This may be due to the presence of the surfactant Tween 80 in the prosurfactosome formulation.

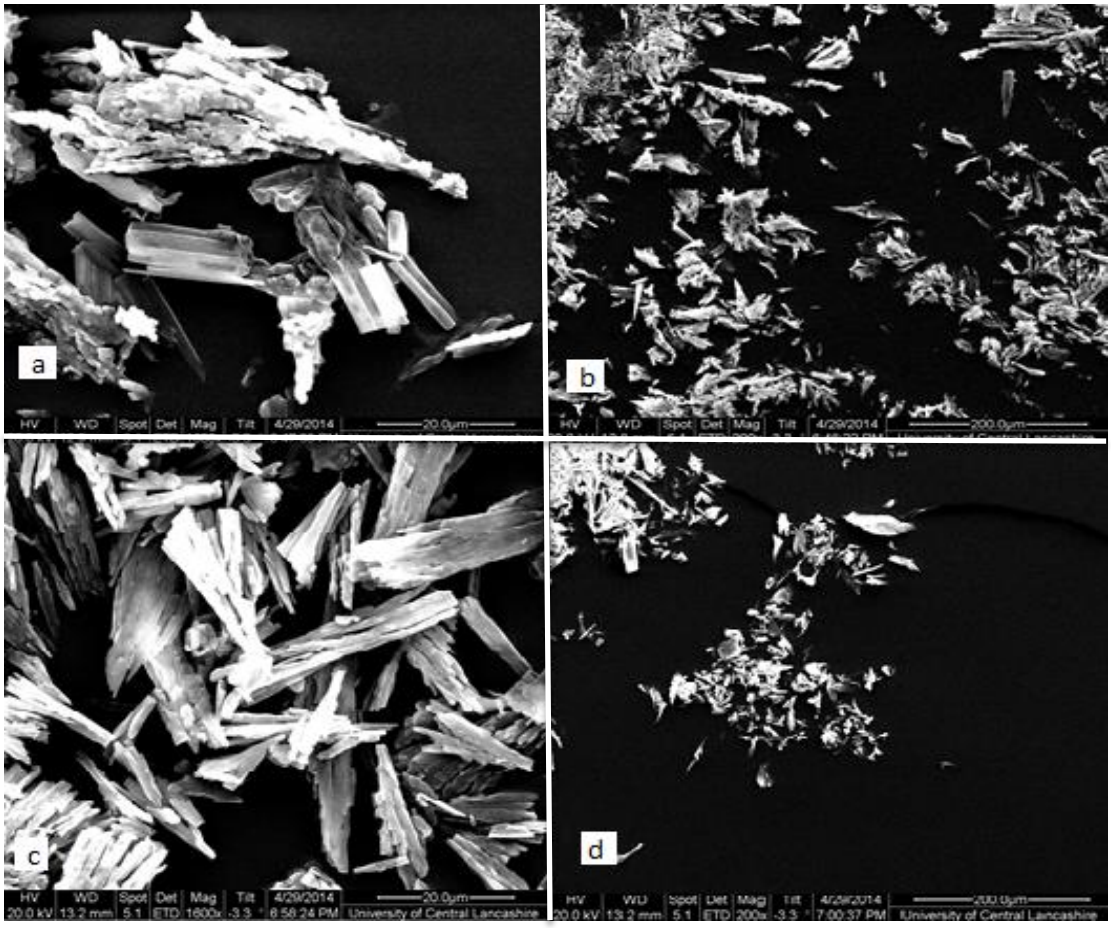

Figure 6.8 SEM of (a) proliposomes after freeze drying (20µm scale) (b) Proliposomes after freeze drying (200µm scale) (c) Prosurfactosomes after freeze drying (20µm scale) and (d) Prosurfactosomes after freeze drying (200µm scale).

6.3.5. X ray diffraction of proliposomes and prosurfactosomes before and after spray drying and freeze drying

6.3.5.1. X-ray diffraction of spray dried proliposome and prosurfactosomes

X-ray diffraction of proliposomes and prosurfactosomes were studied before and after spray drying as shown in Figure 6.9 and 6.10. As shown in Figure 6.9, peaks of proliposomes before and after spray drying resemble mannitol peaks as mannitol is the most abundant and dominating component of the formulation. Sharp peaks of mannitol show its crystallinity (Figure 6.9 a). BDP is semi-crystalline which is shown by the broad and less distinct peaks (disordered state) (Figure 6.9 c). Drug is in very small quantity as compared to other components; hence, they are not detected by X-ray diffraction. Drug peaks are not visible in the proliposomal formulations before and after freeze drying. The proliposomal formulation before spray drying is also crystalline which is shown by the sharp separated peaks (Figure 6.9 b). However, the peaks of proliposome with BDP, PM of proliposome and BDP and empty proliposome after spray drying did not show a highly crystalline structure (Figure 6.9 d-f). This is shown by the broad and less distinct peaks. An amorphous hump was also observed in the XRD graph obtained from the instrument software. Therefore, it can be concluded that proliposomes have lost their crystallinity as a result of spray drying and could possibly be described as semi-crystalline. Similar results of reduction in crystallinity after spray drying was observed by M. Dixit *et al.* when piroxicam nanocrystals were spray dried (Dixit et al., 2010). Corrigan et al have also observed that spray drying may give increased amorphous content (Corrigan et al., 1984, Corrigan et al., 2004). The presence of crystallinity of proliposomes even after being spray dried is possibly due to the crystallisation of mannitol as discussed in chapter 4 section 4.3.7 (Yu et al., 1998, Yoshinari et al., 2002).

As observed in SEM images (Figure 6.7) of spray dried particles, they were spherical and porous. Porous materials could be amorphous as the particles are not in perfect repetitive order. The rapid solidification via rapid solvent removals also leads to increased amorphous content of the material (Dixit et al., 2010). Spray drying of liposomal dispersion involves rapid removal of water and its conversion to solid spray dried proliposomes.

The reduced crystallinity could lead to enhancement of solubility and dissolution of spray dried proliposomes. The tendency of amorphous materials to absorb moisture also leads to instability (Andronis et al., 1997).

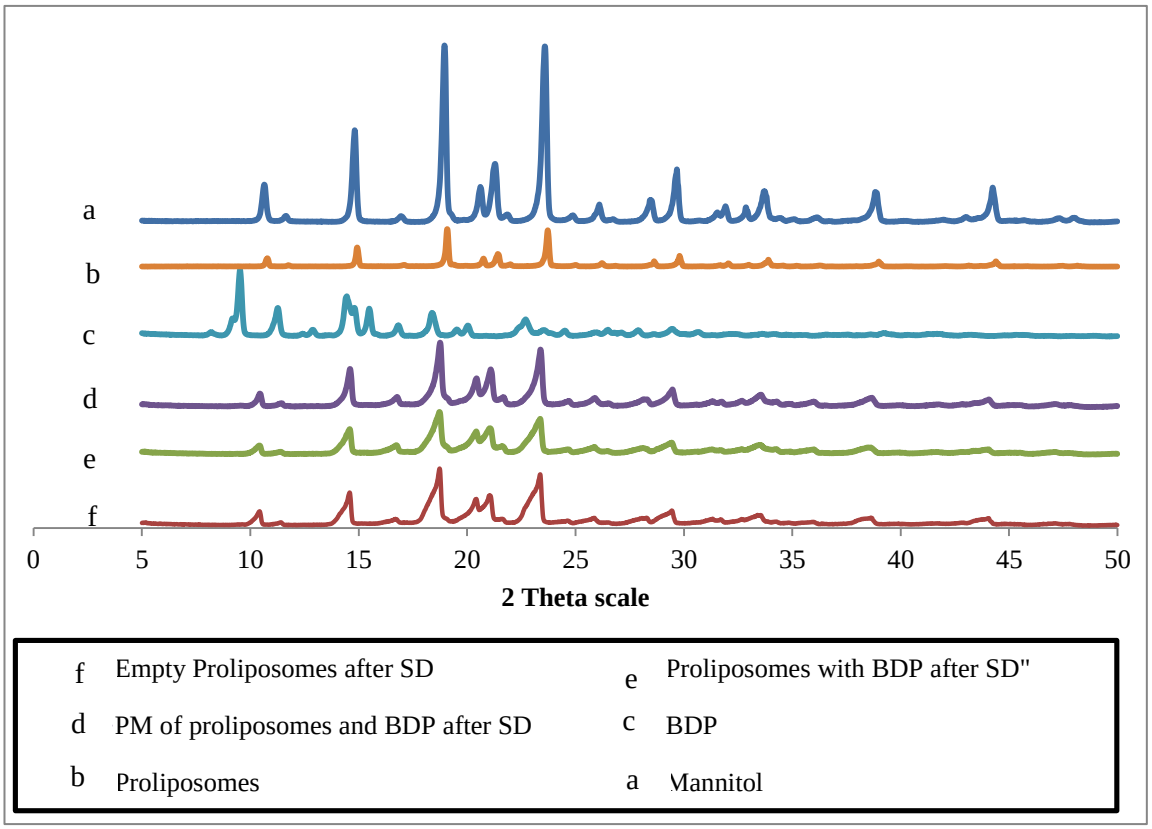

Figure 6.9 XRD of (a) Mannitol (b) Proliposomes before SD (c) BDP (d) PM of proliposomes and BDP after SD (e) Empty Proliposome after SD (f) proliposome with BDP after SD.

Similar change in crystallinity was observed with prosurfactosomes after spray drying as observed with proliposomes in Figure 6.10. Crystallinity of prosurfactosomes decreased as a result of spray drying, thus, increasing their dissolution and decreasing their stability.

