University of Central Lancashire

Nanotechnology in pulmonary drug delivery: Compressible proliposome formulations for nebulization

Mr Iftikhar Khan

PhD Thesis

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In the Name of Allah, the Most Gracious, the Most Merciful

"The ink of the scholar is more holy than the blood of the martyr"

- Prophet Muhammad (Peace be upon him) -

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I would like to dedicate this thesis to my parents

"Awal Khan & Dilruba Khan"

Every happiness is incomplete without you;

You left a void that can never be filled

ABSTRACT

Liposomes are promising carriers for the encapsulation of drug molecules and subsequent delivery to the respiratory tract. The advantages of liposomes as carriers have been widely reported in literature, there are however associated disadvantages such as stability issues when suspended in aqueous media. This has been remedied partially by proliposome formulations (i.e. dry formulation of lipid with carbohydrate carrier). The aim of this work was to develop a novel solid dosage form of "proliposome tablets" (a precursor of liposomes), entrapping beclometasone dipropionate (BDP) as a model hydrophobic drug for the treatment or prophylaxis of asthma.

A novel proliposome formulation method was developed in this study called the "Slurry" method, which is readily scalable to an industrial level. Soya Phosphatidylcholine (SPC) was utilised as the phospholipid component in combination cholesterol, in addition to four carbohydrate carriers (i.e. Lactose monohydrate (LMH), sucrose, sorbitol and D-mannitol), in three formulation ratios (i.e. 1:5, 1:10 and 1:15 w/w lipid to carrier) for the preparation of proliposome powders and tablets. Two dispersion media; deionised water (DW) or deuterium oxide (D_2O) were used to generate liposomes from the formulated powders and tablets. Transmission Electron Microscopy studies confirmed that the generated liposomes from the proliposome formulations were multivesicular liposomes. In literature, the entrapment efficiency values in DW are recorded to be in excess of 80%. Using D_2O, a novel separation method was developed, through which the entrapment efficiency of BDP in the generated liposomes was found to be in the range of 19.69 - 61.91%, showing great disparity in entrapment efficiency when compared to entrapment in DW. BDP entrapment efficiency was found in the following order of carriers used: Sorbitol > Sucrose > D-mannitol > LMH.

D-mannitol and sorbitol-based powder formulations were assessed in terms of flowability via tapped density (17.81 $\pm$ 4.46%) and angle of repose (25.62 $\pm$ 1.08°), classifying sorbitol as possessing good to excellent flowability. Upon tableting of sorbitol and D-mannitol, formulations at lipid to carrier ratios of 1:10 and 1:15 w/w; were viably compressible into tablets. Furthermore, sorbitol-based tablets at a ratio of 1:15 w/w were observed to be the only carrier-based formulation, using the automated function of the Minipress tablet machine (20 $\pm$ 2 tablets/minute with a compression force of 3.00 - 4.50 kN). The manufactured tablets were tested in accordance with British and US Pharmacopeial standards. Nebulization studies of formulated proliposome powders and tablets (sorbitol and D-mannitol-based) were conducted to identify the most efficient formulation for pulmonary delivery. Tablets manufactured from sorbitol-based proliposome powders (1:15 w/w ratio) offered the shortest nebulization and sputtering times

(24.18 ± 0.16 and 2.42 ± 0.54 minutes), with a high output rate (180.20 ± 0.31 mg/minute). Liposome size for the 1:15 w/w lipid to carrier ratio, prior to nebulization, for sorbitol tablets was 5.85 ± 0.86 μm. Moreover, this size was smaller than the liposomes found in the nebulizer reservoir i.e. (10.38 ± 1.11 μm). Based on entrapment efficiency, tableting and nebulization studies, sorbitol-based proliposome formulations at the 1:15 w/w lipid to carrier ratio; a six-month stability study was carried out under various conditions (Room, Fridge and Hot temperature i.e. 22 ± 1°C, 6 ± 1°C and 40 ± 1°C), and testing (size, charge, entrapment efficiency and a set of quality control tests) at monthly intervals. Liposome size increased dramatically from the control sample size (5.9 ± 0.7 μm), to (9.19 ± 0.81 μm) at 40°C on month six. The entrapment efficiency dropped significantly at 40°C decreasing from 57.43 ± 9.12% (control tablets) to 32.56 ± 5.47% at the sixth month of Hot temperature storage. This was posed to be due to degradation of the lipid under high temperature.

These research findings established sorbitol-based proliposome formulations (in a 1:15 w/w lipid to carrier ratio) to be the most appropriate for tableting on an industrial scale. Proliposome formulation and entrapment efficiency studies identified a novel method of liposome separation and proliposome manufacture. Nebulization and stability studies indicated that proliposome tablets are a viable option for pulmonary drug delivery eliciting high stability when sorbitol was used carbohydrate carrier in proliposome.

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ACKNOWLEGEMENT

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On the partial completion of one phase in life, I move to the next. To my fiancé, Hina Khan, I look forward to marrying you and building our future together, I could not have found a better life partner had I tried.

LIST OF ABBREVIATIONS AND ACRONYMS

API	Active pharmaceutical ingredient
AOR	Angle of repose

BDP	Beclomethasone dipropionate
β	Beta
BP	British Pharmacopeia
COPD	Chronic Obstructive Pulmonary Disease
DW	Deionised water
D_2O	Deuterium oxide
DSC	Differential scanning calorimetery
DLPC	Dilauroyl Phosphatidylcholine
DMPC	Dimyrestoyl phosphatidylcholine
DPPC	Dipalmitoyl phosphatidylcholine
DPIs	Dry powder inhalers
EPC	Egg phosphatidylcholine
FPF	Fine particle fraction
FDA	Food and Drug Administration
GIT	Gastrointestinal tract
g	Gravity
GMP	Guanosine monophosphate
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
Kg	Kilogram
KN	Kilonewton
KV	Kilovolts

LMH	Lactose monohydrate
LUVs	Large unilamellar vesicles
LDV	Laser Doppler Velocimetry
MCC	Microcrystalline cellulose
MHz	Megahertz
µm	Micrometers
MLVs	Multilamellar vesicles
mm	Millimeter
M	Molar
MVLs	Multivesicular liposomes
N	Newton
NSAIDs	Non-steroidal anti-inflammatory drugs
nm	Nanometers
pMDIs	Pressurised metered dose inhalers
PEG	Polyethylene glycol
QC	Quality control
QDs	Quantum dots
RBF	Round bottom flask
RPM	Revolutions per minute
SEM	Scanning electron microscopy
SNPs	Solid nanoparticles
SPC	Soya phosphatidylcholine

SUVs	Small unilamellar vesicles
TD	Tapped density
TBT	Tracheo bronchial tree
θ	Theta
3D	Three dimensional
T_m	Transition temperature
USP	United State Pharmacopoeia
(V/V)	Volume /volume
VMD	Volume median diameter
W/O	Water in oil
W/V	Weight/volume
W/W	Weight by weight
XRD	X-ray diffraction

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CHAPTER 1: INTRODUCTION

"People sleep to dream; I cannot sleep because of my dreams"

- Abdul Kalam -

The term "Nanotechnology" was first introduced in 1974 by Taniguchi, describing this field as having innumerate potential applications (Taniguchi, 1974). However, for mankind, though unrealised at the time, nanotechnology had been introduced eons earlier, in 500 BC in the forging of steel-based materials. Nanotechnology is an exceptionally vast field, and therefore it has been subdivided into many sub-areas, such as; nanoengineering, nanophysics and nanomedicine.

Whilst alternate non-medical-based fields such as engineering have seen remarkable progression through the introduction of nanotechnology, the milestones achieved in nanomedicine have been no less astounding. Though, the question must be posed, what is actually inferred by nanotechnology? A simple definition refers to nanotechnology as the manipulation of atoms or molecules ranging in size from 1 to 100 nm (nanometres) (Mansoori and Soeliman, 2005).

In the preceding decades with reference to medicine, drug delivery has developed as a prominent field, allowing for the specific or localised targeting of virtually any component of the body. The introduction of nanotechnology has diversified the specificity of this approach, allowing for cellular and even cellular organelle targeting (e.g. DNA targeting). Aside from targeting, nanomedicine has demonstrated improvements in solubility and stability of active pharmaceutical ingredient (API). Nanomedicine-based systems have been used for *in-vivo* imaging in combination with simultaneous drug delivery and monitoring (Key and Leary, 2014).

There are a number of nano-based systems under continuous interest and investigation in medical research, such as dendrimers, which were introduced by Fritz Vogtle and co-worker in 1978 (Buhleier et al., 1978). Dendrimers typically have a size between 10 - 100 nm and are hyper-branched with three dimensional nanoscale synthetic polymers (Wiener et al., 1994, Bawarski et al., 2008). Alternatively, colloidal semi-conductor nanocrystals termed Quantum dots (QDs) have been developed and implemented to enhance *in-vivo* imaging. Typically these are between 2 - 10 nm in diameter and synthesised from semi-conductor materials, including; indium phosphate, indium arsenide and cadmium selenide etc. (Bawarski et al., 2008). Moreover, Solid nanoparticles (SNPs) have also shown great promise in nanomedical research. They are made from biodegradable materials such as lipids, proteins or polymers (Mo et al., 2007) with a size range of 10 - 1000 nm; and are used in drug delivery (Bawarski et al., 2008).

Nanocarriers with affinities for hydrophobic and hydrophilic environments or materials have seen much research. Through this research it has been established that many of these carriers pertain the ability to entrap both hydrophilic and hydrophobic compounds; which is highly desirable. Non-ionic surfactants which form micelles are key examples of such carriers, possessing hydrophilic and hydrophobic moieties. These colloidal carriers are commonly used in

pharmaceutical formulations particularly for water soluble and insoluble agents, possessing a size of less than 50 nm in diameter. These nanocarriers allow for the modified release of entrapped drug as well use *in-vivo* imaging (Bawarski et al., 2008).

Whilst micelles are notably advantageous carriers, liposomes allow greater versatility in drug delivery owing to their unique structure, i.e. multiple concentric bilayers formed from phospholipids. This structure allows for the entrapment of drugs for localised delivery or systemic targeting. The benefits associated with drug entrapment in liposomes include modified drug release, miniscule size (allowing for specific organ or cellular targeting) and provision of large surface area, all of which contribute to enhanced drug delivery (Radhakrishnan, 1990, Mufamadi et al., 2011). These carriers have been successfully introduced into the medical market and continue to revolutionise and shape the future of medicine.

1.1. Introduction to liposomes in drug delivery

The introduction of new technologies and techniques in the field of biotechnology, biomedical science, medicine and pharmaceuticals, has instigated revolutionary developments in recent years through the design of novel therapeutic agents (i.e. drugs) (Sharma and Sharma, 1997, Hess et al., 2011b). However, research on identifying apposite delivery systems of these drugs is ongoing, with the continuous aim to improve the performance of existing APIs. Scientists from various disciplines have attempted to invent new technologies to deliver macromolecules. For example, aerosol formulations were used to target respiratory diseases to achieve local effect via pulmonary epithelium, reaching the lower lung and offering the possibility for absorption into the systemic circulation (Dunbar et al., 1997).

Though there are vast improvements and novel developments in drug delivery, formulations are of little use without patient acceptability. A survey regarding self-injection was performed on 40 diabetic patients, 75% of these patients showed anxiety towards self-injecting (Bashoff and Beaser, 1995). Anxiety or dislike associated with a particular delivery is unsurprisingly linked to poor compliance, and consequentially poor treatment outcomes. The oral route has been identified as the most accepted and common route of drug administration. However, the hostile environment of the GIT (Gastrointestinal Tract), i.e. high variances in pH and presence of a plethora of enzymes, may make drug delivery through this route challenging (McConnell et al., 2008). In addition, the first pass metabolic effect may result in the elimination of large quantities of drug following oral administration. Both the environment and the presence of first pass

metabolism may directly or inadvertently influence bioavailability, again influencing treatment outcomes.

The pitfalls of the GIT, as well as disadvantages with alternate methods such as intravenous or subcutaneous administration, have resulted in much research in the potential of the lung as a drug delivery site, for local and systemic action. The lung is known to provide a large surface area, and is regarded superior for rapid drug absorption, owing to its high vascularity, allowing for quick systemic availability and avoidance of first pass metabolism (Todo et al., 2004). Depending upon particle size as well as general physiological conditions, particles may deposit into the Tracheobronchial Tree (TBT), by the following mechanisms: Impaction, sedimentation and interception or Brownian diffusion (Johnson, 1997).

Liposomes were first described in 1965 by Bangham and co-workers, who studied phospholipid chemistry and their correlation with biological cells and membranes (Bangham et al., 1965). Living cells require lipids, proteins and nucleic acids to maintain their survival. Various lipids are utilised as building blocks of bio-membranes; these are key components for the formation of liposomes (Bergstrand, 2003). Liposomes are considered a promising technology and are seen to be a milestone in the field of Bio-nanotechnology; particularly due to their ability to act as a nanocarrier, entrapping both hydrophilic and hydrophobic molecule, for drug delivery (LeDuc et al., 2007).

The non-irritant and relative safety of lipids in the form of liposomes maximises the concentration of therapeutic agents in a given target area (Parthasarathy et al., 1999). The amphiphilic nature of liposomes enhances their diverse use as nanocarriers for drug delivery (Bergstrand, 2003). Formation of liposomes is attributed to the property of spontaneous aggregation of phospholipids, which is commonly termed as self-assembly (Bergstrand, 2003). However, the instability of liposomes due to hydrolysis and oxidation of phospholipids is a serious limitation, for which a number of approaches have been adopted (Kensil and Dennis, 1981, Vemuri and Rhodes, 1995, Riaz, 1995).

1.2. Composition and properties of liposomes

Phospholipids are the primary component of liposomes; these are either naturally derived, or artificially synthesised. However, the unique amphiphilic property (Figure 1.1) and the ability of self-assemble into liposomes (Figure 1.3 and Figure 1.4), make phospholipids in the form of

liposomes highly appropriate for encapsulation and delivery of therapeutic agents (i.e. both hydrophobic and hydrophilic) (Ganderton, 1999).

1.2.1. Phospholipids

Phospholipid molecules are comprised of a phosphate group (a molecule of phosphoric acid), diglycerides and an organic molecule (choline) (Figure 1.1Figure 1.1). The diglyceride component is a glyceride composed of two fatty acid chains, which are covalently bonded to a glycerol molecule via an ester link. The three hydroxyl groups (-OH) in glycerol ($C_3H_8O_3$) are responsible for the affinity of phospholipids towards water, whereas the glycerol acts as a backbone, as it is attached to both the phosphate group and fatty acids (hydrocarbon chains) (Vemuri and Rhodes, 1995). Hydrocarbon chains are either saturated or unsaturated, and they represent the hydrophobic moiety of the phospholipid molecule. Thus, phospholipids are amphiphilic molecules which assemble themselves in water in accordance to this inherent affinity. The amphiphilic characteristics of phospholipids make them highly applicable in a multitude of fields (Dolovich, 1999), by encapsulating hydrophobic drug molecules within the concentric bilayers and hydrophilic agents in the central core (Figure 1.1), which is formed when phospholipids are assembled into liposomes in the presence of aqueous phase. A single lamellar or bilayer structure is formed when a moiety (tail) of one of the fatty acid layers faces the moiety (tail) of another layers and the polar head groups face the aqueous phase (Figure 1.3 and Figure 1.4).

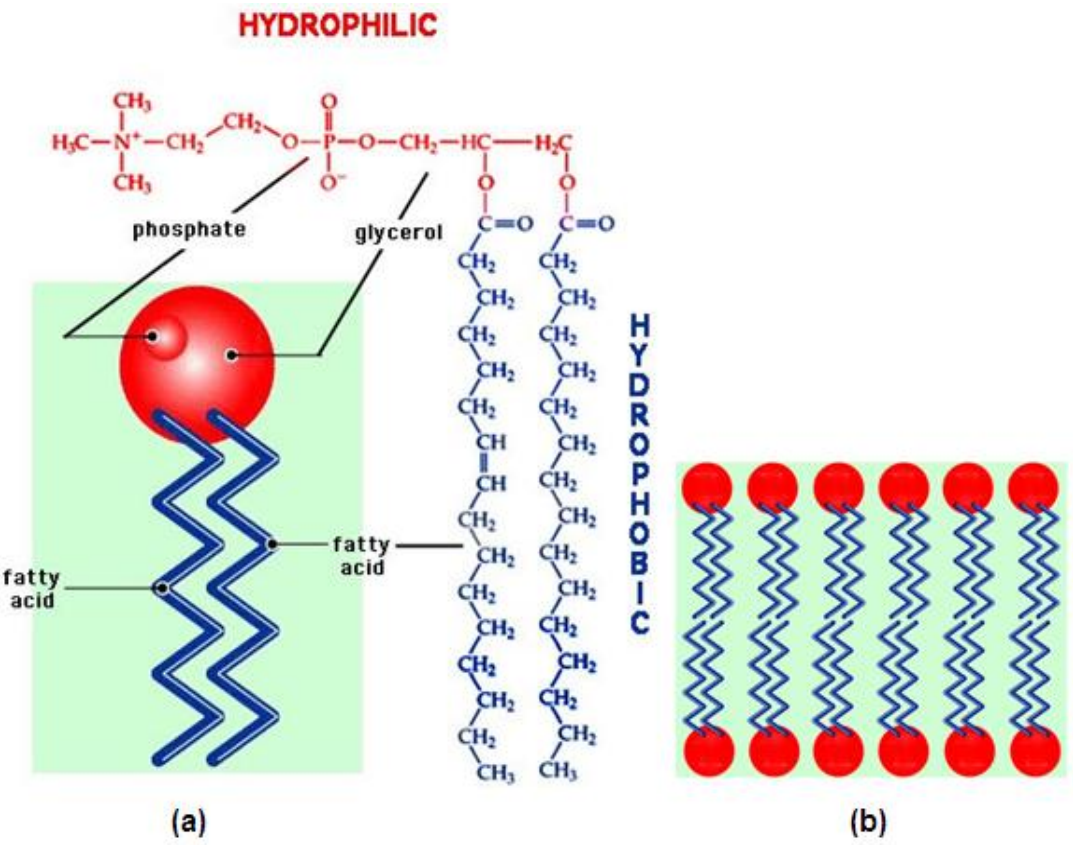


Figure 1.1: Phospholipid structure, (a) showing a generic phospholipid, containing hydrophilic and hydrophobic moieties, (b) representing a typical bilayer structure. (*http://textbookofbacteriology.net/themicrobialworld/Structure.html*)

As discussed, phospholipids are either naturally or synthetically derived. Naturally available phospholipids include: Egg phosphatidylcholine (EPC) and Soya phosphatidylcholine (SPC). Whilst synthetic phospholipids include the commonly used phospholipids: Dimyrestoyl phosphatidylcholine (DMPC) and Dipalmitoyl phosphatidylcholine (DPPC).

1.2.2. Cholesterol

Cholesterol may also be included in the liposome structure (Figure 1.2), enhancing the stability of the vesicles, and minimising the permeability of water-soluble materials across the bilayer membranes. Thus, liposomes are made from phospholipids and cholesterol, in order to prolong the retention time of the liposome-entrapped drug. Cholesterol also enhances the rigidity of liposomes by filling the gaps between the assembled phospholipid molecules, reducing drug leakage (Kirby et al., 1980).

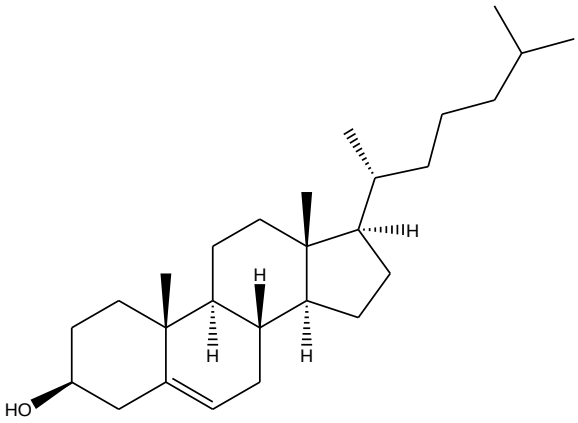


Figure 1.2: Structure of cholesterol

1.2.3. Self-assembly

The phenomenon of spontaneous self-assembly of phospholipids into liposomes was first observed by Bangham et al., (1965). Amphiphiles dissolve as monomers in aqueous solution and as their concentration increases, they begin to assemble themselves (Figure 1.3) (Jesorka and Orwar, 2008). In the presence of water, these amphiphiles arrange and position themselves in thermodynamically favourable arrangements, typically in the form of sheets (Israelachvili et al., 1977) as shown in Figure 1.3.

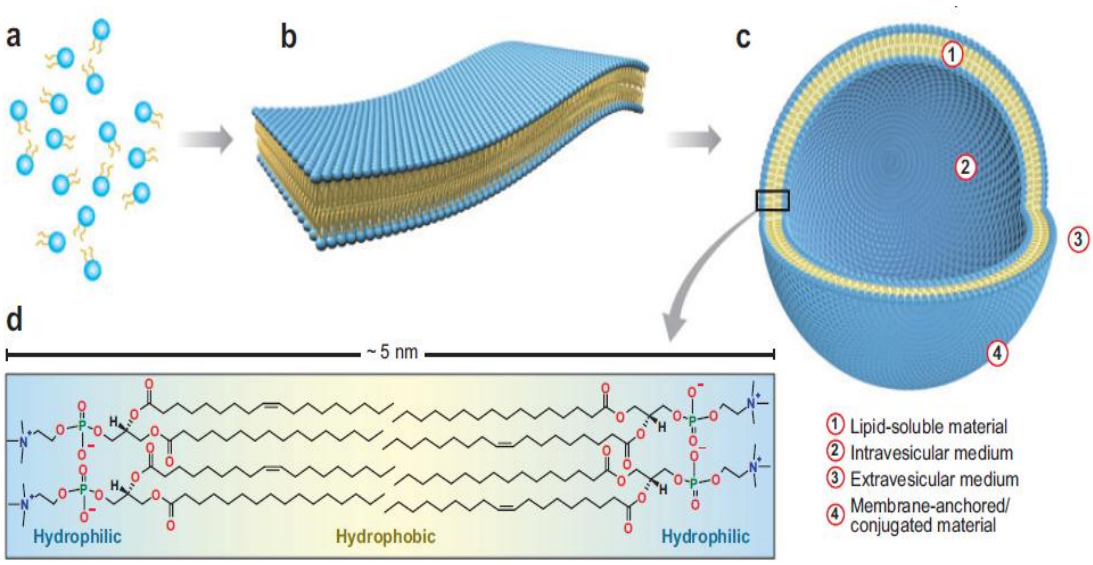


Figure 1.3: A schematic presentation of liposome self-assembly in aqueous phase: (a= Dispersion of phospholipid molecules, b= Sheet of phospholipids, c= Liposome formation, d= Phospholipid chemical form and their arrangement) (*Jesorka and Orwar, 2008*)

The high entropy of the system is thought to be responsible for the formation of amphiphile sheets, this is due to the force of interaction caused by the aqueous phase (water) with the hydrophobic chains (lipophilic moiety) (Bergstrand, 2003). The flat sheets bud off and convert into geometrical shapes slowly forming a smooth outer surface (Figure 1.4). For self-assembly to occur energy is required; which can be supplied by various means of agitation or heat (Dolovich, 1999, Jesorka and Orwar, 2008).

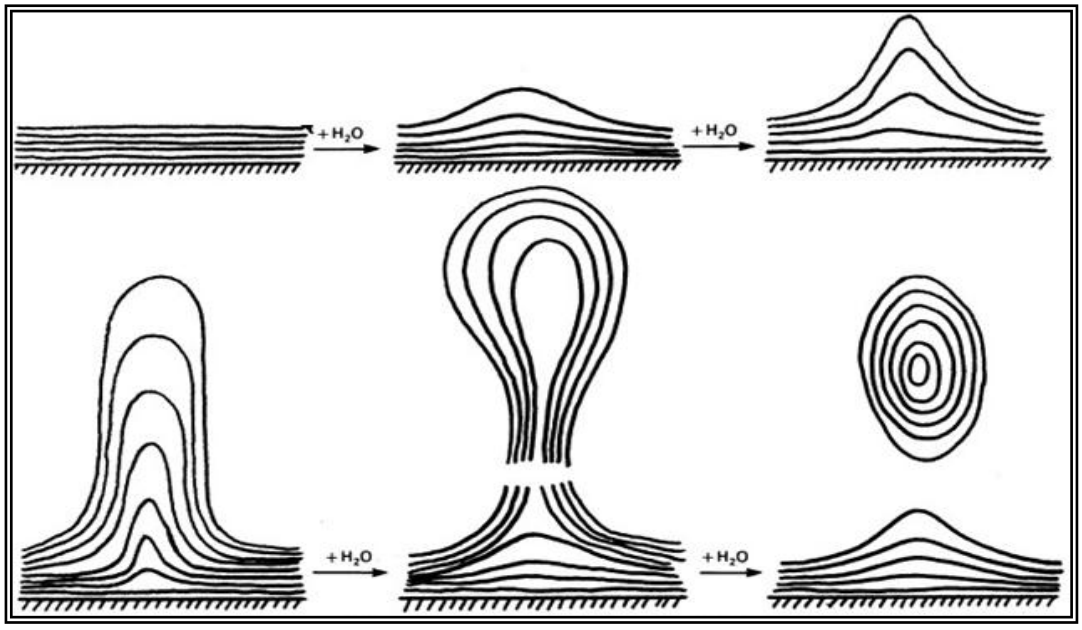


Figure 1.4: A schematic presentation of a multilamellar liposome forming, illustrating how the sheets of concentric layers bud off and form liposomes (*Lasic, 1988*)

1.3. Liposomes

Liposomes are spherical microscopic vesicles, composed of single or multiple concentric bilayers of phospholipids; formed by self-assembly of amphiphilic molecules into vesicular structures. These have the ability to entrap both hydrophilic (in the central core) and hydrophobic molecules (within the concentric lipid bilayers) (Figure 1.1) (Payne et al., 1986a, Payne et al., 1986b, Sharma and Sharma, 1997, Blazek-Welsh and Rhodes, 2001, Darwis and Kellaway, 2001). Liposomes are formulated from both synthetic and/or natural phospholipids which may be used in combination with polymers or cholesterol (Sharma and Sharma, 1997). The size of liposomes falls typically in the range of 20 nm to 20 µm (micrometers) (Taylor and Morris, 1995). Liposomes are biodegradable and biocompatible, making them highly suitable for drug delivery. A variety of

pharmaceutical agents such as antineoplastic agents, antimicrobial drugs, vaccines and steroidal drugs have been formulated in liposomes, to aid and manipulate drug delivery (Gregoriadis and Florence, 1993). Liposomes are considered to be highly reliable carrier vesicles in terms of their ability to protect the entrapped drug from degradation *in-vivo*. These also possess the ability to prolong the therapeutic effect of the API, by sustaining its release profile (Kirby et al., 1980, Szoka and Papahadjopoulos, 1980). Each type of phospholipid possesses a specific phase transition temperature (T_m) and should be hydrated above this to form liposomes. At this particular temperature, an ordered/packed gel state of the phospholipid converts into a less ordered/packed crystalline state, where phospholipid bilayers of liposomes become more flexible and leaky, and as a consequence are able to entrap greater proportions of API (Taylor and Morris, 1995, McMaster, 2007).

1.4. Classification of liposomes

Liposomes may be classified either on the basis of their size, morphology or number of lamellar membranes. Based on their number of bilayers, liposomes may be further classified as large unilamellar, small unilamellar, multilamellar and oligolamellar (Figure 1.5).

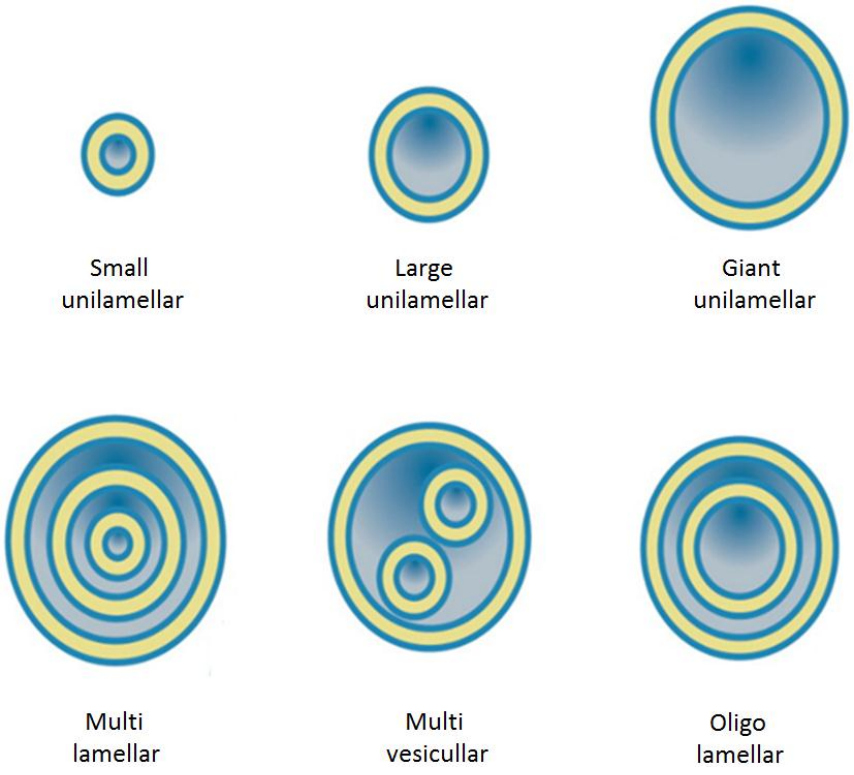


Figure 1.5: Classification of liposome on the basis of lamellarity (*Jesorka and Orwar, 2008*)

1.4.1. Small unilamellar vesicles

Small unilamellar vesicles (SUVs) are typically found within a size range of 20 - 100 nm (Lasic, 1988). Method of preparation is paramount in dictating the type of liposome formed, for SUVs, the solvent injection method can be employed (Vemuri and Rhodes, 1995). Through this method an ethanolic solution of phospholipid is added by injection into a warm aqueous medium (Batzri and Korn, 1973). The temperature is maintained above the phase transition temperature (T_m) to ensure formation of unilamellar liposomes. This method typically produces liposomes with a size approximating at 25 nm (Batzri and Korn, 1973, Pons et al., 1993).

Probe sonication is an alternative method which may be implemented in the formulation of SUVs. Through this method, a probe sonicator generates sound waves which are utilised to reduce the size of liposomes. A disadvantage of this method is the associated generation of heat, which may be adequately controlled by intermittently transferring the vessel containing liposome suspension to crushed ice, in order to reduce the potential of degradation due to excessive heat (Akbarzadeh et al., 2013).

1.4.2. Large unilamellar vesicles

Large unilamellar vesicles (LUVs) consist of a single phospholipid bilayer, offering high entrapment capability of hydrophilic materials in the aqueous central core of liposomes. In terms of size, LUVs are notably larger than their SUVs counterparts, ranging from 0.1 - 1 µm (Szoka and Papahadjopoulos, 1980, Lasic, 1988). The method of production implemented is referred to as reverse phase evaporation. This method has been demonstrated to yield entrapment efficiencies ranging from 60 - 65% (Paternostre et al., 1988, du Plessis et al., 1996). Via reverse phase evaporation, a water in oil (lipid) emulsion (w/o) is formed, by the addition of aqueous buffer to an organic phase. Subsequently, a rotary evaporator is utilised to remove the organic solvent under reduced pressure. The remaining water and lipid phase are then sonicated to obtain a gel which contains LUVs (Vemuri and Rhodes, 1995).

1.4.3. Multilamellar vesicles

Multilamellar vesicles (MLVs) (Figure 1.5) were first described by Bangham et al., (1965) and were identified to consist of multiple concentric bilayers of phospholipids, falling within a size range of 0.1 - 20 µm. A conventional or thin film method is used to prepare the MLVs spontaneously, by simple hydration followed by shaking (Bangham et al., 1965, Brown, 1973, Szoka and Papahadjopoulos, 1980, du Plessis et al., 1996).

Within a round bottom flask (RBF), lipids are dissolved in an organic solvent, subsequently; a rotary evaporator is used to evaporate the organic solvent under reduced pressure. Following evaporation of organic solvent, the thin lipid film formed is hydrated by addition of aqueous phase (e.g. a buffer solution), conducted at a temperature above the phase T_m. This is followed by manual agitation in order to obtain MLVs. Hydration time plays a crucial role in obtaining liposomes, as the entrapment may be enhanced by slower hydration and moderate mixing (Vemuri and Rhodes, 1995).

1.4.4. Multivesicular liposomes

Multivesicular liposomes (MVLs) are formulated using a method where organic solvent is evaporated from chloroform-ether spherules (i.e. spherical body), suspended in water. These spherules contain a number of water droplets, which contain the API. Upon organic solvent evaporation, the API is trapped into the liposome structure (Kim et al., 1983).

MVLs preparation involved three main steps. Firstly, the lipid phase is mixed with natural oils such as triolein in a glass vial, followed by the addition of chloroform and diethyl ether. The aqueous solution is prepared by dissolving API, sucrose or salt solution. This aqueous phase is then slowly added to the lipid containing solution with gentle manual agitation (shaking). Further shaking is carried out upon vortex mixing in order to form a w/o emulsion. Secondly, a chloroform-ether spherules were formed by placing a sucrose solution only in a glass vial and then w/o emulsion prepared were poured into this sucrose solution followed by vortex mixing in order to make liposomes (Kim et al., 1983). Lastly, the chloroform-ether spherules in sucrose solution become suspended and then with the help of nitrogen and constant shaking organic solvent are evaporated (Hess, 2000) and the liposome formed is removed via centrifugation (Zhong et al., 2005).

1.5. Stability of liposomes

The stability of liposomes is vital at every stage including formulation, storage, administration, and drug release. Stability studies are performed to check the qualitative and quantitative properties of these formulations. There are several known issues with liposomal stability, these are: sedimentation and aggregation of vesicles, and leakage of therapeutic agent (Wong and Thompson, 1982). Factors such as pH of medium, buffer type and solvent composition may also affect the stability of liposomes.

1.5.1. Chemical stability of liposome components

Liposome components (i.e. the phospholipids) show varying degrees of instability. For example, phospholipids are known to hydrolyse and oxidise (Hunt and Tsang, 1981). This is known to occur due to the unsaturated fatty acid component of these phospholipids, which undergo oxidation (Riaz, 1995), which may deleteriously effect the permeability of liposomes and subsequently, shorten the shelf-life of the formulation (Vemuri and Rhodes, 1995). Importantly, the aqueous medium in which liposomes are suspended, renders them unstable during storage. The peroxidation of phospholipid may be minimised by the addition of anti-oxidants during formulation, such as butaylated hydroxytoluene or α-tocopherol; these preparations however, are UV sensitive, thus should be shielded from direct light (Vemuri and Rhodes, 1995). Hydrolysis of the lipid causes the production of lyso-PC (lyso-Lecithin), which increases the permeability of liposomes (increasing drug leakage). Consequently, it is vital to maintain the level of lyso-PC at a minimum (Kensil and Dennis, 1981, Riaz, 1995, Vemuri and Rhodes, 1995).

1.5.2. Physical stability of liposomes

Physical properties of liposomes are crucial, and are evaluated via measuring the particle size distribution and visualising the microscopic appearance of the liposomes. Several problems associated with physical stability of liposomes, include: liposome aggregation, fusion and drug leakage. Liposome physical properties may be studied using several methods (Vemuri and Rhodes, 1995). Typically Electron microscopy and light scattering techniques have been employed in order to study the size of liposomes (Szoka and Papahadjopoulos, 1980). However, the

associated problems with physical stability, can be greatly decreased by formulating liposomes in dry form; for example, by using freeze drying, spray drying or proliposome formulations.

1.5.3. Freeze drying of liposomes

Freeze drying (lyophilisation) of liposomes minimises lipid hydrolysis during storage. This process also increases the stability and shelf life of partially frozen liposomes (Mahmoud Nounou et al., 2005). Leakage of the entrapped drug from liposomes as a result of the stress exerted on liposomes during freezing and/or water sublimation, is a drawback of the freeze drying process. This can be reduced by the addition of lyoprotectants (e.g. carbohydrates) prior to freeze drying (Mahmoud Nounou et al., 2005, Stark et al., 2010). Many different lyoprotectants such as sucrose, lactose, trehalose or other sugars can be used to protect the liposome bilayers during freeze drying. Although freeze drying is employed to manufacture stable liposomes for long-term storage, the process itself can damage the liposomes during the formation of ice crystals (during the freezing stage) or application of vacuum (during the drying stage). The lyophilised dry cake of liposomes can be converted into a liquid dispersion of liposomes by rehydration in aqueous solvent. Ideally, the rehydrated liposomes should not change in size, and the entrapment efficiency of the drug should be maintained or improved.

1.5.4. Spray drying

Spray drying is a technique where a solution containing all the ingredients including API is converted into fine dry powders, producing higher stability formulations (Johnson, 1997). Carbohydrates such as sucrose, mannitol, lactose and trehalose can be used as excipients, when spray drying is employed to manufacture dry liposome formulations (Naini et al., 1998). The use of spray drying technology is not limited to the formulation of dry liposomes i.e. proliposomes. Thermo-labile molecules in combination with carbohydrates can be converted into fine powders, owing to their small droplets size in the heating chamber of the spray dryer. This allows drying to occur within 100 milliseconds to a second; thus degradation of these sensitive macromolecules during drying is potentially reduced (Johnson, 1997). However, it is worth mentioning that parameter selection plays a key role in obtaining high quality, spherical shaped particles upon

spray drying (Johnson, 1997). Proliposomes are another approach that can be used to manufacture stable liposome precursors as an alternative dry liposome formulation.