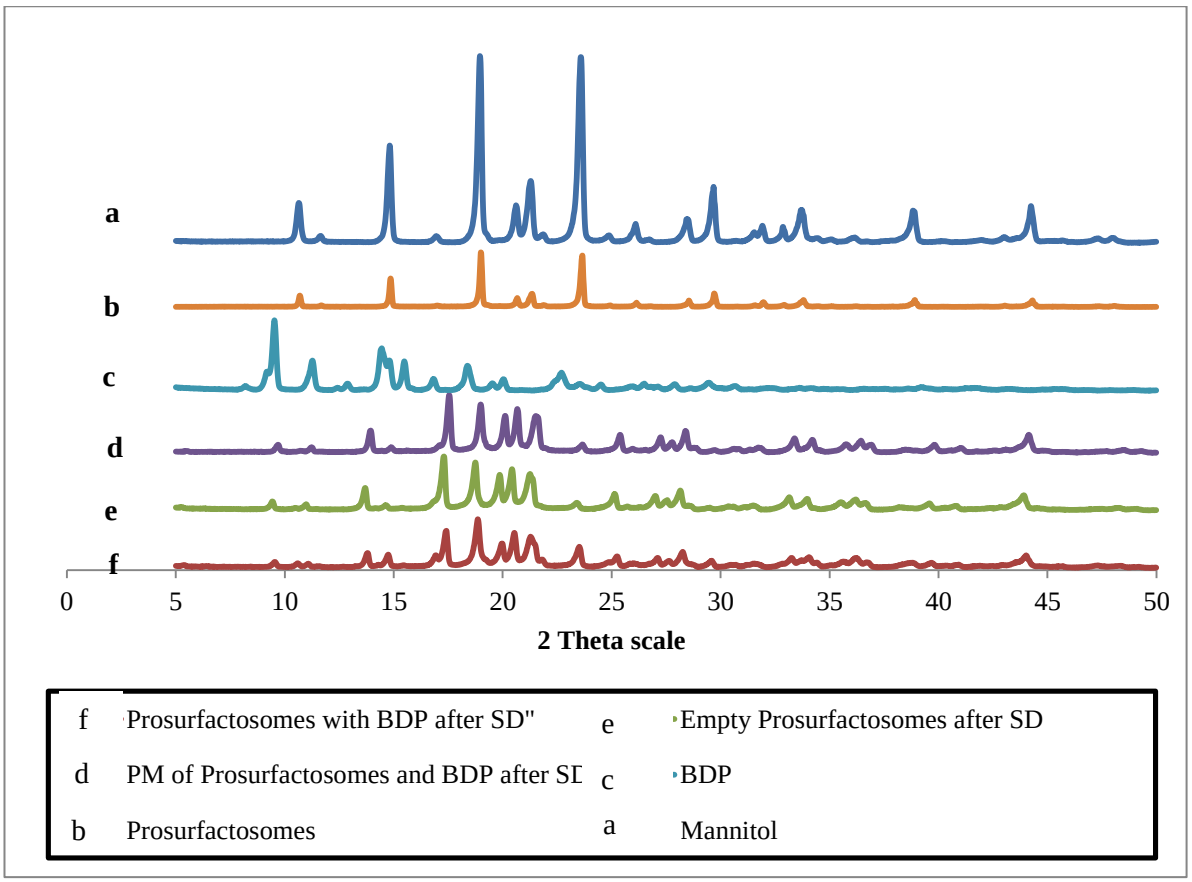

Figure 6.10 XRD of (a) Mannitol (b) Prosurfactosomes before SD (c) BDP (d) PM of prosurfactosomes and BDP after SD (e) Empty Prosurfactosomes after SD (f) Prosurfactosomes with BDP after SD.

6.3.5.2. X-ray diffraction of freeze dried proliposomes and prosurfactosomes

X-ray diffraction of proliposomes and prosurfactosomes were studied before and after freeze drying as shown in Figure 6.11 and 6.12. As shown in Figure 6.11 (a-f), peaks of proliposomes before and after spray drying resembled mannitol peaks as mannitol is the most abundant and dominating component of the formulation. Sharp peaks of mannitol show the crystallinity of the powder (Figure 6.11 a). Similar observations as discussed in section 6.3.5.1 were found for proliposomes before freeze drying, BDP, PM of proliposomes and BDP, empty proliposomes and proliposomes with BDP. The freeze dried proliposomes lost their crystallinity after freeze drying, thus, becoming semi-crystalline. BDP being in very small quantity as compared to other components of the formulation is not visible in the proliposomal peaks. Being less crystalline may help freeze dried proliposomes to easily reconstitute on hydration (Andronis et al., 1997).

As observed in Figure 6.8, SEM of freeze dried particles revealed porous and flake-like needle shaped particles. Porous materials are amorphous as the particles are not in perfect repetitive order. Hence, an XRD study of freeze drying is supported by SEM images.

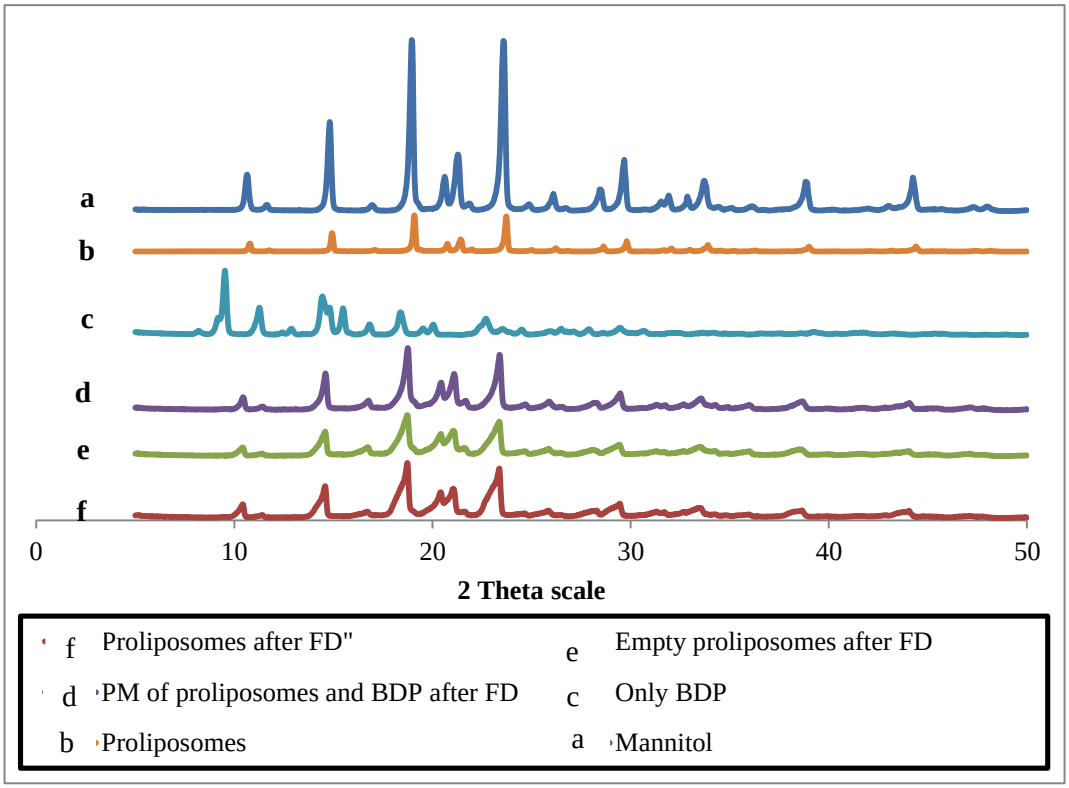

Figure 6.11 XRD of (a) Mannitol (b) Proliposomes before FD (c) BDP (d) PM of proliposomes and BDP after FD (e) Empty Proliposome after FD (f) proliposome with BDP after FD

Similar changes in crystallinity were observed with prosurfactosomes after freeze drying as observed with proliposomes as shown in Figure 6.12. Crystallinity of prosurfactosomes decreased upon freeze drying, thus, increasing their dissolution ability and decreasing their stability.

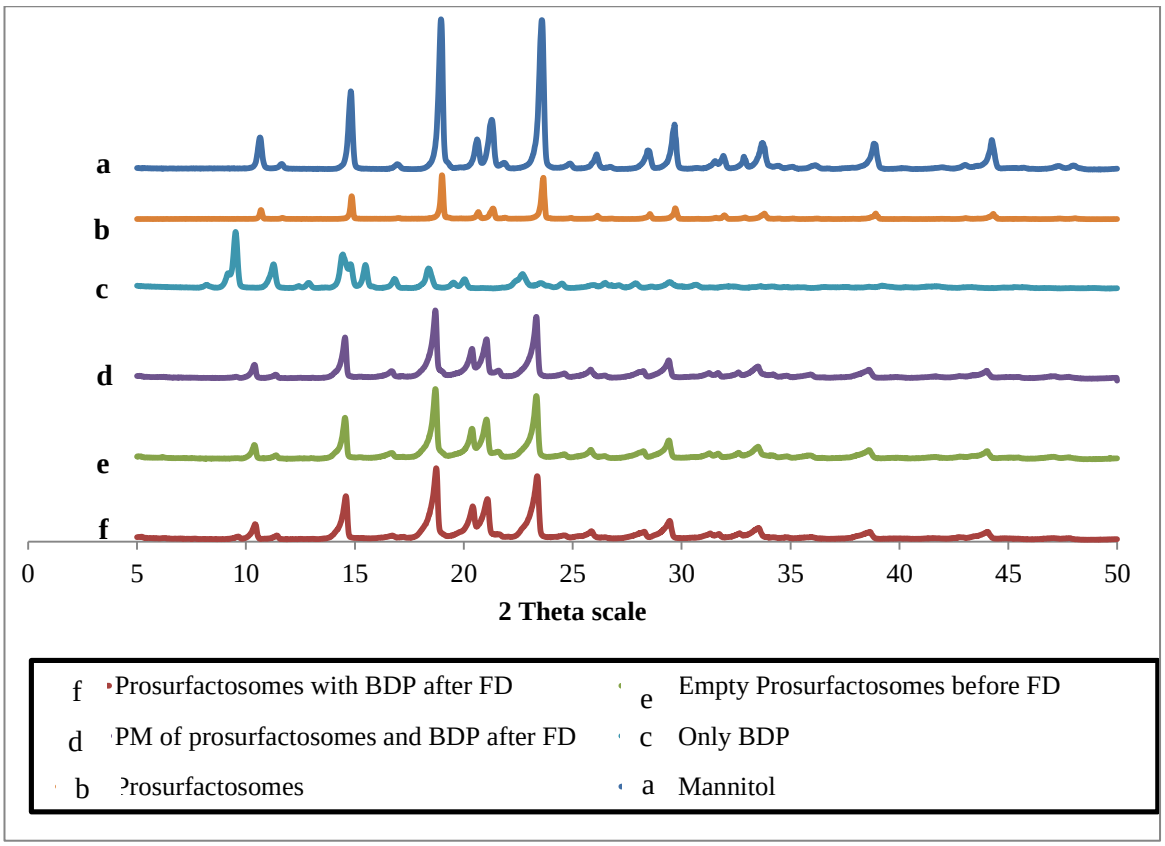

Figure 6.12 XRD of (a) Mannitol (b) Prosurfactosomes before FD (c) BDP (d) PM of prosurfactosomes and BDP after FD (e) Empty Prosurfactosomes after FD (f) Prosurfactosomes with BDP after FD.

Hence, From XRD analysis it can be concluded that spray drying and freeze drying reduces the crystallinity of proliposomes and prosurfactosomes, thus making them semi-crystalline. They tend to become more amorphous. Mannitol being the most abundant material in the formulations, all the peaks of proliposomes and prosurfactosomes resembled the peaks of mannitol.

6.3.6. Stability studies of proliposomes and prosurfactosomes for 12 weeks

The stability of proliposomes and prosurfactosomes were studied. For this study the vesicles were stored in different environment like 40ºC (in incubator), room temperature and 2-8°C. The important factors determining the stability of vesicles like pH, VMD, span, zeta potential and entrapment were studied. The comparison between proliposomes and prosurfactosomes was made to determine the best formulation and condition in terms of formulation stability. Readings were taken every 2 weeks for a

period of 12 weeks. The proliposomes and prosurfactosomes were hydrated before the experiment to form liposomes and surfactosomes respectively.

This is preliminary research where only one reading of each week was taken. To confirm the result and to perform statistical analysis three readings for all vesicles and for all factors must be considered. Week 0 represents the freshly prepared samples.

6.3.6.1. pH of liposomes and surfactosomes generated from proliposomes and prosurfactosomes respectively over 12 weeks

To study the stability of proliposomes and prosurfactosomes in terms of pH, analysis of its acidity and basicity was performed. In Figure 6.13, the pH behaviour of proliposomes in different environments is shown. It was observed that in the initial 4 weeks, the pH of liposomes made from proliposomes was neutral (between 6.5 and 7.5). From week 6 the acidity of liposomes started to increase (i.e. pH decreased). It was observed that liposomes stored in 40ºC were becoming acidic in a rapid rate. At the end of 12 weeks the pH decreased to 4.1. By contrast, for liposomes stored in room temperature (around 18-22ºC) the pH was 4.3. It was also observed that liposomes stored at 2-8°C were becoming acidic in a relatively slow rate, since after 12 weeks the pH became 5.2. Liposomes stored at refrigerator were more stable than the liposomes stored in 40°C and room temperature. The decrease in pH of the liposomal dispersion is due to the hydrolysis of phospholipid to form free fatty acids and lysophospholipids (Tseng et al., 2007a, Ravi and Singh, 2012). Fatty acids produced made the formulation acidic on storage. This results show that liposomes kept in 2-8°C slowed the breakdown of phospholipids. Hence, from this preliminary study it can be concluded that proliposome should be stored in refrigerator at 2-8°C to minimise their hydrolysis instability.

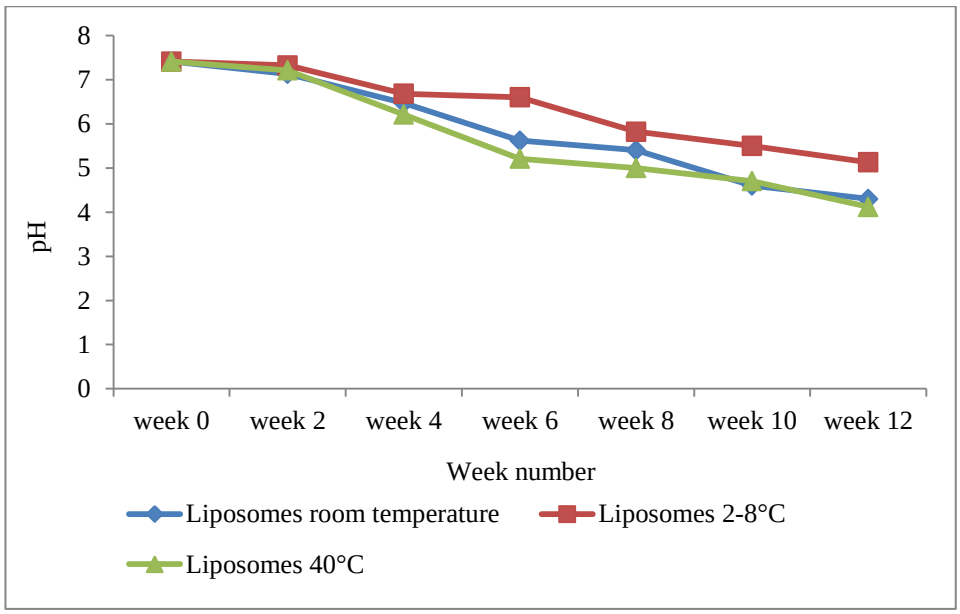

Figure 6.13 pH of liposomes prepared from proliposomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks.

In Figure 6.14, the pH behavior of prosurfactosomes stored in different environments is shown. It was observed that in the initial 2 weeks the pH of surfactosomes made from prosurfactosomes was between 7.5 and 6.5. From week 4 it was observed that the acidity of surfactosomes started to increase. It was observed that liposomes stored in 40ºC were becoming acidic in a rapid rate. Vesicles stored in room temperature followed it. At the end of 12 weeks the pH of surfactosomes stored in 40°C decreased to 3.8. This was followed by surfactosomes stored at room temperature (around 18-25ºC) where the pH was 4.2. It was also observed that vesicles stored 2-8°C was becoming acidic in a relative slow rate. After 12 weeks the pH was 5.2. It behaved relatively better than the vesicles stored in 40°C and room temperature. This is due to the breakdown of phospholipid into its components: glycerol, fatty acids and phosphate group. Fatty acids produced made the formulation acidic on storage. This results show that vesicles kept in 2-8°C slowed the breakdown of phospholipids. Similar results were obtained in another stability study of liposomes where the pH of the liposomes lowered with decrease in temperature (Berg, 2010). Hence, from this study it can be concluded that prosurfactosomes remained the best when stored in refrigerator at 5-6°C.

Hence, from this preliminary study it can be concluded that proliposomes and prosurfactosomes stored at 2-8°C were least unstable in terms of pH compared to the

vesicles stored in room temperature and 40°C. Both formulations started to become acidic on storage.

It was also observed that prosurfactosomes became more acidic than proliposomes when kept in the incubator at 40°C. To check this behaviour of phospholipids, the proliposomes and prosurfactosomes were placed at 40°C for 24 hours to check the pH. The pH of liposomes dropped from 7.53 to 5.11 whereas the pH of prosurfactosomes dropped from 7.38 to 3.45. Hence, this shows that proliposomes are more stable than prosurfactosomes in terms of pH when stored in 40°C.

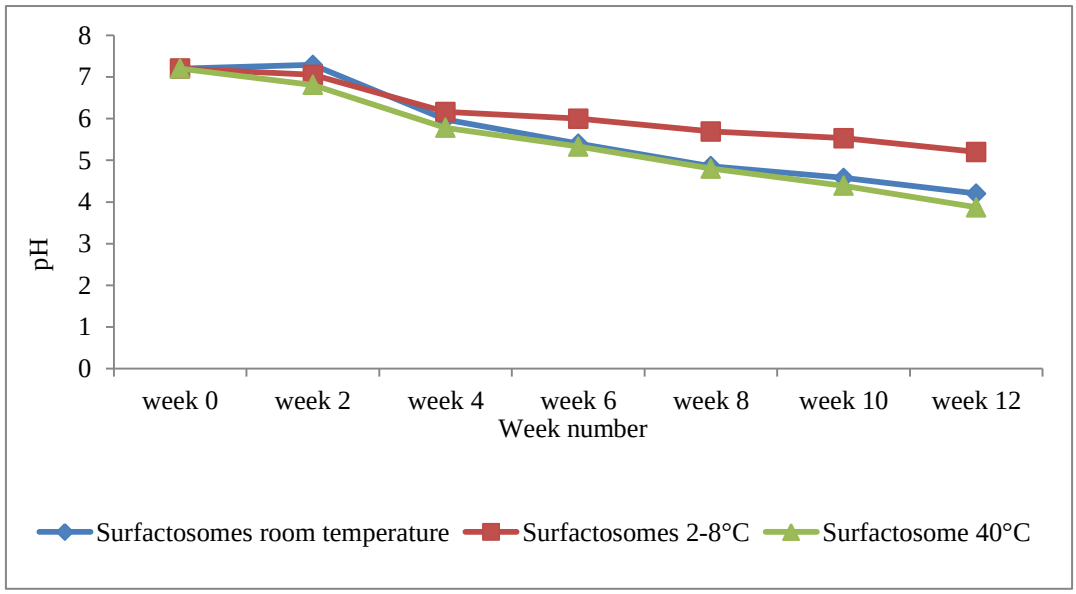

Figure 6.14 pH of surfactosomes prepared from prosurfactosomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks

6.3.6.2. VMD of liposomes and surfactosomes in 12 weeks

To study another parameter of stability of proliposomes and prosurfactosomes VMD analysis was conducted. In Figure 6.15 the VMD of liposomes prepared after hydration of proliposomes stored in different environments like 40°C, room temperature and 2-8°C were analysed. Except for week 4 and 6, the VMD of all vesicles was less than 6µm which was similar to the VMD of the freshly prepared samples. Hence, the VMD of liposomes remained unchanged in the end of 12 weeks. Thus, from this study it can be concluded that proliposomes are stable in all three conditions for the period of 12 weeks, when particle VMD was the determinant stability parameter.