1.5.5. Proliposomes

In general, liposomes may have a range of instability manifestations, such as aggregation and fusion, phospholipid oxidation and hydrolysis. To overcome these problems and minimise instability, a novel approach of liposome preparation is, the use of proliposomes (Payne et al., 1986a, Payne et al., 1986b). As these limitations are due to the instability of liposomes in aqueous medium, formulation in the absence of aqueous media may resolve this issue. Proliposomes are dry formulations which are prepared by coating lipids (phospholipid and/or cholesterol) onto carbohydrate carrier particles. These may then be converted into liposomes by the addition of water (i.e. by hydration) above the phase T_m of the phospholipid component, which causes dissolution of the carbohydrate carrier, and formation of multilamellar liposomes. The hydration step can be performed prior to administration of the formulation (Payne et al., 1986a) to avoid destabilisation of liposomes. High water solubility (during hydration), free flowability and low toxicity are the main overriding factors in choosing the carbohydrate carrier in proliposome formulations. Carbohydrates, including lactose monohydrate, sucrose, sorbitol, mannitol, fructose and glucose were investigated for their suitability as carriers for proliposome formulations (Payne et al., 1986a).

1.6. Preparation of proliposomes

1.6.1. Particulate-based proliposomes

Carrier selection is vital in controlling and maintaining desired properties such as powder flowability and porosity, after the preparation of particulate-based proliposomes. Carbohydrate carriers may be selected on the basis of their solubility, ability to accommodate or uptake lipid, particle size and porosity. Generally, the chosen carbohydrate carrier is transferred to a RBF and affixed to a rotary evaporator under reduced pressure (Payne et al., 1986b, Payne et al., 1986a). An organic solution containing API, phospholipid and/or cholesterol is then injected at set intervals in small portions (e.g. 0.5 - 1 ml each), via a feed-tube onto the carbohydrate carrier

particles. The organic solvent is then evaporated to form a thin film over the carrier surfaces (Jesorka and Orwar, 2008), yielding proliposomes which may then be stored at -18°C for subsequent use upon need. The dry proliposome formulation may then be converted into liposomes by addition of aqueous phase above the phase T_m of the phospholipid, prior to administration (Elhissi et al., 2006a).

Proliposome manufacture can be scaled up using a number of techniques, including: spray drying (Alves and Santana, 2004), fluidised-bed coating (Chen and Alli, 1987, Kumar et al., 2001) and fluid-energy micronisation (jet-milling) (Desai et al., 2002, Desai et al., 2003). In fluidised-bed coating, to ensure coating on particles is uniform, carbohydrate powders are sieved to maintain particle size uniformity, prior to transfer to the drying chamber of the fluidised-bed apparatus. The phospholipid component is then dissolved in an organic solvent and a spray gun is used to spray the resultant solution over nonpareil beads (carbohydrate powder). The resultant coated carbohydrate carriers are then allowed to dry at room temperature (Chen and Alli, 1987). Once again, liposomes are readily prepared through hydration of the formed proliposomes, by the addition of water. The hydration process is identical for proliposomes which are prepared through fluid-energy micronisation (a process which passes the liposome components through an air-jet forming micronised proliposome particles) (Desai et al., 2002, Desai et al., 2003).

1.6.2. Solvent-based proliposomes

Proliposomes may be prepared using ethanol as a solvent comprising the lipid phase (phospholipid and cholesterol). In this method, liposomes are generated by the addition of aqueous phase to the ethanol solution containing dissolved phospholipid, with or without cholesterol, followed by shaking (Perrett et al., 1991). The addition of aqueous phase in combination with shaking causes the transformation of proliposomes into liposomes.

This approach was first introduced by Perrett et al., (1991), and was modified by increasing the amount of cholesterol in the lipid phase and also ethanol to phospholipid ratio, to facilitate the dissolution of lipid (Perrett et al., 1991, Elhissi et al., 2006). Initially, cholesterol and lipid are dissolved in ethanol in a glass vial, which is then stored for 1 minute at 70°C. An isotonic solution (e.g. 0.9% sodium chloride) containing therapeutic agent is then added into the ethanolic lipid solution, liposomes were then generated by vortex mixing or manual shaking.

1.7. Liposome applications

There are a number of liposomal products available on the market in various dosage forms, for a myriad of diseases. Whilst it is established that liposomes are prone to instability, particularly in aqueous media, there are alternative dosage forms where stability is less problematic. This offers longer formulation shelf-lives, preserving pharmacological action and efficacy of the drug. A number of liposomal products which are currently available on the market have been listed below to highlight their wide application and utilisation.

1. ***Myocet***: A doxorubicin entrapped liposomal formulation, available as 50 mg doxorubicin hydrochloride. EPC and cholesterol are used as the lipid component of the liposome. It is ordinarily administered as an infusion over 60 - 90 minutes. Doxorubicin is a chemotherapeutic drug utilised for the treatment of breast cancer, ovary cancer and myeloma. It possesses a shelf-life of approximately 18 months and after the reconstitution its physical stability is only ensured for 8 hours at 25°C, and up to 5 days when stored between 2 - 8°C (eMC, 2014, Macmillan, 2014).

2. ***Lipodox***: Lipodox also contains doxorubicin hydrochloride; however the liposomes are coated with polyethylene glycol (PEG) chains. This dramatically increases the half-life of the product to up to 55 hours in humans. This product is available in the form of an infusion (2 mg/ml doxorubicin). It is most commonly used for breast cancer and requires storage between 2 - 8°C to ensure stability (DailyMed, 2012).

3. ***AmbiSome***: An amphotericin containing liposome formulation, which is available as a 50 mg sterile powder for infusion (after reconstitution contains 4 mg/ml of amphotericin B), has been used successfully for fungal infections. The product holds a substantial shelf life of 4 years, and after reconstitution is stable for up to 24 hours at 25°C, and for 7 days when stored at 2 - 8°C (eMC, 2012).

4. ***DepoCyte***: An injectable sterile liposome suspension of cytarabine, is indicated in the therapeutic treatment of lymphomatous meningitis (a life threatening condition or cancer of the immune system). This is available in a 50 mg dose for intrathecal use only, and it requires storage between 2 to 8°C (DailyMed, 2011).

5. ***Lipoplatin***: Is another anticancer formulation containing liposome entrapped cisplatin, used in the treatment of pancreatic cancer (for which there is a high mortality rate). The formulation is targeted directly at the tumour site, to minimise healthy cell exposure to the cytotoxic API. Liposomes with a 110 nm diameter (which are smaller in size than red blood cells) are noted and utilised for their enhanced performance and drug activity (Boulikas, 2004, Regulon, 2012).

1.8. Pulmonary System

Drug delivery to the lungs via inhalation has gained and sustained much popularity in the treatment of localised pulmonary diseases. The respiratory route has been identified as structurally promising for drug delivery, owing to the large surface area (up to 100 m^2) of the pulmonary organ (Figure 1.6). In addition to this, the thin mucosal membrane (0.1 - 0.2 µm) present in the pulmonary system, makes the lung a desirable site for systemic absorption and offer a means for evading the hepatic first pass metabolic effect (Huang and Wang, 2006). For many centuries the pulmonary route has been used in the treatment of cough (e.g. by using *Atropa belladonna*) as well as for the delivery of anaesthetics and nicotine in tobacco.

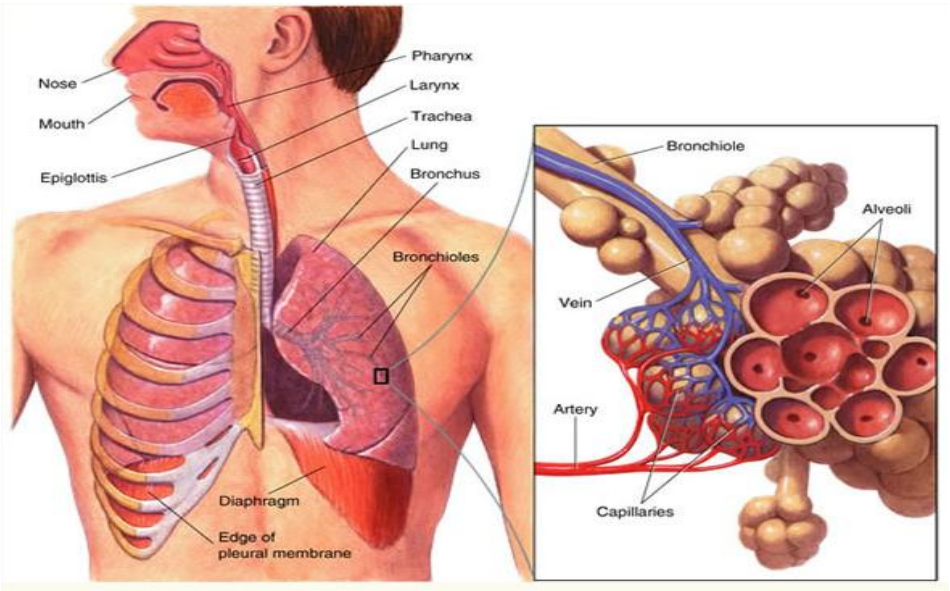


Figure 1.6: A schematic representation of the respiratory system, highlighting various sections (Human Respiration, Excretion and Locomotion). Taken from (*http://www.goldiesroom.org/Note%20Packets/13%20Human%20Other/00%20Human%20Other%20Systems--WHOLE.htm*)

The respiratory system consists of two main sections, the upper and lower respiratory tract. Air enters through the nostrils where it is moistened, filtered and warmed. Particles entering through the nose may become trapped by the mucus (secreted by goblet cells) of the nose. Cilia move particles from the respiratory tract towards the outside to be cleared via swallowing or expectoration. Air travels from mouth to pharynx, which consists of the nasopharynx, oropharynx and hypopharynx (Bassett, 2005, Van De Graaff et al., 2009), followed by the larynx and the trachea. The trachea is a flexible cartilage, which splits the respiratory tract into two parts; the right and left lung; the left lung is then divided into two lobes, whereas the right lung is divided into three lobes (Bassett, 2005) (Figure 1.6). The lungs are divided by carina into primary bronchi, referred to as the first generation of the Tracheo bronchial tree (TBT). This leads to the secondary bronchi (lobar bronchia or second generation), and in turn the third generation are commonly known as bronchioles (Bassett, 2005, Van De Graaff et al., 2009). Bronchioles lead to the terminal bronchioles where gaseous exchange occurs. The final destination is the alveolar region (which contains alveolar sacs), particles less than 2 µm in diameter are believed to be able to reach the alveolar area (Stahlhofen, 1980).

1.9. Pulmonary diseases

1.9.1. Asthma

Asthma is a chronic inflammatory disorder of the respiratory system, associated with airway hyper-responsiveness or obstruction of airflow. Broadly termed, asthma may be described as a respiratory disorder that leads to episodic struggle during breathing; additional symptoms include wheezing, coughing and chest tightness.

The exact cause of asthma is unknown due to a multitude of triggers which may cause airway constriction. Asthma is known to affect populations worldwide, with circa 300 million people documented as suffering from the disease state. Globally, every 1 in 250 deaths is accounted for by asthma (Masoli et al., 2004). In the UK only, over 5 million people are affected by asthma (Asthma, 2001). Although the mortality rate has been reduced significantly in the last two to three decades (Sidebotham and Roche, 2003), this is thought to be due to better clinical management, particularly through regular preventative treatment using inhaled corticosteroids (Gupta et al., 2006). However, the most common cause of death is due to inadequate assessment of the

severity of obstruction of airways of patients, in addition to inadequate treatment using oral or inhaled corticosteroids.

The two main established characteristics of asthma are bronchoconstriction and airway hyper-responsiveness. Hyper-responsiveness is the tendency of the respiratory system to respond to airway triggers, causing an asthmatic attack. Narrowing of the airway causes an obstruction in the airflow known as bronchoconstriction. Highly common allergens which instigate asthma attacks are found in homes, in the form of dust mites (present in carpets, bedding and other household items). Additional triggers include pollen from flowers, grass and seeds, which may be responsible for seasonal asthma. Drugs are also known to induce asthma, including β-blockers and prostaglandin synthetase inhibitors. β-blockers are potent enough to have this effect even when administered ocularly, potentiating enough β_2-receptor blockade to cause bronchoconstriction. Finally, aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) have also been noted as a source of bronchoconstriction (Gibbs and Cripps, 2012).

Mast cells (known for their role in allergy and anaphylaxis) are for the most part stable in the body, but may be activated by signal transduction of antibodies known as Immunoglobulin E (IgE). During mutation, T-helper 2 cells activate Interleukin 4 and 5, which transmit to B-cells to produce plasma cells, responsible for the production of IgEs. Mast cells have receptors for IgE referred to as IgE-receptors. The IgE has two parts, an upper region which binds to the antigen (foreign substance), whilst the lower part binds to the mast cells via IgE-receptors. The two IgEs bind to the receptors separately, yet do not show any hypersensitivity reaction until the allergens (e.g. pollens, dust mite etc.) cross-link the two IgEs present on the mast cells. These allergens may trigger an immune response in the mast cells by increasing the concentration of cyclic GMP (Guanosine monophosphate) inside the cells, acting as a secondary messenger. Then cyclic GMPs send signals to the nucleus of the mast cells, causing the cells to then begin to rearrange their cytoskeleton (made of proteins) structure. Subsequently, the granules inside the mast cells start migrating. At this point mast cells are causative for the release of several mediators, namely histamine and heparin, which cause vasodilation and smooth muscle constriction, resulting in bronchoconstriction (Gibbs and Cripps, 2012).

Asthma involves bronchoconstriction and inflammation, therefore treatment often aims to reduce inflammation and bronchoconstriction. Salbutamol sulphate (often given under the well-known brand of Ventolin, manufactured by GlaxoSmithKline) is used as a short acting β_2-adrenergic receptor agonist, and is employed to initiate and increase bronchodilation. Beclomethasone dipropionate (commonly seen under the brands of Clenil and QVAR, manufactured by Cheisi and

Teva) is a glucocorticoid steroid that is utilised in the prophylaxis of asthma, by reducing the inflammation of the respiratory airways (Gibbs and Cripps, 2012).

1.9.2. Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is characterised by a limitation of airflow into the lungs. This is a progressive condition and is associated with an abnormal inflammatory response by the lungs towards noxious particles and/or gases (Pauwels et al., 2001). COPD is an umbrella term covering the following disease states: Chronic bronchitis, chronic obstructive lung disease and emphysema (Cripps and Gibbs, 2012).

COPD is the fourth leading cause of death worldwide, and the fifth in the UK. It is estimated that 2 million people carry undiagnosed COPD in the UK (Hubbard, 2006). Furthermore, it is predicted that by 2020, COPD will be the third most common cause of death and the sixth significant cause of disability. This disease is more prevalent in populations which are exposed to atmospheric pollution or individuals who are coal miners and foundry workers by profession (Cripps and Gibbs, 2012).

COPD is known to effect various compartments of the lung and to varying degrees, differing from individual to individual. The paramount factor in the development of COPD is exposure to tobacco smoke, as well as other additional noxious particles (such as dust, chemical fumes, irritants and gases), which are responsible for causing an inflammatory response in the lungs. In smokers, these responses are capable of destroying tissues and impeding or impairing the tissue repair mechanism. One of the main causes of COPD is chronic inflammation of the airways and pulmonary vasculature, which further enhance the concentration of macrophages, neutrophils and T-lymphocytes, followed by an increase of eosinophil during exacerbations. Various inflammatory mediators are released, including; interleukin-8 and leukotriene B4, which induce lung damage.

COPD destroys the walls of the alveoli and the elastic fibres (both on alveoli and bronchioles) by proteases (secreted by immune cells). The normal alveolus (single part of alveoli) consists of elastic fibres, outer wall epithelia and surfactant cells; it is these fibres which recoil and relax during respiration. In the inside alveolus, alveolar macrophages are present which clean up and protect the alveolus during infections or diseases. Blood supply is in close proximity to the alveolus, where oxygen from the alveolus is exchanged with carbon dioxide.

Normally the alveolus releases anti-proteinases which control the activity of proteases. These proteases and anti-proteases are kept in a particular balance. Proteases are destructive while anti-proteases inhibit the action of proteases. Emphysema is usually caused by the imbalance of these two enzymes. The slow process of destruction of the alveoli's elastic fibres is mainly caused by the inhalation of toxins, typically cigarette smoke or air pollutants. These toxins initiate an immune and inflammatory response. As discussed, alveolar macrophages are present in the alveolus and their exposure to these toxins from cigarette smoke may cause the release of inflammatory mediators, such as Interleukin-6, Interleukin-8, Interleukin-1 and Tumour necrosis factor-α. These mediators enhance the immune response, therefore, the interleukin and TNA-α may increase the concentration of neutrophils in the affected area, and this process is referred to as chemotaxis. These neutrophils subsequently begin to secrete proteases, mainly elastase, which destroys the elastic fibres. In addition to the inflammatory mediators, macrophages also release metaloprotease which causes tissue damage. Macrophages and neutrophils are the main producers of proteases; these are elastase and metaloprotease in the area, which cause damage to the surrounding tissues. These mediators will continue to produce proteases if exposed to continuous toxin inhalation. Moreover, the T-lymphocytes coming to the area in the blood may cause further damage to the tissues, possibly through the T-cell mediated apoptosis. Finally, collagens begin to deposit in the tissue which may induce fibrosis.

Air trapping, is another condition which individuals with emphysema suffer from. In this condition, following one inhalation, air becomes trapped and is difficult to exhale. Normally, during inhalation the alveoli expand, as the elastic fibres relax. During exhalation the elastic fibres release the air from the alveoli with ease. But in the case of emphysema, the alveoli expand with force as elastic fibres are damaged. Moreover, due to the loss of elastic fibres on the bronchioles, these become narrower during inhalation. Consequently, the narrowing of bronchioles during exhalation, may cause the air to be trapped due to the impaired elastic fibre function, making exhalation difficult.

Therapy consists of physiotherapy and artificial ventilation; these measures have not shown a significant improvement in the progression and symptoms of COPD. However, suitable treatment with drugs may improve quality of life; reduce hospital admissions and even premature death. Drugs may relieve the symptoms of COPD; however at present they do not reverse the damage caused to the lungs.

1.10. Beclomethasone dipropionate

In the prophylaxis of asthma, a number of steroids are commonly used in inhaled therapy, including BDP. BDP is widely used and available under a number of brands and different devices. It belongs to a class of steroids known as glucocorticoids (Figure 1.7), and is available under brands such as QVAR, Clenil and Asmabec, used in the prophylaxis of asthma. This steroid is also used in the treatment of rhinitis and is commonly found under the brand names of Beconase and Alanase for nasal administration.

BDP is a synthetic steroid or analogue of Cortisol (hydrocortisone) and induces an anti-inflammatory and immunosuppressive effect in the body. It is observed in solid form as a white to off-white powder (Sigma-Aldrich, 2014), which is freely soluble in ethanol and acetone, to lesser extent soluble in chloroform, and poorly soluble in water.

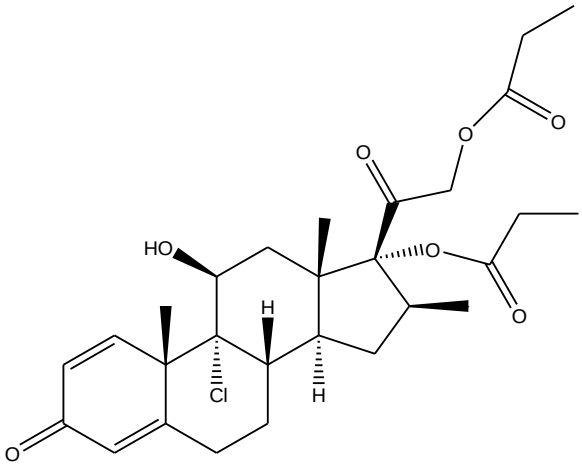


Figure 1.7: Structure of Beclomethasone dipropionate

Ordinarily, Cortisol is released from the adrenal cortex (located on the top of the kidneys) in response to stress. BDP like cortisol, freely passes through the cytoplasm of the cell, where it combines with protein-receptor complexes. These complexes are then translocated into the nucleus. Once in the nucleus, BDP binds to specific-response elements, resulting in protein and RNA synthesis (Greenstein and Greenstein, 2007), the effects which are noted from steroid treatment are exerted through the action of the proteins formed from BDP binding in the nucleus (Greenstein and Greenstein, 2007).

BDP has been successfully entrapped into liposomes as demonstrated by a number of research articles (Waldrep et al., 1994, Saari et al., 1999, Batavia et al., 2001, Darwis and Kellaway, 2001).

The hydrophobic nature of the BDP molecule, gives it an inherent affinity for the fatty acid chain of the phospholipid molecules, causing entrapment in the liposome bilayers.

The incorporation of steroids in the bilayer of liposomes depends upon the molecular structure and the structure of both the phospholipid and the steroid. This incorporation into the liposomal bilayer can be investigated by Differential Scanning Calorimetery (DSC) (Elhissi et al., 2006a). The effect of phospholipid used on resultant steroid containing liposomes, has been demonstrated by varying phospholipid type. Dilauroyl Phosphatidylcholine (DLPC) requires a lower temperature for phase T_m, which yields a higher quantity of BDP incorporation in liposomes. The entrapment of BDP is much greater in MLVs compared to SUVs (Darwis and Kellaway, 2001). In addition, the entrapment of steroids is relatively low in the bilayer which may be due to its geometric structure, potentially causing an inhibition in its interaction with the bilayers (Benameur et al., 1993, Montenegro et al., 1996).

1.11. Pulmonary drug delivery and inhalation devices

The usage of the respiratory system as a route for drug delivery is highly advantageous due to its non-invasive nature. Historically, the pulmonary route has been utilised for inhalation therapy in order to treat symptoms such as cough, by the smoking of leaves of the plant *Atropa Belladonna*, some 4,000 years ago (Grossman, 1994). Many eons later, in the 19th and 20th century, tobacco combined with stramonium powder was smoked in the form of cigarettes in the treatment of asthma (Labiris and Dolovich, 2003).

Though primitive, these examples were the foundation of the highly advanced and developed treatments using the pulmonary route as observed today. Many advances in pulmonary delivery have occurred, particularly in terms of design of inhalation devices for the treatment of asthma and other respiratory disorders. Pressurised metered dose inhalers (pMDIs), dry powder inhalers (DPIs) and nebulizers are currently the most popular devices for inhalation therapy (Bridges and Taylor, 1998, Telko and Hickey, 2005). Formulations designed for delivery via these devices may be aerosolised to the lungs, either in the solid phase as micronised powders, or in the liquid state as solid dissolved or suspended in isotonic aqueous solutions (Telko and Hickey, 2005).

DPIs and pMDIs differ from nebulizers, relying on the incorporation of API in an aqueous solution or suspension. Moreover, nebulizers are more commonly used in a secondary care settings (i.e. hospitals), with little patient input. Prolonged drug delivery is easily and rapidly achieved using

nebulizers, which may add another advantage (Clark, 1995, Telko and Hickey, 2005). Moreover, greater lung deposition is achievable via nebulization, and the elimination of patient co-ordination is highly advantageous. Moreover, multiple dosing is required when DPIs or pMDIs are employed, whilst nebulizers rely on prolonged single sessions of passive inhalation.

1.11.1. Pressurised metered dose inhalers

Glancing over history, modern techniques of aerosol delivery saw surfacing during 1956, when Dr. George Maison (president of the Riker Company) was told by his 13-year old asthmatic daughter (Susie) that her bulb atomizer leaked, and felt that it was like using her mother's hair spray (Clark, 1995, Rubin and Fink, 2005). In conjunction with Charles Thiel and Irving Porush (two other researchers) he developed what is now recognised as the very first pMDI (Terzano, 2001, Oliveira et al., 2010). Following the invention of this model, they quickly began to harness these technologies and techniques in order to develop innovative models, which would accentuate delivery through inhalation (Grossman, 1994). By today's generation, pMDIs are quirkily referred to as "puffers" by their users, and have an established position in the treatment of pulmonary disorders such as asthma and COPD (Dolovich and Dhand, 2011).

Devices such as pMDIs are not wholly free from pitfalls, for instance, the prolonged use of pMDIs may cause irregularities in drug delivery, or a so called "tailing-off" effects. Another key limitation is the level of skill and co-ordination required for the operation of pMDIs, and improper use ultimately results in inconsistent dose deposition in the lungs (Ganderton, 1999, Rubin and Fink, 2005). Whilst pMDIs are very commonly used as pulmonary drug delivery devices, the reliance on the patient's ability to use the correct inhalation technique is great, and leaves a high opportunity for poor treatment outcomes, if the skill level is simply not there (Ramlal et al., 2013).

1.11.2. Dry powder inhalers

DPI devices typically contain formulations which are comprised of micronised drug particles, loaded onto carbohydrate carrier particles. Inhalation results in the liberation of particles from the carriers, and deposition in the central or lower regions of the respiratory tract. Modified-release applications of DPIs are known to be achieved using dried powders of liposomes or

niosomes (made from non-ionic surfactants), which are prepared through freeze drying (Schreier and Bouwstra, 1994) or spray-drying (Goldbach et al., 1993).

A premium advantage of DPI formulations over pMDIs is that they are propellant-free, and their ability to deliver substantially larger doses is higher (McCallion et al., 1996, Dolovich, 1999). Moreover, the dry formulation of the drug is noted to enhance the drug stability, again providing added benefit to the formulation (Chougule et al., 2007). However, negative aspects are also present when using a DPI, since the micronised drug particles are noted for their highly cohesive properties. This possibly results in particle aggregation, compromising efficient delivery and consistent dosing (Labiris and Dolovich, 2003). Inefficient delivery is characterised by particle deposition in the upper respiratory tract, as oppose to the central or lower compartments of the lungs (Clark, 1995). As a device, there are also notable disadvantages with DPIs, namely the requirement for a high inspiratory force for activation of the device and dose delivery. For this reason, many children and elderly patients are unable to benefit from DPIs due to the inspiratory force required to operate them, in short, they are unable to apply the high negative pressure required during inhalation (Dolovich and Dhand, 2011).

1.11.3. Nebulizers

Drug delivery via small aerosol droplets through nebulization is well-established (Dhand, 2003). Nebulizers may be described as devices which are capable of delivering comparatively large volumes of drug dispersions or solutions, in a continuous fashion to the lungs, in the form of an aerosol (Colacone et al., 1990, Elhissi and Taylor, 2005, Lin et al., 2011). This is commonly done for the prophylaxis or treatment of respiratory disorders (including asthma and COPD). In terms of drug delivery, nebulizers possess the potential to be vastly more efficient than pMDIs and DPIs. In contrast to pMDIs and DPIs, nebulizers do not require multiple daily dosing, or have required specific modes of inhalation (Hess, 2000). Additionally, for elderly and young patients, inhalation via nebulization is more convenient, requiring minimum effort and co-ordination than comparative techniques (Taylor and McCallion, 1997, Ramlal et al., 2013).

A key benefit of nebulizers is the high drug concentrations which may be effectively delivered to the respiratory tract (Hess, 2000). However, this effectiveness is heavily dependent upon a number of factors including: particle size, formulation surface tension and viscosity, nebulizer design and patient inhalation patterns (i.e. rapidity and depth of breathing). There has been extensive research conducted on the delivery of controlled release formulations using nanocarrier

systems, such as liposomes and niosomes. Literature has reported that liposomes and niosomes of small particle sizes (i.e. 1 - 5 µm) are likely to reach the deep lung (Máiz Carro and Wagner Struwing, 2011). Small drug particles which are dispersed in the nebulizer solution have the ability to be incorporated into nebulized droplets; as a consequence, it is highly desirable to use fine dispersions, if suspensions or emulsions are to be used for nebulization. Laser diffraction is the most common technique employed to measure the size of aerosol droplets from liposome dispersions, as identified by Bridges and Taylor (1998) (Bridges and Taylor, 1998). In terms of devices, there are innumerate models of nebulizers which are currently available in the market, and are used in medical environments. However, these may be broadly grouped into three different types of nebulizers, based upon their mechanism of action or operation; these are Air-jet, Ultrasonic and Vibrating-mesh nebulizers (Elhissi and Ahmed, 2011, Khan et al., 2013).

1.11.3.1. Air-Jet nebulizers

Air-jet nebulizers are a T-shaped inhalation devices which are comprised of a mouthpiece, a medication plastic bottle; and an air compressor (Waldrep and Dhand, 2008) (Figure 1.8). The conversion of liquid medication into fine small droplets occurs by forcing compressed gas through the nebulizer via a narrow "venture" nozzle (Kendrick et al., 1997, Gaga et al., 2012). Flow rate is an influencing factor on the size of the droplets produced (Arzhavitina and Steckel, 2010).

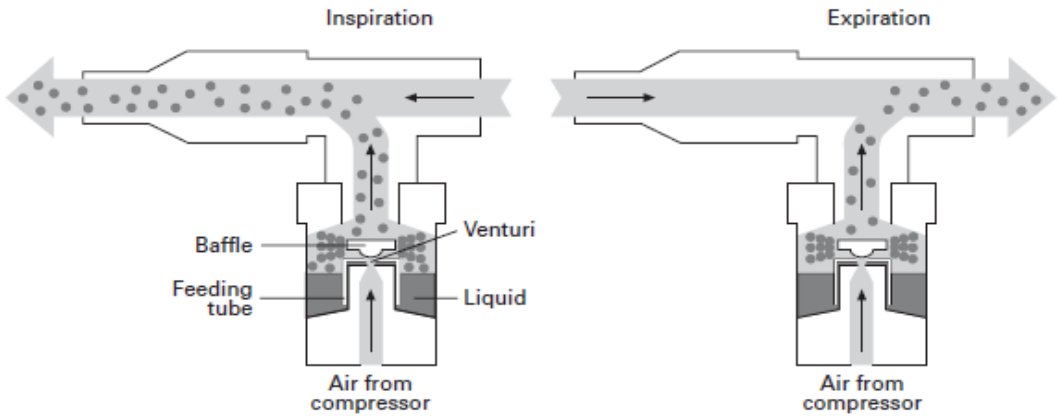


Figure 1.8: A diagram representing the operating principle of a conventional air-jet nebulizer (*O'Callaghan and Barry, 1997*)

On close examination of the device, the mouthpiece is connected to the plastic bottle which is filled with the liquid drug formulation. Compressed gas is supplied though a plastic tube via what is known as the "venturi" nozzle of the plastic bottle (Niven and Brain, 1994, O'Callaghan and

Barry, 1997, Ghazanfari et al., 2007) (Figure 1.8). This nozzle has a diameter ranging between 0.3 and 0.7 mm (millimetres) (McCallion et al., 1996). As the liquid is propelled through the "venturi" nozzle and collides with the baffle of the nebulizer, large sized droplets are reduced in size, which are subsequently propelled as an aerosol cloud through the nebulizer to the lungs.

Open vent nebulizers are known to operate in a similar fashion to air-jet nebulizers. Here, compressed gas produces a negative pressure on top of the medical fluid, causing smaller droplets to form upon impaction with the baffle of the device, resulting in their release for inhalation. Differently, however in the open vent model, a small open vent is a design feature of the chamber, which allows for additional air flow to propel the smaller droplets for inhalation by the patient. Moreover, open vent nebulizers may reduce nebulization time and potentially enhance the formulation deposition in the lower airways (Barry and O'Callaghan, 1999). The presence of additional air flow has not been proven to aid in particle size reduction (O'Callaghan and Barry, 1997).

A further alternative form of air-jet nebulizers are breath assisted/enhanced open vent nebulizers, these are similar in principle to open vent nebulizers with several differences. Firstly, during inspiration, smaller droplets are pushed to the patient with added air flow, also during inspiration a valve which is positioned near the mouthpiece opens and closes the open vent which is on top of the nebulizer (Barry and O'Callaghan, 1999). The loss of aerosol is comparable to that of a conventional nebulizer, as nebulization is continuous. However, the nebulization time is shorter than that of conventional air-jet nebulizer, yet longer than open vent nebulizers (O'Callaghan and Barry, 1997).

1.11.3.2. Ultrasonic nebulizers

The second broad group of nebulizers are ultrasonic nebulizers, which have a special mechanism of generating energy via ultrasonic sound waves, utilising piezoelectric crystals (Flament et al., 1996, Leung et al., 1996, Khatri et al., 2001, Rau, 2002). The generation of waves causes the production of high frequency vibrations ranging from 1 to 3 MHz (Megahertz). The result is the formation of a fountain, from which aerosol droplets may detach and be released from the device for inhalation (Clark, 1995, Labiris and Dolovich, 2003, Ghazanfari et al., 2007, Najlah et al., 2013). Droplet size is dependent on frequency intensity; the higher the frequency, the smaller the aerosol droplets generated (Arzhavitina and Steckel, 2010).

Droplet size may vary at different points on the fountain generated, for example, at the apex of the fountain, larger droplets are formed. Contrastingly, at the bottom of the fountain, smaller droplets are generated. Larger droplets are deflected by the baffle and then recycled for further atomisation into smaller droplets, which are more suitable for nebulization. Modern models of ultrasonic nebulizers are equipped with speed controlled fans *in situ*, to facilitate the movement of the aerosol towards the mouthpiece for inhalation by the patient (O'Callaghan and Barry, 1997).

Additionally, the portability of recent nebulizer designs is generally superior to that of older models, both in terms of weight and size of the nebulizer. This is simply possible through battery operation of new devices. Two mechanisms have been proposed overall for the production of aerosols by ultrasonic nebulizers (Avvaru et al., 2006). The first is the capillary wave mechanism, and the second is cavitation bubble formation (Taylor and McCallion, 1997, Bridges and Taylor, 1998).

In the capillary wave mechanism, a high level of vibration frequency causes the formation of a capillary jet from the nebulizer liquid. Liquid crests may form and then break with the continuous formation of smaller nebulizer droplets (Taylor and McCallion, 1997). The second theory, which is known as bubble formation, uses low frequency energy which is produced by piezoelectric crystals, that are used to create bubbles inside the formulation. These bubbles then burst forth on the surface, as their internal pressure equalises with the external pressure, resulting in generation of small droplets for inhalation (Taylor and McCallion, 1997).

1.11.3.3. Vibrating-mesh nebulizers

The third and final type of nebulizer is known as the vibrating-mesh nebulizer, this is known to comprise of a plate having micro-sized tapered holes, which vibrate in order to produce slow moving small aerosol droplets for inhalation (Waldrep and Dhand, 2008, Elhissi et al., 2013a). The process aims to minimise the residual volume of nebulizer liquid at the end of nebulization time (Dhand, 2002). Vibrating-mesh nebulizers may be further classified into two sub-types, namely: passively vibrating-mesh nebulizers and actively vibrating-mesh nebulizers (Newman and Gee-Turner, 2005).

A common example of a passively vibrating-mesh nebulizers is the Omron MicroAir NE-U22 device, which is known to employ a piezoelectric crystal producing high energy vibrations (Dhand,

2002). The generated vibrations are then transmitted via a transducer horn to the perforated plate. This induces what is referred to as "passive" vibrations of the plate, in order to essentially extrude the medical fluid through the plate openings, causing the formation of small droplets suitable for nebulization (Elhissi et al., 2013a, Najlah et al., 2013). Nebulization devices such as the Omron NE-U03 and Omron MicroAir NE-U22 are known to employ this mechanism of operation (Elhissi et al., 2007, Ghazanfari et al., 2007). The Omron NE-U22 nebulizer is noted to be overall more efficient than NE-U03 in producing smaller droplets with a narrow size distribution and high aerosol output (Waldrep and Dhand, 2008).

The second type of vibrating-mesh nebulizers, namely the actively vibrating-mesh devices are commonly found under the branded product Aeroneb Pro nebulizer. This differs from the passive nebulizer, as it employs a "micropump" technology which supplies a direct electrical current to the mesh plate. The mesh in the device is comprised of a dome-shaped aperture containing around 1,000 small holes (Ghazanfari et al., 2007), which are surrounded by a piezoceramic vibrational element. The nebulizer fluid is positioned above the mesh plate and the given power supply causes the mesh to move in a vertical fashion by several micrometres, in order to generate slow moving aerosol droplets (Dhand, 2002, Hess et al., 2011a). Aeroneb Go and Aeroneb Pro are two nebulizers available that utilise the same principle of operation. However, in general, Aeroneb Pro nebulizers are recommended for patients who are on mechanical ventilation. In this instance, the nebulizer is affixed to the aspiratory limb of the ventilator circuit, to generate a maintained nebulization effect (Waldrep and Dhand, 2008).

DPIs, pMDIs and nebulizers are by far the most commonly used devices for pulmonary drug delivery. DPIs and pMDIs are very much commonly used via self-administration, whilst nebulizers are equally more common for use in secondary care settings. It is clear that three various groups of nebulizers are widely used in the medical field, and each are associated with their own individual benefits and pitfalls. However, air-jet nebulizers show a great variety in terms of the types of nebulizers, which are available in the market for use, making them appealing for research in nebulized formulations.

1.12. Mechanisms of particle deposition

The respiratory system is responsible for supplying and exchanging essential molecules, which are crucial to cell life. As there is a massive surface area and little filtration upon inhalation, foreign particles may enter the respiratory system. The foreign particles which are detected may be

removed by alveolar macrophages in the region, unless they are too small to be recognised. The aerodynamic diameter of the particles is vital at influencing their direction and deposition profile into the pulmonary airways (Bennett and Smaldone, 1987). An alternative method of ensuring drug deposition is presented by utilising the patient breathing pattern and the physical characteristics of the airway (e.g. lung geometry) (Carvalho et al., 2011). In fact, deposition of particles in different areas of the respiratory tract (upper, central and alveolar sections) is dependent upon the size and flow rate of the inhaled particles (Labiris and Dolovich, 2003, Pilcer and Amighi, 2010).

Mechanism by which particles deposit in the respiratory tract (Figure 1.9) are inertial impaction, sedimentation and Brownian diffusion (Carvalho et al., 2011).