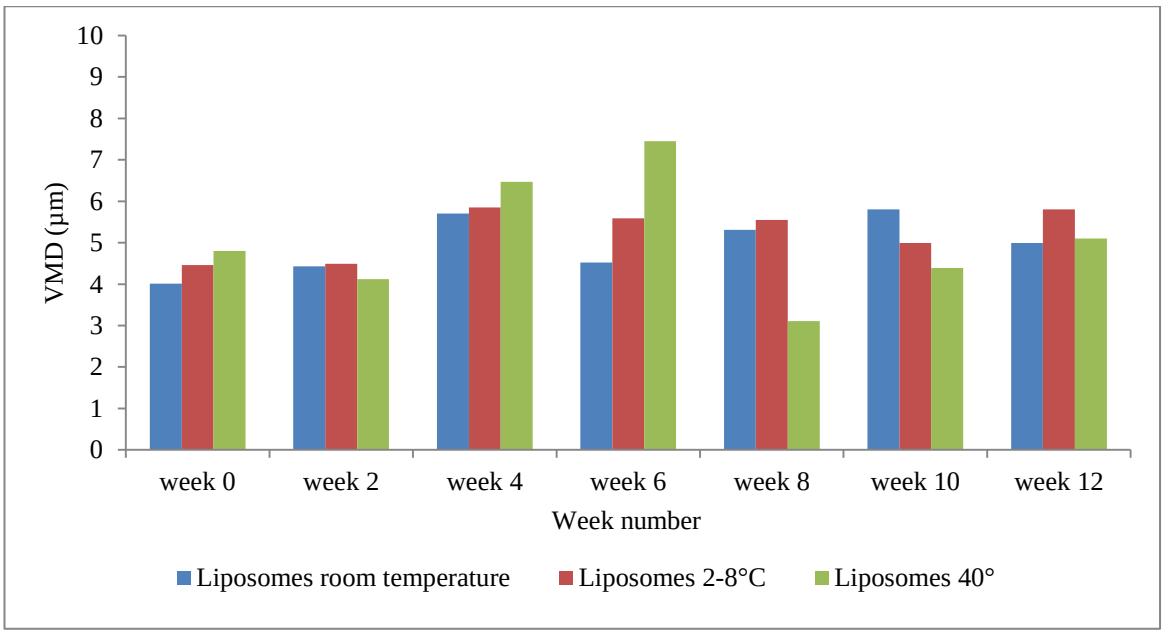

Figure 6.15 VMD (size) of liposomes prepared from proliposomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks.

In Figure 6.16 the VMD of surfactosomes prepared from prosurfactosomes in different environments like 40°C, room temperature and 2-8°C was analysed. It was observed that VMD of vesicle stored at room temperature started has increased from week 4. The trend for particle size increase for 2-8°C was less than that of vesicles stored at room temperature. VMD of vesicles became more than 10µm by this time for those stored in room temperature. By the end of 12 weeks, the VMD of surfactosomes at room temperature reached 19µm and for those stored at 40ºC the VMD was as large as 21.1µm. It was also observed that surfactosomes stored in refrigerator were more stable and small in size than those stored in room temperature and 40°C as the size did not go beyond 13µm. However, the VMD of vesicles increased after on storage which is attributed to aggregation of vesicles due to their instability. Prosurfactosomes remains least unstable when they stored at 5-6°C.

Hence, from this study it can be concluded that prosurfactosomes have more tendency to aggregate as compared to proliposomes when hydrated.

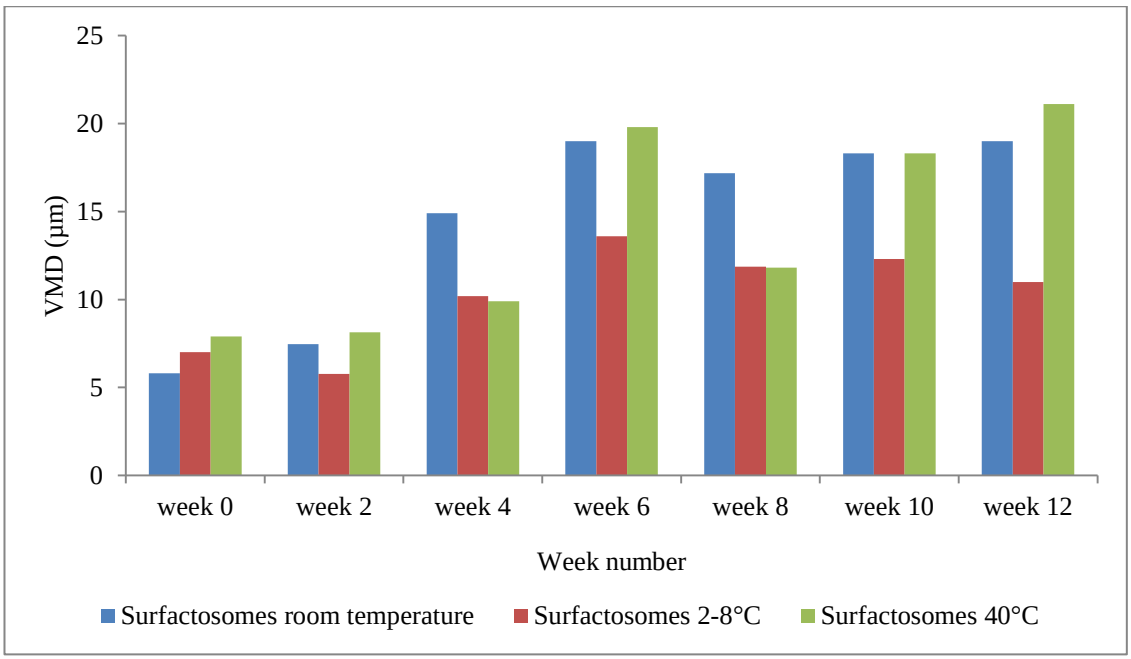

Figure 6.16 VMD (size) of surfactosomes prepared from prosurfactosomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks

6.3.6.3. Span of liposomes and surfactosomes after 12 weeks of storage at different temperatures

Span of surfactosomes and liposomes was determined in all 3 environments: Room temperature, 2-8°C and 40ºC. The span of proliposomes stored in all three environments were analysed as shown in Figure 6.17. It was observed that the span of vesicles started to increase after week 2. The span of proliposomes stored in 40°C increased rapidly from week 8 which was followed by liposomes in room temperature. The span of proliposomes stored in 2-8°C increased gradually till week 12. The span of liposomes by the end of week 12 increased largely as compared to week 0. The span of proliposomes stored in room temperature was 3.22. Proliposomes stored in 40ºC had a span value of 4.9 and of that stored in 2-8°C had a value of 3.88. Hence, from this preliminary study it can be concluded that proliposomes stored in 40°C had the largest span in 12 weeks. The span kept increasing due the tendency of liposomes to aggregate on losing its stability.

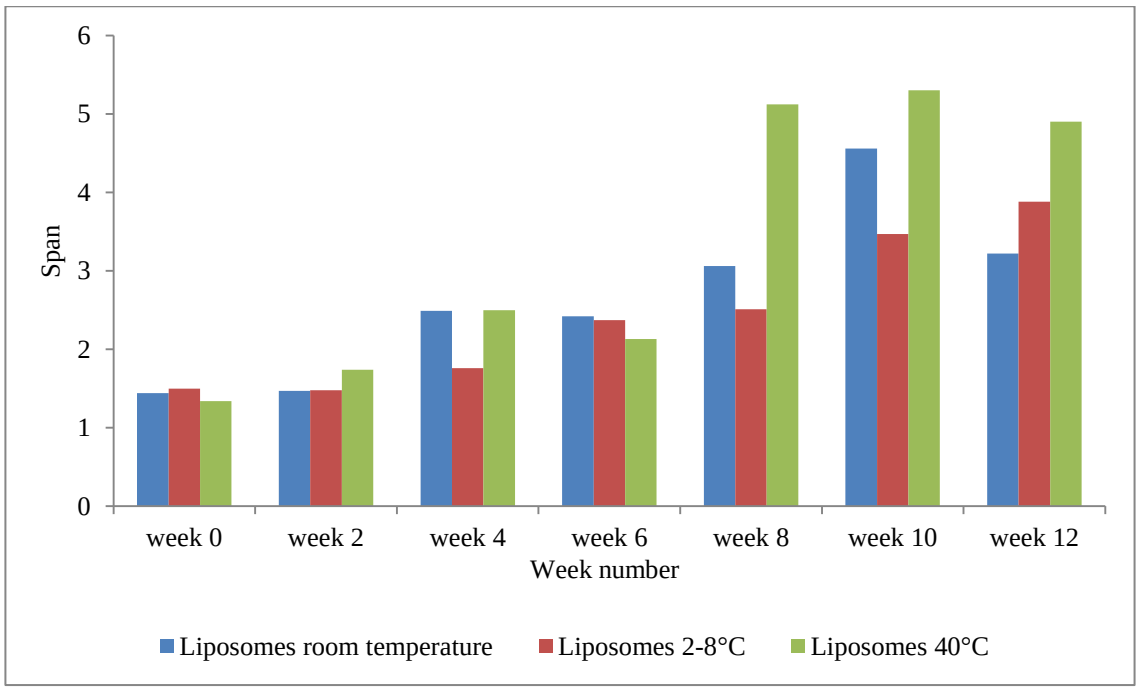

Figure 6.17 . Span of liposomes prepared from proliposomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks

The span of surfactosomes prepared from prosurfactosomes was studied as shown in Figure 6.18. It was observed that span of vesicles increased after week 0. The span of surfactosome was high even in week 0 for prosurfactosomes stored in 40ºC. It was observed that from week 4 the span of vesicles increased to more than 5 in all 3 environments. This indicates the instability of surfactosomes on being stored more than 4 weeks and this is much higher than the span of liposomes. Similar results were observed in the previous studies in chapter 4 section 4.3.4 where surfactosomes showed higher span with more vesicular aggregation.

Hence, it can be concluded that proliposomes produce more stable vesicles with lower span than prosurfactosomes on being stored for a long time. Uniformity in vesicles was maintained with least instability in proliposomes.