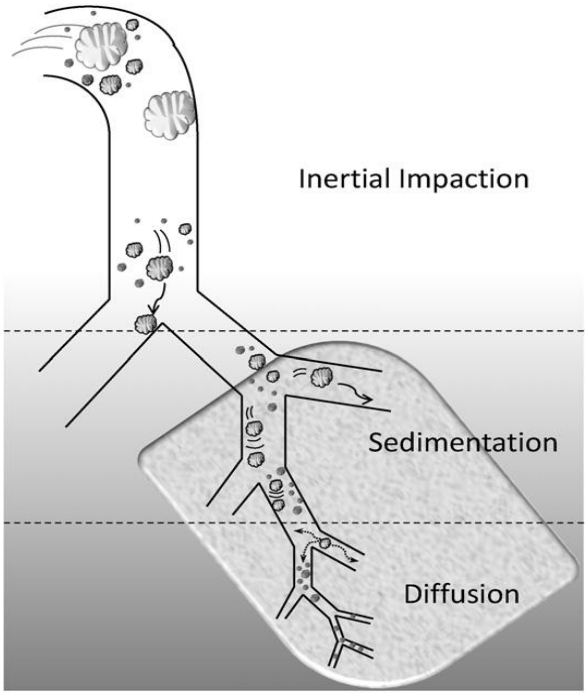


Figure 1.9: A schematic presentation of particle deposition into different regions of the respiratory tract (*Carvalho et al., 2011*)

1.12.1. Inertial impaction

The mechanism responsible for particle deposition in the upper respiratory tract is dependent upon particle velocity, mass, size and particle density (Pilcer and Amighi, 2010). During inhalation, particles enter with high speed, which may restrict their direction/route or flow, and cause deposition in the upper regions of the respiratory system. Impaction rate in the upper respiratory tract is higher for particles with a size in excess of 5 µm (Darquenne and Prisk, 2004, Pilcer and

Amighi, 2010) (Figure 1.9). Moreover, the deposition of particles by inertial impaction increases if they possess a higher density, or enter into the respiratory tract with high velocity.

1.12.2. Sedimentation

Sedimentation may be defined as time dependent deposition (Darquenne and Prisk, 2004, Carvalho et al., 2011), where the particles move in accordance with gravity in both the central and alveolar regions of the pulmonary system. Delaying exhalation (i.e. breath holding) can increase the sedimentation rate due to the longer residence time in a given area of the pulmonary system (Byron, 1986). In sedimentation, particles move by their gravitational force in the absence of inspiration and expiration (Carvalho et al., 2011) (Figure 1.9). Particles with the size range of 0.5 - 5 µm are more likely to deposit by sedimentation (Stahlhofen, 1980, O'Callaghan and Barry, 1997, Darquenne and Prisk, 2004, Pilcer and Amighi, 2010).

1.12.3. Brownian diffusion

Brownian diffusion is the mechanism by which small particles reach with random motion to the deepest region of the respiratory tract (i.e. the alveolar region) (Carvalho et al., 2011) (Figure 1.9). However, low velocity particles, or those with a large volume of aerosol result in an increased lung deposition (Pavia et al., 1977, Newman et al., 1982). The velocity and particle size decrease as particles pass from the upper respiratory tract to the lower regions. When the particles finally reach the alveolar region, the velocity ceases to zero. The sizes of particles reaching this region are typically less than 0.5 µm (Darquenne and Prisk, 2004, Pilcer and Amighi, 2010).

1.13. Clearance of deposited particles

Particles deposited in the pulmonary system may be completely cleared or translocated into other parts of the respiratory tract by two mechanisms. One mechanism is the mucociliary transport system in the TBT, where insoluble particles are cleared faster, often within a day (fast phase). The other is clearance of particles from the alveolar region, which can take from days to months (Lippmann et al., 1980, Asgharian et al., 2001).

The ciliary cells are located on the epithelium starting and extending from the terminal bronchioles to the larynx; these cells possess a rhythmical movement, which propel the particles slowly from terminal parts of the respiratory system to the upper respiratory tract. From here, they may be expelled out from the respiratory system by sneezing, blowing of the nose or coughing, or they may be swallowed and pass through the gastrointestinal tract (GIT) (Lippmann and Schlesinger, 1984). In contrast to insoluble particles, the active or passive system in the lungs may absorb soluble particles through the mucous layer into systemic circulation (McClellan and Henderson, 1995). Insoluble particles are actively cleared by alveolar macrophages from the peripheral or alveolar region. The absence of mucociliary clearance decreases the clearance of particles from the respiratory tract, particularly insoluble particles (Lippmann et al., 1980, Asgharian et al., 2001,).

Evidently there is a large body of literature illustrating the vast amount of research conducted in the liposome field. The wide variety in terms of liposome types and formulations ignites the interest of many scientists in the drug delivery field to explore the potential of liposomes further. There are however notable pitfalls associated with the formulations currently under investigation or available commercially. With particular reference to the respiratory system, there is a large potential of liposome formulations to offer enhanced therapy in the treatment and management of a number of respiratory disorders. A pulmonary formulation which is in the latter stages of Food and Drug Administration (FDA) approval includes; Arikace (the trade name for an Amikacin (an aminoglycoside), a liposomal preparation for inhalation) used in the treatment of cystic fibrosis, is currently under phase III trials for approval by the FDA. This formulation is currently delivered by an eFlow nebulizer system from Pari Pharma at a dosage regime of 560 mg daily (Clancy et al., 2013).

Currently available respiratory treatments focus heavily upon devices which utilise pre-formulated aerosols and dry powders (also in capsules), however there is yet to be a tablet formulation which is solely designed for pulmonary drug delivery. This research aims to address the aforementioned issues, by the development of a novel proliposome tablet formulation for use in pulmonary drug delivery.

1.14. Working hypothesis

The formulation of proliposomes in a novel tablet dosage form will provide improvements in proliposome stability as well as aid in their large-scale manufacture of liposome precursors, which can potentially be used for pulmonary delivery via nebulization.

1.15. Project Aim

This project aims to study and develop proliposome formulations for pulmonary drug delivery using several carbohydrate carriers at various lipid to carrier ratios, for production in the form of proliposome tablets.

1.16. Objectives

1. To investigate four different carbohydrate carriers (i.e. lactose monohydrate, sucrose, sorbitol and D-mannitol) and assess their appropriateness for use in proliposome formulations.
2. To analyse the effect of different lipid to carbohydrate carrier ratios (i.e. 1:5, 1:10 or 1:15 weight/weight) on the entrapment efficiency in liposomes.
3. To Investigate the separation (time and speed) of BDP-entrapped liposomes and unentrapped or free BDP crystals using two different dispersion media (i.e. DW and D_2O) via centrifugation and light microscopy.
4. To determine the entrapment efficiency in both DW and D_2O by employing High performance liquid chromatography (HPLC) and identifying which medium can provide more reliable results.
5. To analyse the surface morphology of the proliposome formulations using Scanning Electron Microscopy (SEM) to study carrier porosity and lipid interaction with carrier surface.
6. To analyse liposome size in comparison to different formulations; comprised of a myriad of carriers and lipid to carrier weight/weight ratios.
7. To quantify the amount of lipid coated onto the carbohydrate carrier surface using the Stewart assay.

1.17. Thesis outline

Chapter 1: An introduction to liposomes; problems associated with their preparation. In addition to an overview of pulmonary drug delivery, with a summary of the aims and objectives of this research.

Chapter 2: Lists detailed general methods utilised within this research.

Chapter 3: Describes novel proliposome preparations, utilising different carbohydrate carriers in various ratios; and their analysis using various techniques and dispersion media.

Chapter 4: Outlines the manufacture of novel proliposome tablets from proliposome powders, and analysis in accordance to pharmacopeial standards.

Chapter 5: Examines nebulization of proliposome powders and tablets, implementing a Two-stage lung model.

Chapter 6: Accounts for stability testing of manufactured proliposome tablets over a 6 month period.

Chapter 7: Lays out derived conclusions from overall research and proposed future work.

Chapter 8: References

CHAPTER 2: GENERAL METHODOLOGY

"If you can dream it, you can do it"

- Walt Disney -

2.1. Materials

LMH, sucrose, sorbitol, ferric chloride and ammonium thiocyanate were purchased from VWR (BDH Prolab), UK. D-mannitol (≥98%), D_2O (1.105 g/ml density and 99.8% purity), BDP (≥99%), phosphotungstic acid and cholesterol (≥99%) were procured from Sigma-Aldrich, UK. SPC (Lipoid S-100) was obtained from Lipoid, Steinhausen, Switzerland. HPLC grade methanol and water were supplied by Fischer Scientific Ltd, UK. Absolute ethanol and chloroform (AnalaR grade) were also purchased from Fischer Scientific Ltd., UK.

2.2. Methods

2.2.1. Coarse carbohydrate carriers

Coarse carbohydrate carriers were utilised in the manufacture of proliposome formulations, each carrier was processed identically, with the exception of sucrose. Prior to use, sucrose particles were reduced as per method outlined by Elhissi et al., (2005 and 2011), a size range of 300 - 500 µm was achieved with additional modification, using a mortar and pestle (Elhissi and Taylor, 2005, Elhissi et al., 2011). These particles were then passed through a sieve (mesh size 300 - 500 µm) to isolate the required particle size fraction (Elhissi et al., 2011). This process was conducted as sucrose particles in this range are optimal for proliposome manufacture. LMH and D-mannitol particles were supplied with a particle size under 250 µm in diameter, sorbitol particles were given in a size in the range of 250 - 700 µm.

2.2.1.1. Lactose monohydrate

Lactose monohydrate (LMH) may be described as a natural disaccharide, and is commonly obtained from milk. Its structure is comprised of one glucose and one galactose moiety. On physical examination, LMH is observed to exist as a white to off-white microcrystalline/irregular powder, comprised of particles pertaining a prism to pyramidal shape (Rowe et al., 2009). LMH is sweet in taste and odourless, and forms aggregates readily in the presence of amorphous lactose (Row et al., 2006). It is also inexpensive and possesses good flowability, offering an explanation

with regards to its wide use in pharmaceutical products available in the market. Additionally, its low moisture content (i.e. 5%) has little impact upon moisture sensitive APIs (Rowe et al., 2009). In terms of tablet manufacture, lactose is utilised as filler and a binder. Properties of lactose-based tablets may improve by the incorporation of microcrystalline cellulose (MCC), in terms of crushing strength and disintegration time (Rowe et al., 2009). Various grades of lactose are utilised in tablet preparation; fine lactose, for example is used in wet granulation for processing of tablets. Conversely, direct compression grade lactose is combined with small quantities of API in tableting, without granulation processing. Spray dried lactose and anhydrous lactose also may be utilised in direct compression methods, as an alternative to direct compressed lactose (Row et al., 2006, Rowe et al., 2009).

2.2.1.2. Sucrose

On physical examination, sucrose is a colorless powder, comprised of crystalline blocks, which are irregular or cubic in shape with a smooth outer surface. Sucrose is commonly found as an excipient in medical confectionary, as well as in pharmaceuticals. It is also used for the purposes of coating, suspending or as a sweetening agent. In tableting, like LMH, sucrose may be utilised as filler, in addition to its role as a viscosity promoter and binder. However, excessive quantities of sucrose in tablets may raise tablet hardness values exponentially, negatively impacting upon tablet disintegration time (Row et al., 2006).

2.2.1.3. Sorbitol

Sorbitol and mannitol are known to share isomerism in terms of structure, in addition to sorbitol being noted for its appearance i.e. a white odourless powder, once again sweet in taste but also hygroscopic in nature (Anh and Kathleen, 2001, Rowe et al., 2009). Unlike sucrose and LMH, sorbitol is porous in structure and is irregular to spherically shaped, possessing needle shaped structures protruding from the particle surface (Newman et al., 1999). In terms of solubility, sorbitol is highly water soluble (2.56 g/ml at 25°C), slightly soluble in methanol and insoluble in chloroform (Wade and weller, 1994).

In terms of reactivity, sorbitol is generally non-flammable and non-corrosive; however it requires storage within air-tight containers. Moreover, sorbitol may retain much of its white colour and is

unlikely to decompose upon exposure to elevated temperatures (Newman et al., 1999). This stability is further reflected in the fact that sorbitol is resistant to fermentation by many microorganisms. As an excipient, sorbitol's absorbance within the GIT is markedly slower than the comparative excipient sucrose. Its wide tolerance in diabetics (unlike sucrose), adds to its preferential wide use in sugar-free liquid preparations, as well as its ability to inhibit the formation of crystals surrounding bottle caps (Newman et al., 1999). The ingestion quantity of sorbitol is capped to 20 g/day however, potentially limiting the mass or volume of preparations which may be consumed (Row et al., 2006).

In food, cosmetics and pharmaceutical industry, sorbitol is extensively utilised, namely due its sweetness which is 50 - 60% the sweetness of sucrose (Row et al., 2006). Though commonly used for its sweetness, the FDA guide outlines a number of products such as rectal, topical and vaginal preparations, intramuscular injections and nasal preparations, where sorbitol is used as an excipient. In the UK, it is found in licenced medications for both parenteral and non-parenteral formulations (Row et al., 2006). Sorbitol, also is a useful excipient in tablet manufacture as a diluent, whether compression is achieved following wet granulation or directly (Row et al., 2006).

2.2.1.4. D-mannitol

D-mannitol is found as a free flowing, odorless powder which is comprised of oblong ellipse crystalline particles, which are ingrained on the surface (Row et al., 2006). It is like many other carbohydrates, sweet in taste, and, like sorbitol, elicits a cooling sensation in the mouth, enhancing its suitability for chewable tablets (Rowe et al., 2009). D-mannitol does not inadvertently affect blood sugar levels, making it suitable and preferable for diabetic formulations (Botez et al., 2003). D-mannitol may also be used in tablet formulations as a binder (10 - 90% w/w), which additionally prolongs disintegration time (Row et al., 2006). Contrastingly, tablets prepared from mannitol and MCC mixture can disintegrate much more rapidly, whilst retaining acceptable levels of tablet strength (Westerhuis et al., 1996). Recently, mannitol has been used increasingly in place of lactose to avoid lactose-intolerance manifestations (Westermarck et al., 1998).

2.2.2. Manufacture of proliposomes via "Slurry" method

In the manufacture of carrier-based proliposomes via the "Slurry" method, carbohydrate carrier particles (i.e. LMH, sucrose, sorbitol or D-mannitol) were placed in a pear shaped RBF (100 ml). SPC and cholesterol (250 mg; 1:1 mole ratio) were dissolved, utilising absolute ethanol (250 mg/0.75 ml) in a glass vial, making a lipid phase (which refers to both SPC and cholesterol together). BDP was employed as a model drug in this study, at a concentration of 2 mole% to the lipid phase. The ethanolic solution containing SPC, cholesterol and BDP was then loaded onto the carbohydrate carriers separately, with the additional ethanol in the pear shaped RBF to form a slurry; this was done to ensure that the lipid phase would form a uniform thin layer/coat on the carbohydrate particles.

Three different formulation ratios were prepared for each carrier; 1:5, 1:10 and 1:15 w/w (weight by weight lipid to carrier ratio) (Table 2.1). Moreover, 250 mg lipid phase consisting of equi-mole ratio of SPC and cholesterol (166.66 mg of SPC and 83.33 mg of cholesterol) and 2 mole% BDP (i.e. 4.48 mg) were kept constant. Only the carrier concentration was varied in the manufacture of proliposome formulations. In the preparation of 1:5 w/w lipid to carrier ratio proliposome formulation, 1250 mg of carbohydrate carrier was added in a pear shaped RBF. Lipid phase was prepared by dissolving the lipid constituents (250 mg) in 0.75 ml of ethanol followed by further addition of alcohol to make a final volume of 4.25 ml.

This ethanolic solution containing lipid phase with or without drug was then poured onto the carbohydrate carrier in the RBF. Similarly, formulation ratios for 1:10 w/w (250 mg: 2500 mg) and 1:15 w/w (250 mg: 3750 mg) were calculated as lipid to carrier ratios in order to prepare proliposomes.

Table 2.1: Proliposome powder formulations prepared utilising various constituents; carrier is representing four carbohydrates particles (i.e. LMH, sucrose, sorbitol and D-mannitol) and each carrier was prepared in three lipid to carrier ratios (1:5, 1:10 and 1:15 w/w). Data are mean ± STD, n=3; P<0.05

Formulations constituents	Proliposome formulations (mg)		
	1:5	1:10	1:15
Carrier	1,250	2,500	3,750
Soya phosphatidylcholine	166.67	166.67	166.67
Cholesterol	83.33	83.33	83.33
Beclometasone dipropionate	4.48	4.48	4.48

The RBF containing the carbohydrate carrier was then attached to a rotary evaporator (Figure 2.1) (Buchi Rotavapor R-114, Buchi, Switzerland) and partially immersed in a water bath, previously adjusted to 45°C (Buchi Waterbathe B-480, Buchi, Switzerland). A vacuum pump (Buchi Vac V-501) was used to evaporate ethanol with a maximum rotation speed of 280 revolutions per minute (RPM).

Complete evaporation of ethanol was performed for two hours. After which, the negative pressure was released and the RBF was detached from the rotary evaporator. A clean spatula was used to scratch and collect the proliposomes powder from the RBF and then placed into a glass vial which was then tightly sealed and stored at -18°C.

Figure 2.1: Buchi rotary evaporator (*Buchi Rotavapor, 2014*)

2.2.3. Hydration of proliposomes

LMH, sucrose, sorbitol or D-mannitol-based proliposome powders prepared in section 2.2.1 were hydrated (30 mg/ml; containing 30 mg of proliposome powder was dispersed in 1 ml of solvent media as a standard ratio) in aqueous solvent, either DW or D_2O. Proliposome powder was placed in 10 ml glass vial and hydration was conducted significantly above the phase T_m of SPC (-20 $\pm$ 5°C) (Lipoid, 2014), and room temperature in this case was appropriate. For 30 mg of proliposome powder, 50 µl of the desired aqueous solvent was added followed by vigorous vortex-mixing (Fision WhirliMixer, Fision scientific equipment, Leicestershire, UK) for 2 minutes. The remaining aqueous phase (950 µl) was added and vortex-mixing was performed for an additional 1 minute, to dissolve the carbohydrate carrier and hydrate the lipid in order to form liposomes. Liposomes were left for two hours to anneal above the phase T_m. On preparation of larger volumes, the concentration of proliposome in aqueous solvent was maintained at 30 mg/ml.

For the hydration of proliposome tablets in nebulizer (Chapter 5), sorbitol or D-mannitol-based proliposome tablets with a 150 mg weight were placed in the reservoir chamber of nebulizer, and 5 ml of DW was added (the ratio of proliposome was kept at 30 mg/ml to make liposome dispersion). The resultant liposome suspension was made in the nebulizer reservoir after 2 minutes of manual shaking. However, approximately 8 minutes of manual shaking was performed to ensure dissolution of D-mannitol-based proliposome tablets. D-mannitol coarse powder exhibited a lower solubility in water than coarse sorbitol (Weymarn, 2002). This was noted in the dissolution time of the respective proliposome tablets (up to 8 minutes for D-mannitol and 2 minutes for sorbitol-based proliposome tablets).

For stability testing (Chapter 4 and 6) of the hydration process, ten proliposome tablets were triturated in a corner of polythene bag with the aid of a pestle, in order to avoid any loss of lipid from the formulation (which may affect the entrapment of drug). The crushed proliposome tablets were subsequently weighed in a glass vial (10 ml) followed by hydration with aqueous solvent to form a liposome dispersion (30 mg/ml; containing 30 mg of proliposome powder was dispersed in 1 ml of solvent media as a standard ratio). Initially, hydration was performed with 50 µl of aqueous solvent, followed by further addition of 950 µl of the aqueous phase. This process was conducted for a total of 3 minutes (mentioned above) vortex mixing. Liposome suspensions were then left for two hours at room temperature to anneal before characterisation.

2.2.4. Scanning Electron Microscopy

Surface morphology of proliposome powders prepared (Section 2.2.1) from carbohydrate carriers (i.e. LMH, sucrose, sorbitol or D-mannitol) utilising various lipid to carrier ratios (i.e., 1:5, 1:10 or 1:15 w/w), with or without the incorporation of BDP, were examined using SEM. Coarse carbohydrate carriers were also analysed to allow for comparison with the proliposome powders. Prior to imaging, all samples were air dried on SEM stubs and gold coated with a sputter coater using a JFC-1200 Fine Coater (JEOL, Tokyo, Japan) for 2 minutes, followed by examination using an SEM (Quanta-200, FEI) at 20 kV (Kilovolt). The surface morphology of the samples was evaluated and compared amongst different formulations.

2.2.5. Separation speed using bench centrifuge for HPLC analysis

Proliposome powders prepared from LMH, sucrose, sorbitol or D-mannitol by "Slurry" method, were hydrated in DW or D_2O in accordance with section 2.2.2 to form a milky liposomal dispersion. The resultant liposomal dispersion (1 ml) was transferred to centrifuge tubes (1.5 ml) and centrifuged (using a Spectrafuge 24D, Jencons-PLS, UK) to separate BDP-entrapped in liposomes (i.e. BDP entrapped in the liposome concentric bilayers) from the unentrapped BDP (both the dissolved and free BDP crystals). For this purpose, a range of centrifugation speeds/gravity (i.e. 11,000 RPM/11,100 g, 12,000 RPM/13,200 g and 13,000 RPM/15,500 g) were applied separately in order to optimise the separation of liposome-entrapped BDP from the unentrapped BDP.

It was observed that in DW, after centrifugation with the aforementioned speeds, liposomes sedimented as a pellet in the centrifuge tubes, and the clear unentrapped BDP (containing fractions of free BDP crystals or soluble BDP in DW) were found as a supernatant fraction. However, in contrast to DW, in D_2O it was established that after bench centrifugation, the liposome dispersion formed three distinct fractions/part; a creamy-white fraction at the top of the tube (containing liposome-entrapped BDP), unentrapped BDP liposomes (containing dissolved or free BDP crystals with lipid fraction) in the middle fraction of the tube and the free BDP crystals forming a spot (bottom fraction) at the bottom of the centrifuge tube (Figure 3.7). These entire fractions, either in DW or D_2O were analysed via HPLC to determine the quantity of BDP.

In addition, Stewart assay (Stewart, 1980) was implemented to investigate the presence of lipid content in the middle fraction, after centrifugation in D_2O. Lower lipid concentrations in the middle fraction, indicated a greater likelihood of the complete separation of liposome dispersion fractions (Section 3.3.5). Therefore, various speeds were employed to achieve the best separation of BDP entrapped in liposomes from the unentrapped fractions.

2.2.6. Separation time using Light Microscopy for HPLC analysis

Light Microscopy (Novex, Holland) was employed to investigate the standard liposomal dispersion in D_2O (post-hydration according to section 2.2.3). Different time intervals were investigated to separate the liposome-entrapped BDP from the unentrapped BDP (including dissolved or free BDP crystals), after using bench centrifuge at 13,000 RPM in accordance to section 3.3.5 and 3.3.6. Centrifugation was conducted for 60 or 90 minutes, before observation of the samples under the light microscope, by taking the portions of upper creamy-white fraction and a spot which appeared at the bottom of the tube in D_2O. On examination of each fraction identified, light microscopy indicated that the free BDP crystals were completely separated in the form of a spot (at the bottom of the tube), from the BDP-entrapped in liposomes (creamy-white supernatant) (Figure 3.7). Both the middle fraction (containing dissolved or free BDP crystals with lipid fraction) and spot containing free BDP crystals were analysed using HPLC in order to establish the entrapment efficiency of BDP in the isolated liposomes (Chapter 3).

2.2.7. Liposome size analysis via laser diffraction

Liposome size, also referred to as volume median diameter (VMD) and size distribution (SPAN) were measured by utilising laser diffraction, employing a Malvern Mastersizer 2000 (Malvern instrument Ltd, UK). Liposome VMD (a form of average which gives liposome size median values) was measured in µm (micrometres). The term SPAN has no unit; however the distribution width was expressed in 10%, VMD 50% and 90% undersize quantities, the SPAN values (Equation 2.1) are calculated as follows:

$$SPAN = \left(\frac{90\%\ undersize - 10\%\ undersize}{VMD} \right) \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 2.1)$$

Size evaluation is achieved via the scattering of laser beams from contact with liposomes at various angles and intensities. These angles of scattered light and intensity are then measured by a series of photosensitive detectors, which are connected to a computer that converts these signals into size and size distribution values. Liposome size and scattered angles are inversely proportion to each other i.e. the smaller the liposome size, the larger the scattered angle and vice versa (Figure 2.2).

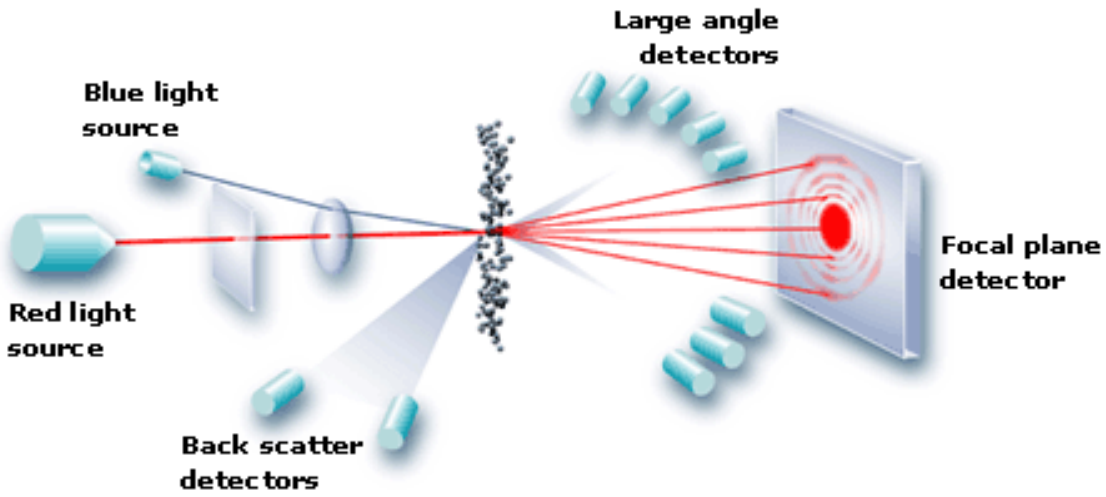


Figure 2.2: Schematic representation of laser diffraction, Malvern Mastersizer 2000 mechanism, (Malvern-Instruments, 2011)

2.2.8. Zeta potential analysis via electrophoretic mobility

Zeta potential (surface charge) of the liposomes was automatically calculated via electrophoretic mobility, using a Zetasizer Nanoseries (Malvern Instruments Ltd., UK). Liposome suspensions and saline solution (0.9% w/v) were combined in a 1:1 v/v (volume by volume) ratio and vortex mixing was conducted for 1 minute. Samples of 900 µl were transferred using a Gilson pipette (Gilson Pipetman, UK) into a cell made from polystyrene latex (Malvern Instruments Ltd., UK). The instrument parameters were set for 1 minute for equilibration, and the temperature was maintained at 25°C (Figure 2.3).

The specialised cell employed for measuring zeta potential has two electrodes built into the polystyrene latex structure. These electrodes are essential for measuring the zeta potential. The liposome dispersion itself is referred to as a conducting medium. On transfer of the dispersion into the cell, liposomes with a positive or negative surface charge migrate towards the opposite electrode, under the influence of the electric field (mechanism used by the Zetasizer instrument) through electrophoretic mobility (Zheng et al., 2009). Laser Doppler Velocimetry (LDV) is the

system which calculates and measures the electrophoretic mobility of liposome vesicles in the Malvern Zetasizer (Figure 2.3). Liposome vesicles pass in front of the light, thus causing the light to scatter. The intensity of scattered light exhibited by the liposomes indicates their velocity or movement in the electric field and is measured by the LDV. These integrations are calculated by the software of the instrument and are displayed as shown in Figure 2.3.

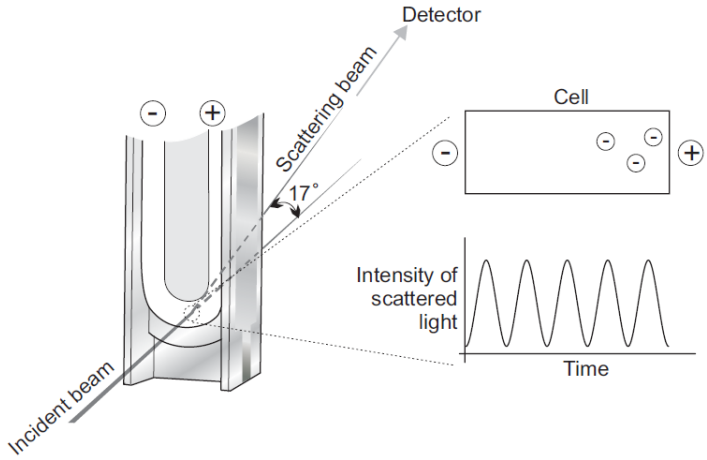


Figure 2.3: A schematic representation of Laser Doppler Velocimetry mechanism in the Zetasizer instrument *(Malvern-Instruments, 2004*)

2.2.9. Lipid quantification using Stewart assay

2.2.9.1. Calibration curve

A calibration curve was constructed using the average of three individual lipid measurements, by transferring 10 mg of lipid phase (comprising SPC and cholesterol in a 1:1 mole ratio) in a pear shaped RBF followed by addition of absolute ethanol to aid dissolution of the lipid. Rotary evaporation was then used to evaporate the organic solvent at a regulated temperature of 45°C, under reduced pressure. A thin film which formed in the pear shaped RBF was then hydrated with D_2O (1 ml) to form a liposomal suspension. Following this, 1 ml of absolute ethanol was added to convert the suspension into an ethanolic solution. This sample was stored overnight at 90°C in an oven to evaporate the solvents, and form a dried thin lipid film; this was then dissolved in 100 ml chloroform.

The ability of lipid to develop colour on contact with ammonium ferrothiocyanate solution (Ferric chloride 13.52 g and ammonium thiocyanate 15.2 g dissolved in DW and made up to total volume

of 500 ml) was harnessed to quantify the amount of lipid in the sample (Stewart, 1980, Elhissi and Ahmed, 2011). Different concentrations of lipid (10 - 200 µg) were used for establishing the calibration curve, however the volume of ammonium ferrothiocyanate was maintained at 2 ml. Additionally, chloroform was added to make the total volume of chloroform to 2 ml in the mixture. The resultant solution was then vortex mixed (Fision WhirliMixer, Fision scientific equipment, Leicestershire, UK) vigorously for 1 minute, followed by 5 minutes centrifugation at 300 g. The upper black/maroonish layer was discarded and the lower chloroform layer containing the lipid (forming a complex with ammonium ferrothiocyanate) was aspirated to estimate the quantity of lipid using a UV-Visible Spectrophotometer at 488 nm (Jenway, 7315, Bibby Scientific Ltd, UK) (Stewart, 1980, Elhissi and Ahmed, 2011).

2.2.9.2. Unentrapped BDP fraction after bench centrifugation

Following preparation of a 5 ml liposome suspension (30 mg/ml, explained earlier) in D_2O, 1 ml of the suspension was centrifuged. For analytical purposes, the unentrapped part (middle fraction of the tube containing lipid content) from the centrifuge tube was isolated as previously described (Section 2.2.5 and 2.2.6) (i.e. adding 1 ml of ethanol followed by overnight drying). The resultant dry film was dissolved using 2 ml of chloroform and an equal volume of ammonium ferrothiocyanate, followed by vortex mixing and bench centrifugation, to measure the amount of lipid by UV-Visible Spectrophotometry. 1 ml of liposome dispersion was used to measure the practical or total yield of lipid, in accordance to the aforementioned method, for comparison to the drug-free liposomes (middle fraction) (Table 3.3, chapter 3).

2.2.9.3. Lipid concentration for different carriers

For analysis of the lipid concentration coated upon each carbohydrate carrier, 1 ml of liposomal dispersion (30 mg of proliposome dispersed in 1 ml, for all three proliposome formulations i.e. 1:5, 1:10 or 1:15 w/w lipid to carrier ratio) in DW or D_2O was taken. The liposome dispersion was transferred to a 10 ml glass vial and was dried overnight in an oven at 90°C. Chloroform and ammonium ferrothiocyanate (2 ml of each) were then added and Stewart assay was performed (Stewart, 1980). Also, the lipid component was quantified by UV-Visible spectrophotometry at a wavelength of 488 nm (Table 3.1, Chapter 3).

2.2.10. HPLC determination of BDP-entrapment in liposomes using DW or D_2O as dispersion media

After determination of the optimum separation speed (13,000 RPM or 15,500 g) and time (90 minutes) of the bench centrifuge for liposome suspensions, HPLC was used to quantify different distinct fraction/layers in both DW and D_2O, depending on which dispersion medium was used. It was observed that in DW, the majority of the liposome-entrapped BDP (i.e. BDP entrapped in the liposome concentric bilayers) and BDP free liposomes were separated into two fractions/layers. The lower layer was found to contain, liposome-entrapped BDP and free BDP crystals which sedimented at the bottom of the centrifuge tube (forming a pellet), post centrifugation. Comparatively, the upper layer was noted to consist of a clear solution containing the remaining supernatant which contained little BDP and liposomes.

However, in D_2O, due to its higher density (1.105 g/ml) than water (1 g/ml), the entrapped and unentrapped drug was clearly separated into three fractions/layers, within the centrifugation tube (Chapter 3, Figure 3.7). The upper creamy-white fraction was found to contain liposome-entrapped BDP, whereas the second fraction was comprised of dissolved or free BDP crystals with traces of lipid in the middle fraction of the centrifuge tube and the third fraction was found to contain free BDP crystals forming a spot at the bottom of the centrifuge tube. Each fraction/layer in both hydration media (DW or D_2O) was diluted in methanol for HPLC analysis (Agilent 1200 HPLC instrument, UK).

2.2.11. HPLC analysis for the determination of BDP

Proliposome powder of 150 mg was taken and hydrated in 5 ml, according to the procedure described earlier in section 2.2.2 to produce liposome suspension (30mg/ml). For total drug content or total drug loading, the 1 ml liposomal dispersion was diluted with methanol to disrupt/dissolve the liposomes and BDP, followed by analysis of the drug quantity using HPLC. However, for the analysis of entrapment efficiency a 1 ml liposome suspension in D_2O or DW was hydrated and annealed for 2 hours. Following this, the suspension was centrifuged at 13,000 RPM for 90 minutes (Section 3.3.5 and 3.3.6).

The two separated fractions/layers in DW and in D_2O were individually diluted with methanol to quantify the amount of BDP in each fraction/layer by HPLC. The following equation 2.2 was used to determine the drug entrapment efficiency:

$$Entrapment\ efficiency\ (\%) = \left(\frac{Total\ drug - Unentrapped\ drug}{Total\ drug}\right) X\ 100.. (Equation\ 2.2)$$

BDP was assayed by employing a mixture of 75:25 (v/v) methanol and DW as the mobile phase at a flow rate of 1.7 ml/minute, and 239 nm was used as the detection wavelength. The temperature was set and maintained at 40°C, with an injection volume of 20 µl. The HPLC column used was a 15 cm X 4.6 mm, C-18, 5-micron column (Agilent technology, USA). The flow rate, mobile phase and injection volume were adapted with some modifications from the methods described by Zeng et al., (2000) and Batavia et al., (2001), due to the difference in HPLC equipment and formulation constituents (Zeng et al., 2000, Batavia et al., 2001).

A calibration curve for BDP in methanol was prepared in the concentration range of 0 - 50 µg/ml and the area under the curve was calculated from the resultant chromatograms.

2.2.12. Tapped density of coarse and proliposome powders

Tapped density measurements were taken in accordance to United State Pharmacopeia (USP) and British Pharmacopeia (BP) standards (BP., 2010d, USP., 2012). These may be described as the measurement, where the bulk density of a powder is noted following a controlled compaction process (often vibration or tapping of the powder container to ascertain the tapped density). Values from this process were used to determine the sedimentation or compactability of a given powder. This information is crucial in determining the suitability of powders for tableting by essentially assessing the powder flow properties.

A graduated cylinder with 1 ml increments and a volume of 50 ml was implemented to test the powdered sample. A set amount of 15 g (either coarse carrier or proliposome powder) was transferred into the cylinder, after which the graduated cylinder was tapped manually on a hard surface for two to three repetitions. Following this process, the initial volume (V_o) of the powder was noted. The cylinder was then affixed to a tapped density tester (Agilent technologies, USA) for automated controlled tapping. The apparatus was then set at a rate of 300 $\pm$ 15 taps per

minute and the height from which the cylinder was tapped (14 ± 2 mm) was fixed. Post tapping, the final volume was recorded (V_f). These values were then inputted into the provided equation 2.3 in order to establish the tapped density of the investigated powder.

The initial method outlined parameters such as 300 repetitions (taps) for all coarse carbohydrate carriers, including; LMH, sucrose, sorbitol and D-mannitol, as well as proliposome powders based upon each carrier. A percentage difference of greater than 2% was found upon using 300 repetitions in order to assess tapped density. As a consequence, tapping repetitions were increased in order to reduce the found percentage difference below 2%.

Tapping repetitions were then increased in set increments i.e. 500, 750, 1250 and 1500 taps. On increasing to 1500 repetitions for a set time of 5 minutes (i.e. 300 ± 15 taps per minute), the percentage difference between 1250 and 1500 taps was reduced below 2%. Thus, all formulations were tapped for 1500 repetitions within a time of 5 minutes.

2.2.12.1. Powder compressibility studies

Usage of tapped density values provides an indication of the inter-particulate interaction influence upon the bulking properties of investigated powders, and ultimately their flow properties (Shah et al., 2008, BP., 2010d, USP., 2012). This can be assessed by determining the difference in the powder volumes following the tapping process.

Compressibility index and Hausner ratio are measures which may be implemented to estimate or predict the flow characteristics of a given powder. Having achieved widespread popularity, compressibility index is recommended as an easy and indirect manner of measuring bulk powder density, moisture content, surface area, size, shape and material cohesiveness, as these are contributing factors in the degree of compressibility.