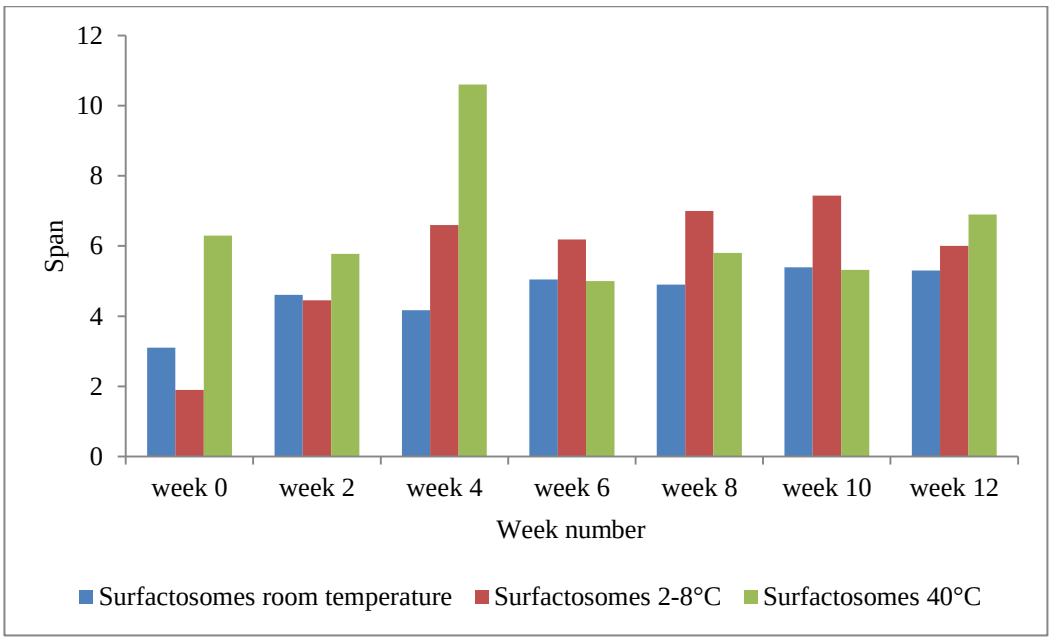

Figure 6.18 Span of surfactosomes prepared from prosurfactosomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks

6.3.6.4. Zeta potential of liposomes and surfactosomes in 12 weeks

The zeta potential (surface charge) of liposomes and surfactosomes prepared from proliposomes and prosurfactosomes respectively were analysed for stability by considering the zeta potential measurements. Proliposomes stored in room temperature, 2-8°C and 40ºC for charge analysis as shown in Figure 6.19. It was observed that zeta potential of vesicle increased with time when compared to that measured for the freshly prepared samples (i.e. week 0). The surface charge of liposomes increased more when the storage temperature was 40ºC than at room temperature and 5-6°C. By the end of 12 weeks it was observed that the charge of liposomes stored in room temperature was -9mV, liposomes stored in 2-8°C was -7.1mV whereas liposomes stored in 40ºC were -13.9mV. Hence, from this study it can be concluded that liposome become least stable with its charge in 40°C and most stable in refrigerator at 5-6°C. In a study by Plessis *et al*.it is stated that the zeta potential alone cannot prove the instability of a vesicle and VMD should always be considered (Berg, 2010). He also concluded that liposomal system should be kept in refrigerator to achieve the best physical stability.

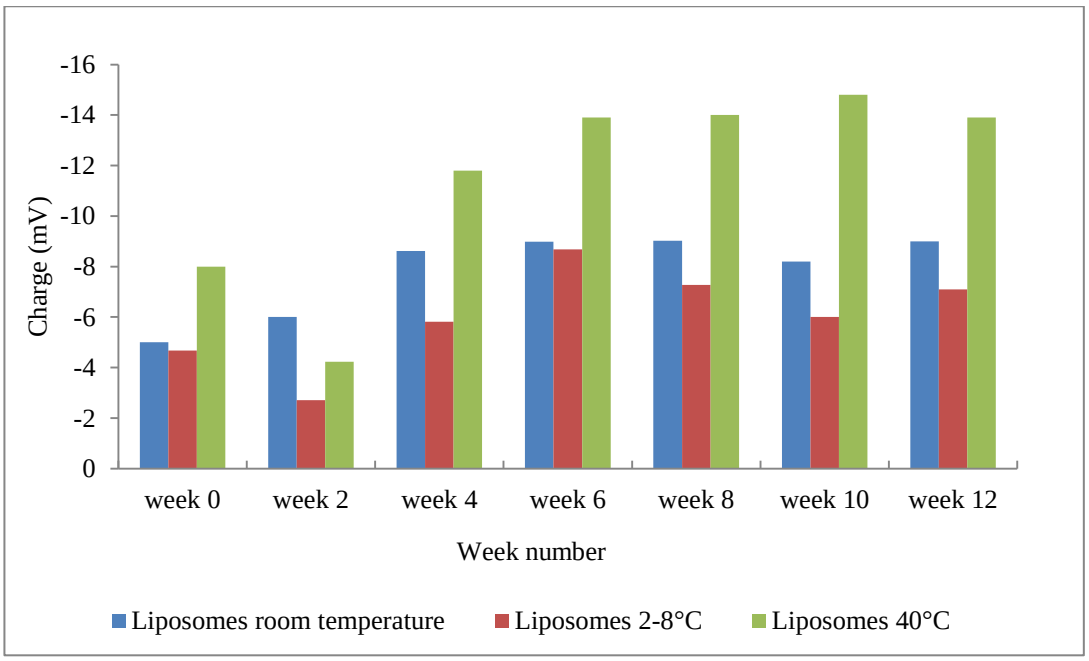

Figure 6.19 Zeta potential of liposomes prepared from proliposomes stored in room temperature, 2-8°C(refrigerator) and in 40°C (incubator) for 12 weeks

The charge of surfactosomes prepared from prosurfactosomes were analysed as shown in Figure 6.20. It was observed that the charge of surfactosomes from week 0 was more than that of liposomes in all three environments: room temperature, 2-8°C and 40°C. It was also observed that the surface charge of surfactosomes has changed throughout 12 weeks. The surface charge gradually increased and by the end of 12 weeks, prosurfactosomes stored in 40°C had a zeta potential value of –17.6mV, prosurfactosomes stored in room temperature had a measurement of -14.32mV and that stored in 2-8°C had a value of -13.7mV. Hence, the zeta potential of surfactosomes has increased with time and with increasing the storage temperature.

Hence, surfactosomes had more intensive surface charge than liposomes as discussed in chapter 4 section 4.3.5. It is stated that higher zeta potential indicates a more stable suspension and lower value indicates colloid instability which could lead to aggregation of vesicles (Ma et al., 2011). The negatively charges vesicles can easily bind to the cationic sites of the cell in the body in the form of cluster for absorption (Henriksen et al., 1994). For both type of vesicles the instability in terms of surface charge increases more in 40ºC than in any other condition used in this investigation.

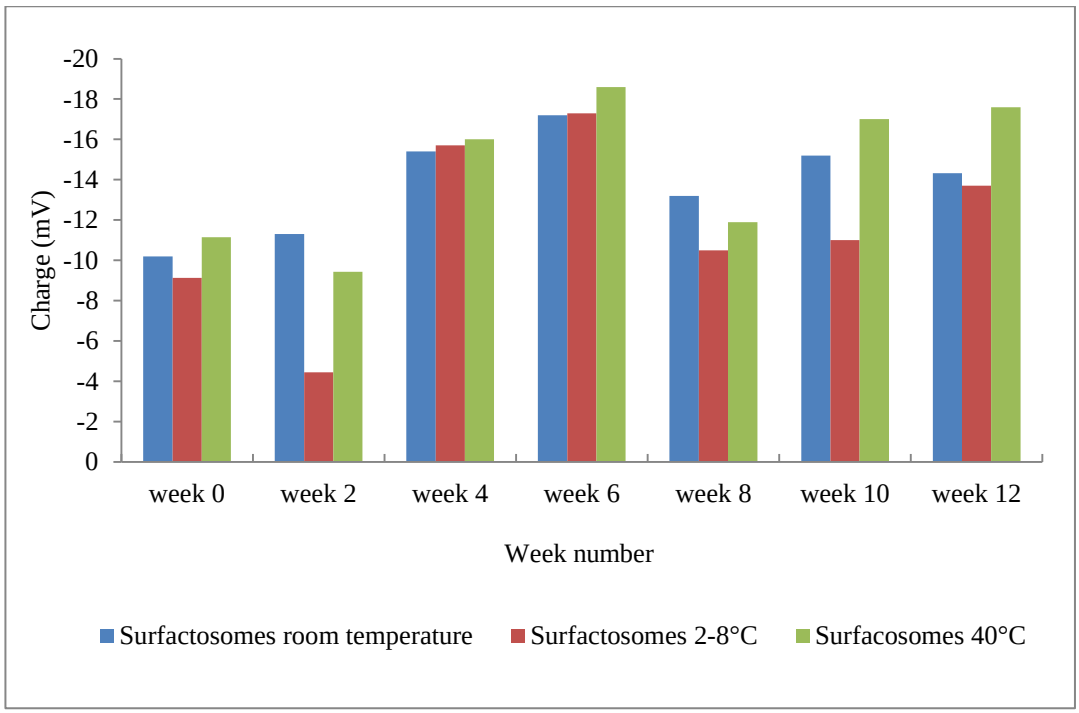

Figure 6.20 Zeta potential of surfactosomes prepared from prosurfactosomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks

6.3.6.5. Entrapment of BDP in proliposomes and prosurfactosomes over 12 weeks of storage

The entrapment of BDP in proliposomes and prosurfactosomes for a period of 12 weeks was analysed. They were stored in three different environments: Room temperature, 2-8°C and 40ºC. In Figure 6.21, the entrapment of BDP in liposomes prepared from proliposomes for a period of 12 weeks was analysed. It was observed that the entrapment of drug decreased with time. The leakage of drug was maximum in proliposomes stored in 40°C and was minimum in proliposome stored in 5-6°C. The initial entrapment of drug in liposome was 53%. By the end of 12 weeks, the entrapment of BDP in proliposomes stored in room temperature was 35%, stored in 40ºC was 30% while that stored in 2-8°C was 40.8%. Hence, drug retention in proliposomes was maximised when proliposomes were stored at fridge temperature (i.e. 5-6°C) and was least when storage was done at 40°C.It has been previously reported that liposomes are more stable when stared in 4-5°C as compared to any other temperature (Gregory, 2006) Muppidi *et al* also studied that liposomes stored at 4°C were more stable than those stored in 24°C and 37°C (Muppidi et al., 2012). This

increase in stability is due to the decrease in fatty acid breakdown at lower temperatures (HERNÁNDEZ-CASELLES et al., 1990). There is inhibition of peroxide formation (oxidation) at low temperatures, thus, increasing liposome stability.