Both compressibility index and Hausner ratio are calculated from values generated from the tapped density process (i.e. V_o and V_f), however both equations utilise these values distinctively differently (BP., 2010d). Comparative degrees of bulk and tapped density may also be assessed using Carr's scale of flowability (Carr, 1965) (Table 2.2) via use of compressibility index (Equation 2.3) and Hausner ratio (Equations 2.4) as follows:

$$Compressability\ Index = \left(\frac{V_o - V_f}{V_o}\right) X\ 100 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 2.3)$$

$$Hausner\ Ratio = \left(\frac{V_o}{V_f}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 2.4)$$

$V_o = Unsettled\ apparent\ volume$

$V_f = Final\ tapped\ volume$

Carr's scale (Table 2.2) exhibits the degree or quality of powder flow in correspondence to values given in compressibility index and Hausner ratio, which indicate powder characteristics.

Table 2.2: Carr's scale of flowability (Carr, 1965)

Compressibility index (%)	Flow character	Hausner ratio
1-10	Excellent	1.00-1.11
11-15	Good	1.12-1.18
16-20	Fair	1.19-1.25
21-25	Passable	1.26-1.34
26-31	Poor	1.35-1.45
32-37	Very poor	1.46-1.59
> 38	Very, very poor	> 1.60

2.2.13. Angle of repose of coarse and proliposome powders

Angle of repose (AOR) is an additional technique which is employed in pharmaceutical industry for powder characterisation, associated with inter-particulate fractions, which is known to restrict particle movement (Shah et al., 2008). AOR may vary drastically from sample to sample, and is known to be influenced heavily by the density of the powder being investigated, as well as surface roughness, size and morphology of the particles (Guo et al., 2014).

For the purpose of this measurement, 15 g of coarse carbohydrate and/or proliposome powder was used. Investigated powders were dropped through a funnel at a set height (2 - 4 cm), allowing

for the formation of a cone. As cone formation occurred, the funnel was elevated between the set height values (2 - 4 cm) in order to maintain constant distance from the cone powder as it formed (BP., 2010d). Any improper or asymmetrical cone formation (attributed to powder segregation and separation) was discarded and the measurement repeated, until uniform cone formation was achieved. A level and smooth surface was ensured for this process.

Upon successful cone formation, the angle formed through the formation process was established using a protractor (Figure 2.4), which was kept parallel to the base of the cone to measure the AOR (Wong, 2002), which may be calculated using the following equation (Equation 2.5):

$$Tan\ (\alpha) = \left(\frac{height}{0.5\ X\ base}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\,..(Equation\ 2.5)$$

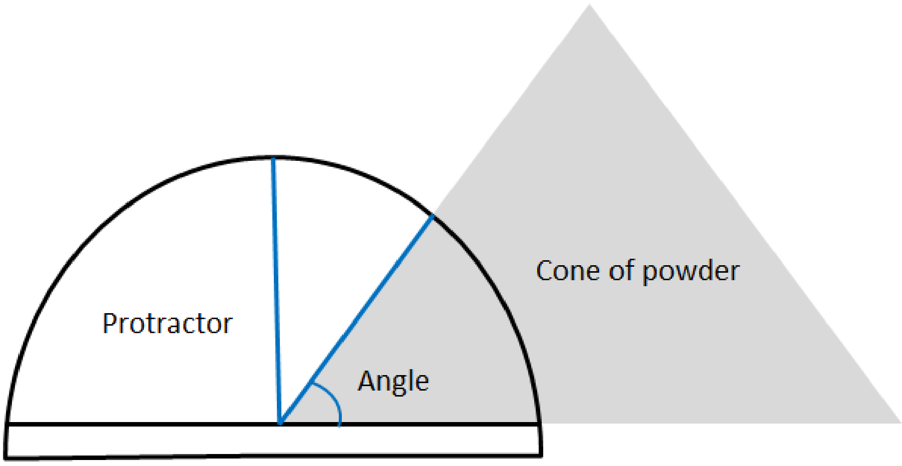


Figure 2.4: Angle of repose measurement from the cone of powder via use of protractor

AOR values generated were compared with set values outlined by Carr's flowability index (Carr, 1965). Excellent flowability was indicated by AOR values which fell in the range of 25 - 30^{o} whereas conversely, very poor flowability was denoted an AOR which fell in the range of 56 - 65^{o} (Carr, 1965, Geldart et al., 2006).

Table 2.3: Flow properties and corresponding angle of repose (Carr, 1965)

Flow property	Angle of repose (degree)
Excellent	25-30
Good	31-35
Fair (aid not needed)	36-40
Passable (may hang up)	41-45
Poor (must agitate, vibrate)	46-55
Very poor	56-65
Very, very poor	> 66

High AOR values are indicative of high inter-particulate resistance and excessive cohesion. This results in a powder with poor flow properties (Wouters and Geldart, 1996, Ileleji and Zhou, 2008). Common causes of high AOR's, are often attributed to the presence of elevated moisture content in powder formulations. These significantly alter the flowability of the powder, preventing smooth movement and giving rise to higher values (Wang et al., 2010). As an analytical technique, AOR is a relatively cheap and valuable tool. Moreover, its simplicity and reproducibility make it desirable for examining powder flowability when compared to alternative methods, which may require more time and preparation (Wouters and Geldart, 1996, Ileleji and Zhou, 2008, Geldart et al., 2009). However, a plethora of different methods have been implemented, leading to different outcomes in terms of the values generated. The method employed within this piece of research is in stringent accordance with current BP guidelines (BP., 2010d).

2.2.14. Direct compression tableting method

Direct compression is a common tablet manufacturing method, which involves the dry mixing of the formulation ingredients followed by compression. This method is ideal for tablet formulations which include moisture or thermo sensitive APIs, as it does not involve the incorporation of any liquid, and heat production is minimal (Armstrong, 1997). This method is also preferable for low dose tablets (Mao et al., 2013). Preferentially, powders which have satisfactory flowability and compactability, are the choice for this method (Villanova et al., 2011). A number of stages comprise the method of direct compression, including the rearrangement stage, deformation stage, compaction and relaxation stage; which are elaborated upon as follows:

- *Rearrangement*: Here the die cavity of the tableting machine is primarily filled with powder (void spaces between particles are still prevalent in this stage).
- *Deformation:* Once self-rearrangement of the particles is complete, they are deformed elastically through the force exerted by compression.
- *Compaction*: Here the elastic limit of the tableting material is exceeded; this form of deformation may be detrimental to the tablet ingredients, causing fracture or fragmentation. The outcome of deformation is dependent upon the properties of the compacting material, compaction force, compaction speed and also particle size. The process of deformation increases the contact area amongst particles, which are loosely arranged causing fragmentation of these particles, and thus an increase in newer surface area.
- *Relaxation*: At this stage, the punch is ejected from the die cavity, allowing for release of pressure generated during the tableting process. Through the process of direct compression, certain excipients exhibit plastic deformation (i.e. microcrystalline cellulose), whereas others such as dicalcium phosphate dihydrate show brittle deformation i.e. fracturing.

There are a number of advantages associated with direct compression as a method for tablet manufacture, entailing:

- Process is associated with minimal instability issues for heat-labile materials (Yuan et al., 2013).
- Little machinery is required to conduct the process, resulting in a shorter manufacturing time and lower energy consumption (Pather et al., 1998, Martinello et al., 2006).
- Fewer numbers of excipients are required for direct compression (Jivraj et al., 2000, Villanova et al., 2011).

The direct compression method is well-established and has been used as a tablet manufacturing method, predominantly for a number of years. Recent developments, however, indicate that there are varying degrees of excipient suitability for this process. Moreover, many of these excipients may be mixed with large quantities of API without impacting upon tablet quality. Lactose and sorbitol are ubiquitous in pharmaceutical industry and are commonly incorporated as excipients in tablet formulations (Rojas et al., 2014). Depending upon the concentration used, excipients may serve a variety of purposes. However, typically the following properties are desirable for the majority of excipients:

- Sufficient flowability and compressibility (Villanova et al., 2011).
- Ideally, particle size distribution should be similar to the API to reduce the probability of component segregation during manufacture.
- Batch variations are undesirable, product consistency from batch to batch to ensure reproducibility is coveted.
- Physical and chemical stability are essential due to the potential exposure to heat, moisture and air (i.e. oxidation).
- Chemical and physical compatibility with additional excipients (which may possibly be included) and packaging material are also properties, which are considered in the selection process of excipients.

2.2.15. Manufacture of proliposome tablets via single punch machine

Proliposome powders formulated via a "Slurry" method (Section 2.2.2) were employed to manufacture tablets using a Riva Minipress MII SA single punch machine (Cenova 4018 Ciudadela, Buenos Aires, Argentina) (Figure 2.5). This machine was either operated manually or automatically (automatically for 1:15 w/w lipid to carrier ratio sorbitol-based proliposome tablets and manually for all other formulations prepared as discussed in section 4.3.4). Small tablets (6 mm in diameter) were prepared using the provided punches, these typically weighed between 62.66 - 91.97 mg with a drug content of 70.18 - 103.01 µg per tablet. Depending upon the required tablet weight, the punches of the machine were adjusted accordingly. Prior to tableting, the required proliposome powder was weighed and transferred to the tablet die, after which either the manual or automated mechanism was used to compact the powder into a tablet.

Figure 2.5: Single punch machine containing different parts for tablets manufacturing (*Prolyse BV, 2013*)

Tablets softness and hardness were controlled by the set compression force (KiloNewton, kN), which could be adjusted manually. Frequent adjustment was not required, as this force was only found to vary when the automated function of the unit was utilised. For the automated tableting, the powder was fed through a funnel (component referred to as the hopper), which guided the required weight of powder to the die. Proliposome tablets (from sorbitol-based proliposome powder with 1:15 w/w lipid to carrier ratio) manufactured through single punch machine via automated route were produced at a rate of 20 $\pm$ 4 tablets per minute, and with a compressive force ranging between 3.00 and 4.50 kN. Manufacturing conditions were maintained for all formulations tested. In between tableting of each batch, the machine punches and die were cleaned with a soft microfiber cloth to revoke any contamination.

2.2.16. Weight variation/Uniformity of weight

Weight variation/uniformity testing was conducted to ensure consistency in batch formulation. This test was conducted as part of Quality control (QC) and is ordinarily conducted during and post manufacture of tablet batches, in accordance to British Pharmacopeial standards., (2010) (BP., 2010e). For the purpose of this test, twenty tablets were selected at random from each manufactured batch; each tablet was weighed individually using a calibrated balance (PI-225DA Denver Instruments, Germany). The mean weight of these tablets was then ascertained using

equation 2.6, and the variance of each tablet from the mean weight was calculated (BP., 2010e, El-Bagory et al., 2012,).

$$Average\ weight = \left(\frac{X_1 + X_2 + X_3 + X_1 + \cdots X_{20}}{20} \right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 2.6)$$

$$X = Weight\ of\ individual\ tablet$$

In accordance with the British Pharmacopeia., (2010), tablets ranging in weight from 80 - 250 mg should possess a variance of less than 7.5%, whilst tablets below 80 mg in weight are permitted a wider range of variance (10%). Moreover, for both weight ranges, no more than two tablets are permitted to deviate from the average weight of the twenty tablets. Additionally, no more than one tablet is permissible to deviate from double the average weight derived from twenty tablets. Deviation outside of these parameters infers a failed batch of tablets, which are unsuitable for use (BP., 2010e).

2.2.17. Disintegration testing

Disintegration testing may be described as the time required for a given tablet to disintegrate into particles, when placed in a specific aqueous medium. An Erweka disintegration tester, (GmbH, type TBH 220D, D-63150 Heusenstamm, Germany) was used to study the disintegration time of tablets. The apparatus comprised of a basket rack assembly, containing 6 open ended transparent glass tubes (3 inches in length, containing 10 mesh screens at the bottom of each tube) (BP., 2010b). This rack assembly was placed in 1000 ml glass beaker filled with water up to 800 ml. Thermostatic arrangement was ensured by maintaining the temperature between 35 and 37°C. The basket rack assembly was adjusted to move vertically into the beaker, being lowered 28 - 32 times into the medium per minute. Six proliposome tablets were placed in six tubes individually, followed by positioning of six small disks (of transparent plastic material) over the tablets, to prevent tablet loss during testing. The distance covered by the assembly to move up and down was approximately 53 - 57 mm (BP., 2010b). Disintegration time was noted for all the tablets tested. According to BP standards, if one to two tablets fail to disintegrate, a repeat test for a further 12 tablets is required. From the total of 18 tablets, 16 must pass the test for the batch to be approved (BP., 2010b).

2.2.18. Crushing strength/Tablets hardness

Following tablet manufacture, proliposome tablets were assessed for hardness. A specialised Erweka hardness tester (GmbH, type TBH 220D, D-63150 Heusenstamm, Germany) was employed to measure the mechanical integrity (breaking force) of the proliposome tablets. Breaking force is dependent upon the geometry and the applied compression force on the tablets. However, they should not be too soft to not to withstand the crushing strength. Uncoated tablets are required to have a hardness range of 4 - 10 kg (kilogram) (1 kg= 10 Newton). Tablets below this range are often soft and are unable to withstand crushing strength. Consequently they are believed to be unsuitable for blister packing, potentially likely to break during shipping. Tablets with high compression force are above this range are associated with longer disintegration times, to the extent that they are liable to fail testing, exceeding the specified disintegration duration of 15 minutes (BP., 2010b). Prior to assessment of tablet hardness, the machine was calibrated with no tablets, followed by assessment of the crushing strength of ten tablets (measured in Newtons).

2.2.19. Friability testing

Friability is deemed to be related to tablet hardness; this test sets out to gauge the ability of tablets to avoid abrasions and fractures during handling, packaging and transportation. Friability testing essentially monitors the weight loss of tablets under controlled handling conditions for a set duration. The extent of friability may be noted from a number of phenomena including; capping and chipping of the tablet, which impacts upon tablet appearance, weight variation and ultimately consumer compliance.

For the purpose of this test, ten tablets were selected at random, weighed and placed into an Erweka friabilator (GmbH, Type TA 120, D-63150, Heusenstamm, Germany). The apparatus is comprised of a drum, with an internal diameter of 283 - 291 mm, and a depth between 36 - 40 mm (BP., 2010c). The drum material is comprised of a transparent synthetic polymer. One side of the drum is removable for loading and removing the tablets. On testing, the drum is attached to the main unit and rotates at an RPM of 25 $\pm$ 1. During each revolution tablets fall in the apparatus, being subjected to handling force (BP., 2010c). Following 100 revolutions or four minutes, the tablets were taken out from the device and cleaned to remove any powder or dust. These tablets were then weighed, analysed and compared to the initial weight for any loss, which should not

exceed 1% (BP., 2010c). The following equation 2.7 allows for the calculation of percentage friability of tablets:

$$Percentage\ Friability = \left(\frac{W_1 - W_2}{W_1}\right) X\ 100 \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ (Equation\ 2.7)$$

Where, W_1 is the initial weight or tablets weight before conducting the friability test

W_2 is the weight of tablets measured after testing was completed

2.2.20. Aerosol deposition studies using a Two-Stage Impinger

The Two-Stage Impinger (TSI) (also known as twin impinger) is an artificial pulmonary deposition model. It consists of two stages (also referred to as the compartments of the impinger). The initial compartment represents the upper respiratory tract, whilst the second compartment represents the lower respiratory tract, and is referred to as the upper stage and lower stage, respectively. Each compartment consists of small sub-pieces, which can be dismantled to wash and collect the API. The cut-off aerodynamic diameter between the upper and lower stage is of 6.4 µm, particles below this size are known as respirable or fine particle fraction (FPF), which deposit into the lower stage of the impinger (Hallworth and Westmoreland, 1987). A flow rate of 60 Litres per minute utilising a flow controller (Model TPK 2000, Copley Scientific Ltd, Nottingham, UK) and the flow meter (Model DFM 2000, Copley Scientific Ltd, Nottingham, UK), is maintained between the compartments (BP., 2010a) (Figure 2.6). DW is employed as a collection medium for aerosol deposition in the upper and lower stage of the impinger (volumes used as 7 ml and 30 ml respectively) (BP., 2010a).

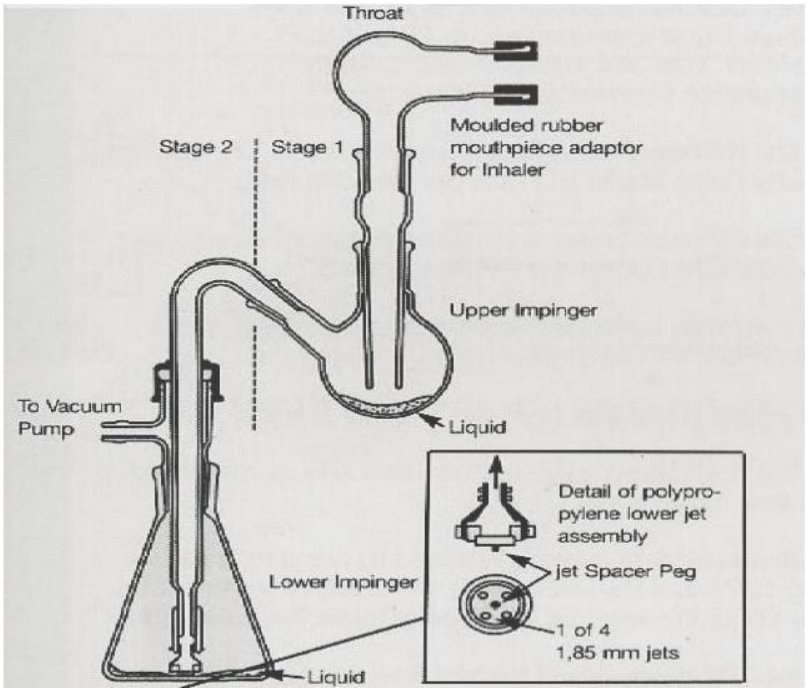


Figure 2.6: A schematic presentation of the Two-stage Impinger (Source: Copley Scientific Limited, UK)

In order to ascertain the concentration of the drug in the impinger stages post nebulization each part of the impinger was dismantled and washed with DW, to make a total volume of 30 ml in a graduated cylinder. Each experiment was performed in triplicate for each individual formulation. The drug concentration in each stage was then analysed via HPLC.

2.2.21. Aerosol droplet size analysis via laser diffraction

Liposome dispersions of 5 ml (Steckel and Eskandar, 2003, Elhissi et al., 2007) were prepared (30 mg/ml; containing 30 mg of proliposome powder/tablet was dispersed in 1 ml of solvent media as a standard ratio) from sorbitol and D-mannitol-based proliposome powders and tablets. The suspension in the nebulizer was clamped in a fixed position, 2.5 cm away from the central laser beam of the Spraytec's laser diffraction apparatus (Malvern Instruments Ltd., UK) (Figure 2.7). A vacuum pump was connected with a suction speed of 60 litres per minute to draw the aerosol across the laser beam. A vacuum was employed to minimise any plume formation (aggregation or fusion of small aerosol droplets into larger droplets) during aerosolisation.