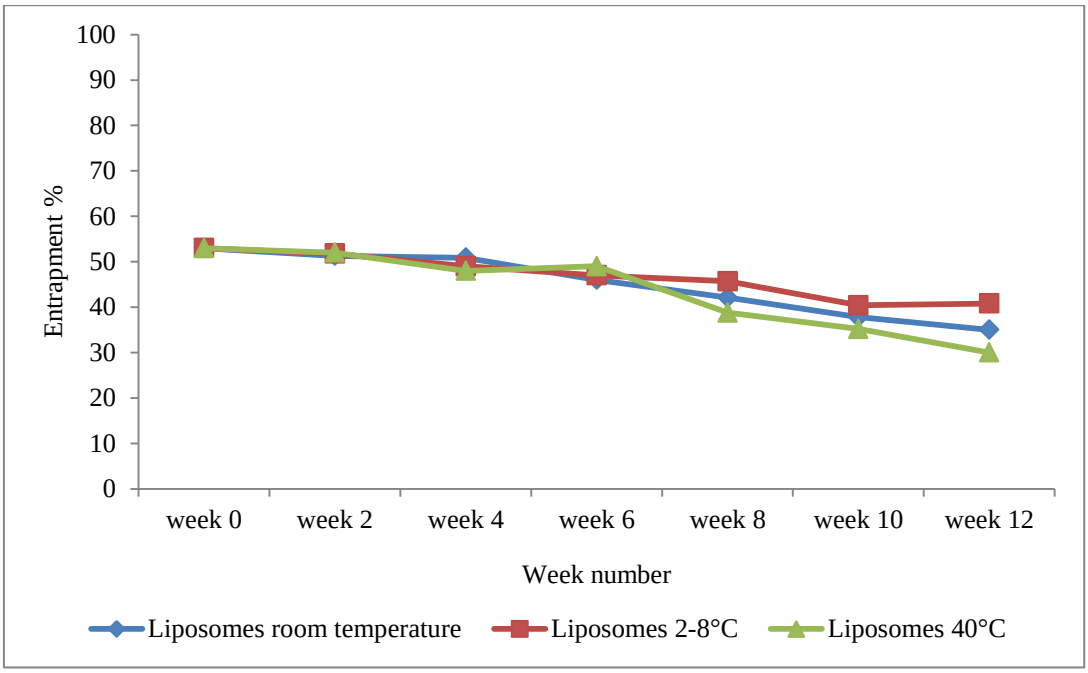

Figure 6.21 BDP Entrapment% of liposomes prepared from proliposomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks

The entrapment of BDP in surfactosomes prepared from prosurfactosomes for a period of 12 weeks was analysed as shown in Figure 6.22. It was observed that the entrapment of drug decreased with time. The leakage of drug was most in prosurfactosomes stored in 40°C and was minimum in prosurfactosome stored in 5-6°C. The initial entrapment of drug in surfactosome was 42%. By the end of 12 weeks, the entrapment of BDP in surfactosomes prepared from prosurfactosomes stored in room temperature was 23.8%, stored in 40ºC was 20.2% while that stored in 2-8°C was 29.3%. Hence, drug retention in prosurfactosomes was at maximum when formulation was kept in the refrigerator (i.e. 5-6°C) and minimum in 40°C. The leakage of BDP is due to the hydrolysis of phospholipids to free fatty acids and lysophospholipids. This can possibly disturb the phospholipid bilayer structure and may lead to leakage of encapsulated material (Tseng et al., 2007a, Ravi and Singh, 2012).

Hence, it can be concluded that the stability of proliposomes and prosurfactosomes was best at 2-8°C and worst at 40°C among the three temperatures investigated.

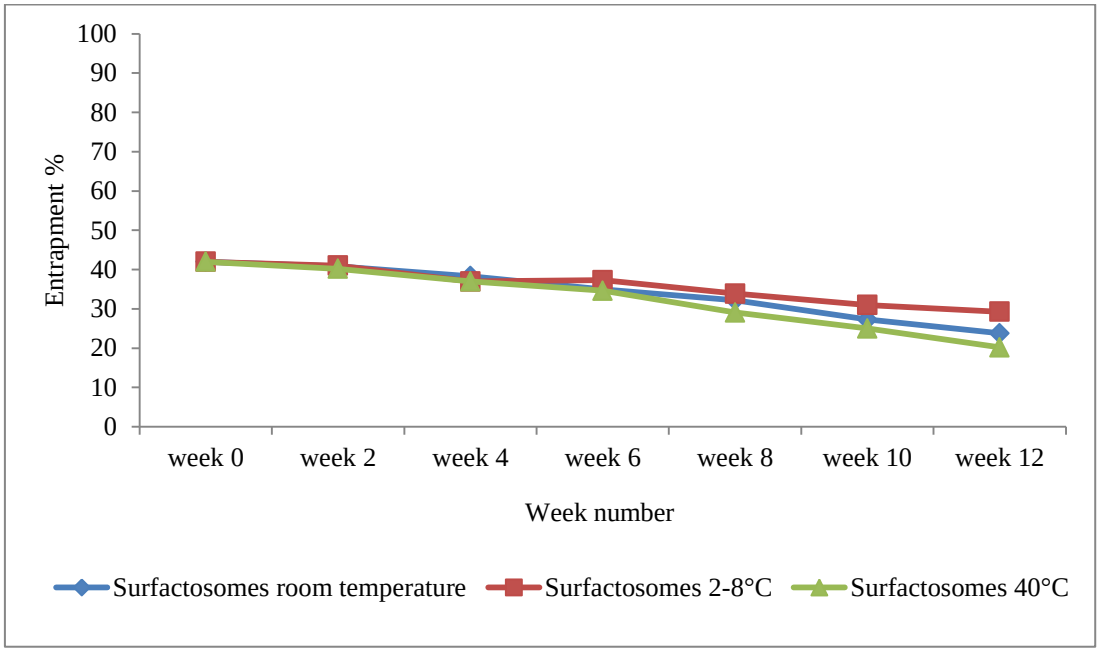

Figure 6.22 BDP entrapment % of surfactosomes prepared from prosurfactosomes stored in room temperature, 2-8°C (refrigerator) and in 40°C (incubator) for 12 weeks

6.4. Conclusion

In this chapter, the stability of proliposomes and prosurfactosomes were analysed using different methods. The VMD, span, charge and entrapment of BDP of these vesicles were analysed.

After the study of spray drying, it can be concluded that there was no significant difference between the VMD and span of liposomes as compared to those of surfactosomes. However, the zeta potential of surfactosomes was significantly greater than that of liposomes after spray drying. It was also observed that there was no significant difference in the VMD and span of both the vesicles before and after spray drying. However, the zeta potential of both the vesicles increased significantly after spray drying. It was found that there was no significant difference between the entrapment of BDP in liposomes and surfactosomes. This concludes that surfactosomes were as stable as liposomes after facing the heat and stress generated during spray drying. The mass output of surfactosomes after spray drying was slight but not significantly greater than liposomes. Hence, it can be concluded that both liposomes and surfactosomes are equally stable to spray drying. Thus, spray drying can be preferred to be used to increase the stability of liposomes and surfactosomes.

After the study of freeze drying it can be concluded that the VMD and span of surfactosomes was significantly larger than the VMD and span of liposomes. The VMD and span of surfactosomes increased significantly after freeze drying as compared to that before spray drying. However, there was no significant difference between the VMD and span of liposomes before and after freeze drying. It was also observed that there was significantly more loss of BDP from surfactosomes than in liposomes after freeze drying. This concludes that liposomes are more stable to freeze drying than surfactosomes.

The SEM studies revealed that mannitol lost its porosity after being coated by SPC and cholesterol in proliposomes and prosurfactosomes. Structure of prosurfactosomes was smoother than proliposomes. SEM revealed that after spray drying, proliposomes and prosurfactosomes were reduced in size and became spherical. They formed homogenous microspheres with reduced surface area. It was also observed from SEM that freeze drying produced small and needle shaped porous proliposomes and prosurfactosomes. The flake-like structure of proliposome and prosurfactosome may be due to the vestige of sublimation of ice in the drying stage.

X-ray diffraction (XRD) concluded that before spray drying and freeze drying proliposomes and prosurfactosomes were crystalline in nature. Mannitol is crystalline and dominated the peaks of proliposomes and prosurfactosomes as it forms the major component. BDP is semi-crystalline and being the minor component does not form the peak in XRD graph of proliposomes and prosurfactosomes. Spray drying of proliposomes and prosurfactosomes decreased its crystallinity. Semi-crystalline structure was signified by broad and less distinct peaks and amorphous hump observed in the XRD. Rapid solidification via rapid solvent removals also leads to increase in amorphous nature. Similar observation was done for prosurfactosomes. Freeze dryer was also observed to decrease the crystallinity of proliposomes and prosurfactosomes. Porous materials as found after freeze drying are amorphous as the particles are not in perfect repetitive order. Similar observation was found for prosurfactosomes after freeze drying. Hence, it can be concluded that spray drying and freeze drying decreases the crystallinity of proliposomes and prosurfactosomes, thus, making them semi-crystalline.

Preliminary stability studies were performed for 12 weeks where the proliposomes and prosurfactosomes were stored in 40ºC (in incubator), room temperature and 2-8°C (in refrigerator). PH studies revealed that proliposomes and prosurfactosomes became acidic on storage after hydration. They were most stable in 2-8°C and least stable in 40°C. This may be due to the breakdown of phospholipid into glycerol and fatty acid on

storage. It was also observed that proliposomes were more stable that prosurfactosomes when stored in 40°C. Study of VMD concludes that proliposomes on hydration are stable on all three conditions after 12 weeks. However, prosurfactosomes on hydration started to increase in VMD due to aggregation after 4 weeks. Prosurfactosome remain most stable when stored in 5-6°C. Study of span concluded that liposomes and surfactosomes formed from proliposomes and prosurfactosomes respectively were more stable when stored in 2-8°C and least stable in 40°C. It was also concluded that proliposomes maintained more uniformity than prosurfactosomes when hydrated after 12 weeks. Zeta potential studies revealed similar results of being stable in 5-6°C. Prosurfactosomes increased their charge more than liposomes in the end of 12 weeks, thus, being less stable. The preliminary studies can also conclude that the stability of proliposomes and prosurfactosomes is best in 2-8°Cand worst in 40°C among the three environments used. Proliposomes were more stable that prosurfactosomes in all three conditions. Overall it can be concluded that proliposomes and prosurfactosomes were most stable in 2-8°C of refrigerator and least stable in 40°C of incubator. Proliposomes were more stable than prosurfactosomes in terms of maintaining pH, VMD, span, zeta potential and BDP entrapment in all three conditions.

Hence this chapter concludes the stability of proliposomes and prosurfactosomes when spray dried, freeze dried and when stored in 2-8°C, room temperature and 40°C for 12 weeks. SEM also reveals the structure of proliposomes and prosurfactosomes before and after spray drying and freeze drying and XRD reveals the degree of crystallinity of both the formulations.

Chapter 7

General conclusions

The aim of this study was to investigate the efficiency of surfactant-enriched vesicles (i.e.surfactosomes) compared to conventional liposomes for potential use in pulmonary delivery via nebulisation. The properties of surfactosomes were investigated in terms of their drug delivery efficiency and stability. Salbutamol sulphate (SBS) and beclometasone dipropionate (BDP) were used as the model hydrophilic and hydrophobic drug respectively. Soya phosphotidylcholine (SPC) was used as the phospholipid with or without cholesterol to prepare liposomes and surfactosomes. The four formulations used in this study were

Proliposomes with cholesterol (1:1 molar ratio SPC to cholesterol)
Proliposomes without cholesterol
Prosurfactosomes with cholesterol (1:1 molar ratio SPC to cholesterol)
Prosurfactosomes without cholesterol

Thin film method and proliposomes technology were both used to prepare liposomes and surfactosomes in this work. The vesicles' efficiency to act as carriers for delivery of drug via nebulisation along with its stability was initially tested using a mini-extruded with polycarbonate membranes 5µm, 2µm, 1µm and 0.4µm. The amount of drug retained by all four formulations after extrusion was studied. These hydrated formulations were nebulised using Aeroneb Pro (vibrating-mesh), Beurer iH50 (vibrating-mesh) and PARI LC plus (air-jet) nebulisers. Effect of cholesterol on liposomes and surfactosomes for drug entrapment and retention was also studied. Finally both liposome and surfactosome formulations were compared for their stability in different conditions like spray drying, freeze drying and when stored for 3 months at different temperatures room temperature, 40°C (in oven) and 2-8°C(refrigerator). The structure of proliposomes and prosurfactosomes were also studied and visualised using SEM, and X-Ray diffraction. TEM was used to visualise structure of liposomes and surfactosomes. The work focused on finding the best formulation for pulmonary drug delivery and to investigate if surfactosomes are better than liposomes in sustaining the different forms of stress and forces applied on them before delivering the drug via nebulisation.

7.1. Comparison between liposomes and surfactosomes formed using thin film method for entrapment of drug before and after extrusion

In Chapter 3, liposomes and surfactosomes with or without cholesterol were prepared using the thin film method. SBS and BDP were used as model drugs. They were studied for their size (VMD), span (size distribution) and entrapment efficiencies. They were even studied for their drug retention capacities on being extruded with different polycarbonate membranes like 5µm, 2µm, 1µm and 0.4µm. Effect of excessive extrusion (51 times) using 1µm polycarbonate membrane on all four vesicles were studied. There was a comparison between un-extruded vesicles and previously extruded vesicles with 1µm.

Initially the VMD and span of vesicles were observed when they were prepared using two organic solvents: chloroform and ethanol. From the study it was concluded that evaporation of chloroform was better than ethanol since alcohol may lead to difficulty in hydration, thus, leading to aggregation or fusion of the vesicles. This resulted in vesicles with large size which are inappropriate for pulmonary drug delivery via nebulisation. Hence, chloroform was chosen for subsequent studies as it could be more appropriate for generating thin films that can be hydrated more readily to form less aggregated vesicles. All vesicles had VMD and span which were desirable to be used in pulmonary drug delivery.

The entrapment studies using the hydrophilic drug SBS showed that all four formulations entrapped similar amount of SBS with vesicles having cholesterol being slightly better that those without cholesterol. This shows that cholesterol is an important component in liposomes and surfactosomes. Similarly the loading efficiencies were similar to all four formulations. However it was observed that liposomes were better than surfactosomes in retaining SBS when being extruded through all sizes of the polycarbonate membranes (5µm, 2µm, 1µm and 0.4µm). For hydrophilic drugs like SBS, liposomes are more resistant to drug leakage than surfactosomes when shearing via extrusion was applied. When the vesicles were extruded 51 times using the mini-extruder with 1µm polycarbonate membrane, it was found that smaller vesicles tend to retain greater proportions of the hydrophilic drug than larger vesicles after extensive extrusion.

The entrapment studies of the hydrophobic drug BDP showed that similar to SBS there was no significant difference in the initial entrapment of BDP in all four formulations. Even the loading efficiencies were similar with no significant difference between the formulations. On extrusion through polycarbonate membranes it was observed that vesicles made using any of the four formulations have retained considerable proportions of BDP. It was noticed that as the pore size of polycarbonate membranes used was smaller, the drug entrapment was decreased. Liposomes with cholesterol retained greater drug proportions than liposomes made without cholesterol, indicating that cholesterol plays an important role in the stability of liposomes. However, for surfactosomes, cholesterol did not have an effect as surfactant was the dominating factor in making it less stable. On being extruded 51 times with mini extruder using 1µm polycarbonate membrane, it was observed that vesicles with cholesterol retain more BDP and liposomes are more stable than surfactosomes. It was also found that smaller vesicles tend to retain more drug than larger vesicles after extensive extrusion.

In this study, chloroform was found to be a better organic solvent than ethanol for preparing liposomes and surfactosomes using thin film method. Ethanol made vesicles to aggregate when it was used as the organic solvent. For hydrophilic drug like SBS liposomes are more stable than surfactosomes while for hydrophobic drug like BDP both liposomes and surfactosomes are good with liposomes being slightly better. It was also concluded that cholesterol is an important component to be incorporated in the vesicular formulation as it increases formulation stability. Liposomes are better than surfactosomes for retaining greater drug proportions (SBS and BDP) after excessive extrusion (51 cycles). Smaller vesicles with size 1µm retained higher proportions of drugs after undergoing extensive extrusion than larger vesicles with size 4-7µm

7.2. Entrapment studies of SBS and BDP for proliposomes and prosurfactosomes using particulate based proliposome technology

In Chapter 4, liposomes and surfactosomes were prepared using particulate based proliposome technology. SBS and BDP were used as the model hydrophilic and hydrophobic drug respectively and mannitol was used as the carbohydrate carrier. Proliposomes and prosurfactosomes were hydrated to form liposomes and surfactosomes respectively. The vesicles were compared for size (VMD), size distribution (span), zeta potential (surface charge) and drug entrapment. Drug retention

on extrusion with polycarbonate membranes 5µm and 2µm was also studied. Effect of cholesterol concentration on drug entrapment and retention by liposomes and surfactosomes was also considered.

The studies revealed a similar VMD and span for liposomes and surfactosomes regardless of cholesterol incorporation. However, it was observed that zeta potential of surfactosomes was more negative than that of liposomes. This is may be due to the presence of Tween 80 along with its impurities like linoleic, palmitic and stearic acids in the surfactosomal formulation. These acids may tend to dissociate on the surface of vesicles, thus, giving a negative surface charge.

The entrapment of hydrophilic drug SBS was very low in all four formulations. It was also noticed that vesicles with cholesterol retained higher drug proportions than vesicles including no cholesterol. Hence, cholesterol is an important component in liposomes and surfactosomes for entrapping hydrophilic drugs like SBS. Proliposome technology unlike thin film method was not appropriate for the entrapment of hydrophilic drug like SBS. Hence, no extrusion was carried out using SBS formulations.

The entrapment of the hydrophobic drug BDP was studied using proliposomes and prosurfactosomes. For both type of formulation (i.e. surfactosomes and liposomes), 3 different formulations were compared: vesicles with only SPC, vesicles with SPC and cholesterol (2:1) and vesicles with SPC and cholesterol (1:1). For liposomes, formulation without cholesterol has shown to provide the highest BDP entrapment. This proves that due to the similar structure of BDP and cholesterol there is a competition for the incorporation of BDP in the lipid bilayers, thus, minimising the BDP entrapment. Increase in the concentration of cholesterol decreased the BDP entrapment. For surfactosomes, formulations with no cholesterol and those with low cholesterol concentration (i.e. SPC and cholesterol; 2:1) were better than vesicles with high cholesterol concentration at entrapping greater proportions of BDP. This may be due to the low competition between BDP and cholesterol to be incorporated in the bilayer and low displacement of BDP by cholesterol when low cholesterol concentrations were incorporated in the formulation. The low entrapment may also be due to the “burst effect” shown by these vesicles. It can also be concluded that there was no significant difference between the entrapment of BDP by liposomes and surfactosomes prepared by proliposome technology in formulations with only SPC and those with 1:1 SPC to cholesterol ratio. However, surfactosomes entrapped significantly more than liposomes in formulations with 2:1 SPC to cholesterol ratio.

It was also realised that when extrusion was performed using 5µm and 2µm polycarbonate membranes, liposomes and surfactosomes with or without cholesterol retained similar proportions of BDP. However, vesicles with cholesterol retained slightly lower BDP that those without cholesterol. This may be due to the excessive rigidity provided by cholesterol; hence making it difficult for BDP to locate within the bilayers. With regard to BDP entrapment, there was no significant difference in all three formulations of liposomes and surfactosomes before and after extrusion through 5µm and 2µm polycarbonate membranes. This indicates that for BDP retention with proliposome technology is good in all formulations with different concentrations of cholesterol in both proliposomes and prosurfactosomes.

TEM studies revealed that liposomes and surfactosomes prepared from proliposomes and prosurfactosomes respectively formed unilamellar vesicles on hydration.

Hence from this chapter it can be concluded that VMD and span of all formulations were similar whereas the zeta potential of surfactosomes were more negative than that of liposomes. It was also noticed that with particulate based proliposome technology there was very low entrapment of the hydrophilic drug SBS. Hence, no further extrusion studies were performed. For entrapment of the hydrophobic drug BDP using particulate based proliposome technology, proliposomes with only SPC was the best formulation. Inclusion of cholesterol decreased the entrapment of BDP in liposomes. Prosurfactosomes with low cholesterol concentration (SPC to cholesterol 2:1) was best in entrapping BDP with low standard deviation as compared to surfactosome with no cholesterol at all or with high cholesterol concentration (SPC to cholesterol 1:1).

7.3. Delivery and retention of BDP by liposomes and surfactosomes when delivered via nebulisation

In chapter 5, liposomes and surfactosomes with and without cholesterol were used to deliver BDP to twin impinger via nebulisers. Aeroneb Pro (vibrating-mesh), Beurer iH50 (vibrating-mesh) and PARI LC sprint (air-jet) nebulisers were used for this purpose The twin impinger was used as an *in vitro* model where the upper stage may collect the fraction of aerosols that are likely to deposit in the upper respiratory tract while the lower stage is known to collect the “respirable” fraction of the aerosol (i.e. the fraction that is likely to deposit in the lower respiratory tract). The delivery and retention of BDP in the four formulations were studied in both stages of the impinger

using the three aforementioned nebulisers. VMD, span and zeta potential of the vesicles before and after nebulisation were also studied from samples collected from the impinger following nebulisation.

From this study it was observed that the VMD and span of liposomes and surfactosomes with and without cholesterol decreased after nebulisation. Decrease in VMD suggests that the vesicles were fragmented due to the extrusion offered by the vibrating mesh nebulisers and the shear forces provided by the air jet nebuliser. The decrease in span suggests the decrease in uniformity of vesicles after nebulisation (i.e. the narrower size distribution). It was found that zeta potential of all vesicles after nebulisation were similar irrespective of the formulation, nebuliser used and stage of the impinger. This indicates that the vesicles may have similar surface properties after being delivered by nebulisation, if these formulations would be considered for in vivo investigations. It was also seen that the vesicles became more negatively charged after nebulisation using all three nebulisers.

On studying the initial entrapment of BDP on all four formulations, it was observed that vesicles without cholesterol entrapped significantly greater BDP than vesicles with cholesterol. This was due to the competition between BDP and cholesterol to be incorporated into the vesicular bilayers because of the similar structure of BDP and cholesterol. After this study it was concluded that surfactosomes without cholesterol was the best formulation to be delivered via all three nebulisers. This is due to the elasticity of the surfactosomes that has maximised the BDP proportioned delivered without considerable leakage. These vesicles fragmented less in the presence of forces generated by the nebulisers compared to other vesicles studied. The absence of cholesterol in the formulation decreased the rigidity and increased the flexibility, thus, delivering maximum BDP.

After concluding surfactosomes without cholesterol to be the best formulation to deliver maximum BDP using all three nebulisers, the best nebuliser suitable to deliver the BDP via other 3 formulations was also analysed. It was concluded that for liposomes with and without cholesterol, Beurer iH50 was the most suitable nebuliser in this study. This was because the Beurer iH50 device delivered maximum BDP to both stages of the twin impinger using liposomes. For surfactosomes with cholesterol PARI LC sprint air jet nebuliser was proved to better than the other two devices at delivering BDP to lower stage of impinger.

This study also proved that using all three nebulisers, higher BDP proportions were delivered to the lower impinger as compared to the upper impinger. However, the air jet nebuliser delivered significantly greater BDP proportions than both vibrating mesh nebulisers to the lower impinger stage.

The BDP retention studies concluded that vesicles with cholesterol retained less BDP than vesicles without cholesterol when nebulised via vibrating mesh nebulisers. This was possibly due to the competition between BDP and cholesterol to be incorporated in the vesicular bilayer. This displaced more BDP during extrusion via nebuliser, thus, retaining lower drug proportions in the vesicles having cholesterol. However, in air jet nebuliser, surfactosomes without cholesterol retained the least BDP after nebulisation. This was possibly due to the excessive fragmentation of this type of vesicles due to the shear force generated by the air jet nebuliser. Absence of cholesterol and presence of surfactant made the vesicles less stable, thus, increasing BDP leakage.

On analysing the aerosol VMD generated by all three nebulisers it was concluded that the nebulisers generated aerosols with larger VMD than liposomes and surfactosomes regardless of cholesterol incorporation. This indicates that the vesicles can be incorporated in the aerosols with least fragmentation, thus, leading to less BDP leakage.

Thus, this study helped to conclude the best formulation of all four formulation studied in this work to be used for maximum delivery of BDP via nebulisation. The formulations with surfactosomes were concluded to be ultradeformable in this study as it delivered maximum drug with less leakage. It also helped to conclude the most suitable nebuliser among the 3 used, for all four formulations and to deliver maximum BDP twin impinger representing the upper and lower respiratory tract.

7.4. Characterisation of proliposomes and prosurfactosomes for stability

In chapter 6, the stability of liposomes and surfactosomes prepared from proliposomes and prosurfactosomes respectively were studied.

On studying the effect of spray drying on liposomes and surfactosomes, it was observed that the VMD and span of the vesicles and initial entrapment of the drug was similar for vesicles before spray drying and those after the drying was conducted. However, surfactosomes had higher zeta potential values than liposomes. After spray dying, there

was no difference in their VMD, span ad BDP entrapment and was similar to that of before spray drying. This shows that surfactosomes were as stable as liposomes to spray dying.

On studying the effect of freeze drying, it was found that the VMD and span of surfactosome increased significantly whereas liposomes had similar VMD and span. BDP leakage was significantly more in surfactosomes compared to liposomes, indicating liposomes are more stable to freeze drying than surfactosomes.

After SEM studies, it was seen that mannitol lost its porosity after being coated by SPC and cholesterol. After spray drying, proliposomes and prosurfactosomes were reduced in size and became porous microspheres. After freeze drying, they became very small needle shaped and had flake-like porous structures. It was also observed that prosurfactosomes apparently had slightly smoother surface than proliposomes, possibly due to the presence of the Tween 80 (surfactant) in prosurfactosomes.

On studying the X-ray diffraction patterns, it was found that proliposomes and prosurfactosomes were more crystalline before spray drying and freeze drying. The crystallinity was dominated by mannitol as they formed distinct peaks. It was found that after spray drying proliposomes and prosurfactosomes became semi-crystalline (i.e. the amorphous content of the powders increased). This was shown by the amorphous hump and broad less distinct peaks. Similar observation was made after freeze drying which led to a decrease in the crystallinity of the formulations.

The stability of proliposomes and prosurfactosomes were studied over 12 weeks where the samples were stored at 2-8°C (in refrigerator), room temperature and 40°C (in incubator). On studying the pH it was observed that the pH of both liposomes and surfactosomes decreased (became acidic) with time and 40°C was the most unfavourable temperature whilst 2-8°C was the most favorable. On studying the VMD it was observed that proliposomes were more stable than prosurfactosomes in all three conditions. The VMD of surfactosomes made from prosurfactosomes increased after 4 weeks whereas for liposomes made from proliposomes the size measurements remained consistent. On studying the span it was observed that the span of liposomes and surfactosomes prepared from hydration of proliposomes and prosurfactosomes exhibited the maximum increase at 40°C and minimum increase at 5-6°C. However, it was demonstrated that proliposomes were more stable than prosurfactosomes in all

three temperatures investigated. Zeta potential studies revealed similar results of being stable in 5-6°C. Prosurfactosomes increased their charge more than liposomes towards the end of the 12 week period of investigation, thus, being less stable. These stability studies can also reveal that the stability of proliposomes and prosurfactosomes was at best at the fridge temperature (5-6°C) and exhibited the worst stability at 40°C. For each temperature, proliposomes were more stable than prosurfactosomes. However, the stability was studied over 12 weeks was only a preliminary study as the experiment was conducted only once. The experiment should be repeated at least 2 more times to validate the above results.

7.5. Study limitations and future work

In this study the characteristics of surfactosomes have been evaluated and compared to conventional liposomes. The efficiency of the vesicles at retaining SBS and BDP after extrusion and delivery of BDP via nebulisation was also studied. However, this study has many scopes to be improved and further testing and experiments are required to formulate a best formulation to deliver hydrophilic and hydrophobic drug to the pulmonary system.

7.5.1. Use of different surfactants

In this study Tween 80 was used as the primary surfactant. However, surfactosomes can be made using other Tw eens as well as Spans. On using other surfactants with different HLB values, there is a possibility of formulating a better surfactosome with high drug entrapment.

7.5.2. Use of different carbohydrate carriers

In this study mannitol was used as the model carrier in formulating particulate based proliposomes and prosurfactosomes. There are many other carriers available like sucrose, sorbitol and lactose. Formulations with these carriers may lead to different entrapment efficiencies of BDP and SBS along with their nebulisation efficiencies. Hence, the experiment can be repeated with other carbohydrate to validate the efficiency of prosurfactosomes.

7.5.3. Use of different phospholipids

In this work, soya phosphotidylcholine (SPC) was the primary phospholipid used. Different phospholipids like egg phosphotidylcholine (EPC), Dimyristoylphosphatidylcholine (DMPC) and dypalmitoylphosphatidylcholine (DPPC) are available and, thus, can be used to prepare liposomes and surfactosomes.

7.5.4. PEGylation

PEGylated liposomes have been studied extensively in drug delivery. PEGylation may increase the size and molecular weight of biomolecules, thus, improving their pharmacokinetics and pharmacodinamics, protecting molecules from enzymatic degradation, reducing renal clearance and limiting immunogenic reactions. Similarly, the surfactosomal formulation can be PEGylated and its effect can be studied.

7.5.5. Use of different hydrophilic and hydrophobic drugs

SBS and BDP were used as the model hydrophilic and hydrophobic drug respectively in this study. However, these findings can be validated by using different drugs available for treatment of diseases other than asthma. On studying the results with other hydrophilic and hydrophobic drug may give a clearer vision about the novel prosurfactosomes and their potential for pulmonary administration.

7.5.6. *In vivo* studies

In this study only *in vitro* experiments were conducted due to lack of time and the need for ethical approval if animals are to be used. However, a more robust conclusion on the efficiency and safety of the prosurfactosomes can be finalised only after conducting *in vivo* experiments using animal models and possibly human volunteers.

7.5.7. Stability studies

In this work the stability of proliposomes and prosurfactosomes was tested using freeze drying and spray drying. However, there can be more methods to compared and test stability of the formulations. The 12 weeks stability study in this work was conducted only once, hence, they should be repeated at least 2 more times to confirm the results.

Chapter 8

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