Figure 2.7: Photograph of the Malvern Spraytec apparatus (*Malvern-Instruments, 2014*)

A Malvern Spraytec apparatus was employed for the determination of aerosol droplet size distribution (Figure 2.7). The aerosol mist from the nebulizer was passed angularly through the laser beam of the Spraytec, and the scattered light intensity was analysed by the detector. Scattered light detected were converted into measurements using the software provided. These scattered light measurements were used to quantify the VMD (also referred to as size), SPAN (size distribution) and fine particle fraction (FPF). In this study, the average aerosol VMD of droplets was determined (50% undersize) as well as their associated SPAN values. FPF makes reference to smaller droplet size of aerosols (i.e. less than 4.5 µm) to determine the mass output (O'Callaghan and Barry, 1997).

2.2.22. Nebulizer performance

Pari LC Sprint air-jet nebulizer (i.e. containing Pari Turbo Boy Master Compressor) (Pari GmbH, Germany) was used in this study, to nebulize liquid dispersions of sorbitol and D-mannitol-based proliposome powders and tablets prepared with a ratio of 30 mg/ml. A vital parameter of nebulizer performance is nebulization time for a given volume, which is the duration from the beginning of aerosol production to complete cessation (including sputtering time) of aerosols (i.e. time to "dryness") (Kradjan and Lakshminarayan, 1985). Moreover, "sputtering time" is described as the phase where normal aerosol formation begins to show sporadic mist formation and erratic sound of nebulizer. During this period of inconsistent mist formation, the nebulizer was tapped

gently to encourage continuous mist formation. This procedure was repeated several times until complete cessation of nebulization occurred.

In this study, all liposome formulations (5 ml; 30 mg/ml) were nebulized (Steckel and Eskandar, 2003, Elhissi et al., 2007) , and connected to a suction pump for aerosol extraction of 60 litres per minute. All experiments were performed in triplicate under an ambient temperature of 22 - 25°C. Both nebulization time and sputtering time were determined for all formulations.

Additionally, aerosol mass output was determined in percentage, calculated gravimetrically by the difference between the weight of the formulation before and after nebulization. The aerosol mass output (%) was calculated in equation 2.8 as follows:

$$\text{Mass output } (\%) = \left(\frac{\text{Weight of nebulized formulation}}{\text{Weight of formulation present in the nebulizer prior to nebulization}}\right) \text{X100} \ldots . \text{(Equation 2.8)}$$

Furthermore, aerosol output rate is defined as the time required for the formulations to nebulize against the complete cessation of nebulization (i.e. nebulization time). Longer nebulization times were associated with lower amounts of nebulized formulation per minute. Aerosol output rate was quantified utilising the following equation 2.9:

$$Aerosol\ Output\ (mg/\text{min}) = \left(\frac{Weight\ of\ nebulized\ formulation}{Complete\ nebulization\ time}\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 2.9)$$

2.2.23. Transmission Electron Microscopy for liposome identification

Both lamellarity and morphological studies were performed for liposomes by using Transmission Electron Microscopy (TEM). In this experiment a liposome suspension was prepared (section 2.2.2), and a drop was placed upon a carbon-coated copper grid (400 mesh) (TAAB Laboratories Equipment Ltd, UK), followed by negative staining with phosphotungstic acid (1% weight/volume). The sample containing liposome vesicles were examined and photographed utilising a Philips CM 120 Bio-Twin TEM (Philips Electron Optics BV, Netherland).

2.2.24. X-ray Diffraction

The extent of BDP crystallinity when formulated as a proliposome powder was assessed using X-ray Diffractometry (X-ray diffractor, D2 PHASER with LYNXEYE, Bruker, Germany). Samples were loaded onto inert polymeric discs and the powder surface was smoothed using a glass slide, in order to maintain homogenous contact between the sample and equipment. The samples were scanned from 5 to 50° with a set scan type, coupled with two θ (theta) using a scintillation counter and 1-dimensional LYNXEYE detector. Upon sample testing, collation of the generated patterned data was used to ascertain the presence or absence of crystalline BDP, in the proliposome powder formulations. Several samples were assessed using X-ray diffractometer (XRD) to allow for data validation, including BDP alone, coarse carbohydrate carriers, and proliposomes of different carriers with BDP (i.e. 1:5, 1:10 and 1:15 w/w lipid to carrier ratio). Moreover, coarse carbohydrate carriers were treated with ethanol alone (as in proliposome formulation but without lipid and BDP) and examined. Additionally, proliposome formulations with 100 mole% of BDP to the lipid phase (here BDP concentration was increased from 4.48 mg for 2 mole% to 244.05 mg for 100 mole%) was used to prepare 1:5 w/w lipid to carrier ratio (utilising LMH, sucrose, sorbitol or D-mannitol). These were also examined for comparison in crystallinity.

2.2.25. Differential Scanning Calorimetery

Differential Scanning Calorimetery (DSC) was employed in this study to further investigate the state of BDP presence in proliposome formulations. The thermal behaviour (absorbance of heat) of the individual ingredient expressed in the form of a peak indicated the endothermic profile and the melting point (onset temperature) of the sample. The onset temperatures of each of these peaks were used to identify specific ingredients. Each individual constituent of proliposomes including BDP, cholesterol and carbohydrate carrier were weighed separately in 40 µl aluminium pans, followed by their introduction into the DSC (DSC 823e, METTLER TOLEDO, Switzerland) instrument. Various temperature ranges were used depending on the excipient properties, each with a set heating rate of 20°C per minute. SPC was not utilised alone, due to its low phase T_m (-20°C). A proliposome formulation containing 2 mole% of BDP (4.48 mg) with respect to the lipid phase (250 mg) was used to examine and analyse the presence of amorphous or crystalline form.

Additionally, a higher amount of 100 mole% of BDP (244.05 mg) to the lipid phase (250 mg) was also investigated to analyse the degree of crystallinity.

2.2.26. Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) was utilised in this research to analyse and identify the structure of organic compounds. In this case BDP was isolated as a spot (post centrifugation of liposome suspension dispersed in D_2O) and dried to eliminate moisture. The resultant dry spot of BDP, was dissolved completely in deuterated chloroform. The dissolved sample was then transferred to an NMR tube and covered with a cap to prevent any evaporation. Care was taken to ensure that the sample tube contained sufficient quantity of both the drug spot and solvent; to increase detection. Prior to use the sample tube was shaken vigorously, to further ensure dissolution.

Following sample preparation, the sample tube was transferred to the sample holder of the NMR unit. Proton (^1H) NMR employed by the NMR (Bruker, Magnetic system 300 MHz/54mm, UltraShield, UK) was conducted to scan the sample; this procedure was repeated in triplicate. The isolated drug spot and pure BDP were compared using NMR by examining the generated spectra, from which the relevant proton peak was identified.

2.2.27. Statistical analysis

One-way analysis of Variance (ANOVA) and Student's *t-tests* were performed as appropriate, to assess the statistical difference using SPSS software. Additionally ANOVA was also utilised with the Tukey test for comparison between variables of more than three groups. P-values below 0.05 were considered as statistically significant. All experiments were performed in triplicate.

CHAPTER 3: SLURRY METHOD PROLIPOSOME MANUFACTURING: GENERATION AND CHARACTERISATION OF LIPOSOME IN DEUTERIUM OXIDE

"Repetition is the mother of all skills"

- Waqar Ahmed -

3.1. Introduction

Liposomes are known to suffer from poor stability in aqueous media; this fact stems from its components i.e. phospholipids, which may undergo oxidation and hydrolysis (Hunt and Tsang, 1981). As a consequence, drug leakage and aggregation of the liposome vesicles may occur (Wong and Thompson, 1982), resulting in marked shortening in the shelf-life of liposome formulations.

Liposomes pertain the ability to entrap APIs; however its degree of entrapment efficiency is dependent upon the properties of the therapeutic agent, phospholipid and carrier type; as well as method of liposome preparation. Advantageously, liposomes possess the ability to entrap both hydrophobic and hydrophilic APIs; enhancing their desirability as drug carriers (Payne et al., 1986a, Payne et al., 1986b, Sharma and Sharma, 1997, Darwis and Kellaway, 2001).

The lamellarity (number of concentric bilayers) of liposomes (i.e. unilamellar or multilamellar) is an influential factor upon the degree of entrapment, of a given therapeutic agent. Large unilamellar liposomes prepared through reverse phase evaporation, have been considered to be highly appropriate for the entrapment of hydrophilic drugs; within the central aqueous core of the vesicle (Szoka and Papahadjopoulos, 1978, du Plessis et al., 1996). Contrastingly, multilamellar liposomes are commonly used to entrap hydrophobic drugs within the lamella of the liposomes (Batavia et al., 2001).

As a solution to oxidation and hydrolysis of phospholipids, associated with liposome stability; proliposome technologies (i.e. a pre-cursor dry formulation of liposomes, which utilises carbohydrate carriers) have been suggested. Both spray drying (Alves and Santana, 2004) and fluidised bed drying (Chen and Alli, 1987, Kumar et al., 2001) are complicated procedures, requiring high pressure and a suitable flow rate; to ensure uniform thin layer formation of the lipid (phospholipid and cholesterol) on the carrier surface. Slow-spray coating or particulate-based proliposome methods (Payne et al., 1986a, Payne et al., 1986b, Elhissi et al., 2006a) involve the injection of 0.5 - 1 ml of an organic solution (containing lipid) every few minutes, over the carrier via an injection feed tube in a rotary evaporator. However, both procedures are associated with phospholipid loss. Injection methods suffer from additional phospholipid and drug loss due to potential leakage or splashing during injection, as this process requires a notable amount of physical force. The development of the novel "Slurry" method (Section 2.2.2) offers a solution to this, as no spraying or injections are needed, resulting in greater economy during proliposome manufacture (i.e. reduce lipid loss).

With regards to the entrapment of BDP in liposomes, highly conflicting findings have been reported in literature. For instance, Batavia et al., (2001) using Dipalmitoylphosphatidylcholine (DPPC) demonstrated that the maximum amount of BDP incorporated into liposomes was 2.52 mole%. Elhissi and co-workers (2006) similarly used a range of 1 - 5 mole% BDP (Elhissi et al., 2006a), and a concentration of 2.24 mole% was reported by Darwis and Kellaway (2001) (Darwis and Kellaway, 2001). High entrapment has typically been noted in literature; for example, Radhakrishnan (1991) claimed 98.8% entrapment when using BDP as a model drug (Radhakrishnan, 1991). This level of high entrapment was re-iterated by Jaafar-Maalej et al., (2011), who again reported a BDP entrapment of 98% (Jaafar-Maalej et al., 2011).

The differences in entrapment reported are perplexing. A possible explanation for these differences may firstly be the conventional methods by which liposomes are generated. Alternatively, these differences may be attributed to the method of proliposome preparation and subsequent generation of liposomes. Secondly, the separation technique or methods where drug loaded liposomes are separated from the unentrapped free BDP may also be a causative factor for the high readings observed across literature.

In order to prepare stable proliposomes, where the lipid phase (a combination of phospholipid and cholesterol in a 1:1 mole ratio) is evenly distributed over the carbohydrate carrier (irrespective of carrier size differences) with minimal lipid phase loss, the novel "Slurry" method was developed. Four carbohydrate carriers (i.e. lactose monohydrate (LMH), sucrose, sorbitol and D-mannitol) were employed, and three different formulations (i.e. 1:5, 1:10 and 1:15 w/w lipid to carrier ratio) were used to prepare proliposome powder formulations.

It has been noted that liposomes and free BDP crystals are characteristically similar in size (Batavia et al., 2001), as a consequence it is perceivable that upon centrifugation both liposomes and free BDP crystals may sediment together. This co-sedimentation may result in false entrapment values, as typically these crystals are not removed from the collected BDP-entrapped in liposomes. It is for this reason that D_2O (a higher density medium than DW) has been implemented as a liposome dispersion medium. In theory, whilst similar in size, liposomes and BDP crystals possess variant densities, which may allow for their complete separation; using an alternatively denser medium than DW.

Following preparation of liposome suspensions in both media, an array of characterisation techniques, including: Laser diffraction, Electrophoretic mobility and HPLC were employed to establish the most ideal formulation ratio and carrier type, in terms of size and entrapment efficiency.

3.2. Methodology

3.2.1. Proliposome powder manufacture via "Slurry" method

Proliposome powders utilising carbohydrate carriers (i.e. LMH, sucrose, sorbitol or D-mannitol) were prepared via the "Slurry" method (Section 2.2.2), in three formulation ratios (i.e. 1:5, 1:10 or 1:15 w/w lipid to carrier ratio). For the 1:5 w/w ratio, one portion of lipid phase (250 mg; containing 1:1 mole ratio of SPC and cholesterol) was dissolved in ethanol, followed by the incorporation of BDP, employed as 2 mole% (i.e. 4.48 mg) to the lipid phase. Five portions of carrier (1250 mg) were taken in a pear-shaped RBF, the ethanolic solution of lipid phase and drug were then added into the RBF containing carbohydrate carrier to form a slurry. This procedure was conducted identically for the other lipid to carrier ratios (i.e. 1:10 and 1:15 w/w), however the carrier concentration was altered (i.e. raised). Subsequently, the organic solvent was evaporated via a rotary evaporator, and the resultant dry proliposome powder was stored at -18°C.

3.2.2. Characterisation of proliposomes and liposomes

Proliposome powders formulated at various ratios (1:5, 1:10 or 1:15 w/w lipid to carrier ratios) were characterised for their surface morphology via SEM (Section 2.2.4).

Liposomes generated from LMH, sucrose, sorbitol or D-mannitol-based proliposomes in DW or D_2O, were characterised for their VMD also referred to as size, and SPAN (i.e. size distribution), via a Malvern Mastersizer (Section 2.2.7). Zeta potential of liposomes was also investigated using a Zetasizer Nanoseries (Section 2.2.8).

3.2.3. Entrapment efficiency

Entrapment efficiency of BDP in liposomes was analysed in two different dispersion media (i.e. DW or D_2O) (Section 2.2.10 and 2.2.11). Spots formed at the bottom of the centrifuge tube, post centrifugation of the liposome suspensions, as well as samples from other fractions (Section 2.2.5 and 2.2.6), were collected and subsequently dissolved with methanol and analysed using HPLC.

3.3. Results and discussion

3.3.1. Surface morphology of coarse carriers and proliposome powders

SEM was employed for the analysis of the surface morphology of the coarse carbohydrate carriers (i.e. prior to loading of the carrier with lipid) and proliposome powders prepared by "Slurry" method (Section 2.2.2), utilising different carbohydrate carriers (LMH, sucrose, sorbitol or D-mannitol); at varied lipid to carrier ratios (1:5, 1:10 or 1:15 w/w).

SEM observation of coarse LMH showed irregular to prism, or pyramidal shaped carriers (Figure 3.1 a), whilst sucrose was found to have an irregular or cubical shape with a smooth surface (Figure 3.2 a). Both types of carrier particles were found to be non-porous. The lipid coating (SPC and cholesterol in a 1:1 mole ratio) over LMH carriers appeared to be of higher density with the 1:5 w/w lipid to carrier ratio (containing one portion of lipid phase and five portions of LMH carrier) (Figure 3.1 b). Intermediate to moderate coating was noted with the 1:10 w/w lipid to carrier ratio formulation (Figure 3.1 c), and partial coating was observed with the 1:15 w/w lipid to carrier ratio (Figure 3.1 d).

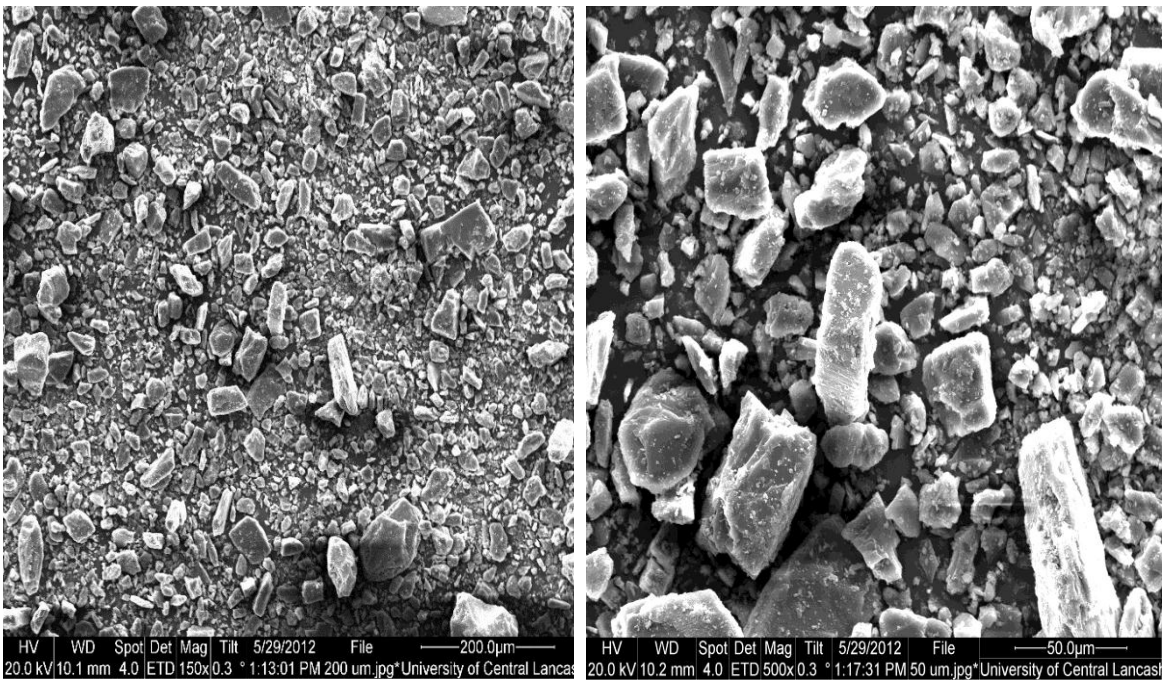
LMH-based proliposomes exhibited a degree of agglomeration following lipid coating (Figure 3.1 b), attributed to the sticky nature of the lipid on the surface of the non-porous carbohydrate carrier (Payne et al., 1986b). Moreover, sucrose-based proliposomes showed a higher degree of agglomeration than LMH-based proliposomes, entailed by their smooth surface morphology. Sucrose surface was densely coated with lipid in the 1:5 w/w formulation ratio (Figure 3.2 b), moderately coated in the 1:10 w/w ratio (Figure 3.2 c); and adequately coated in the 1:15 w/w lipid to carrier ratio (Figure 3.2 d). A high degree of stickiness was also demonstrated by sodium chloride-based proliposomes, when EPC was used as phospholipid (Payne et al., 1986b).

The tendency of lipid to concentrate in the form of a thin layer/film on the carbohydrate carriers was investigated and substantiated by Stewart assay (Stewart, 1980), performed for all the formulation ratios (i.e. 1:5, 1:10 and 1:15 w/w lipid to carrier ratio) (Section 2.2.9 and 3.3.2). It was found that the 1:5 w/w formulation ratio contained greater amounts of lipid than the 1:10 and 1:15 w/w ratio. The trend with respect to lipid concentration for both sucrose and LMH-based proliposome powders has been shown in the following order of descending lipid content:

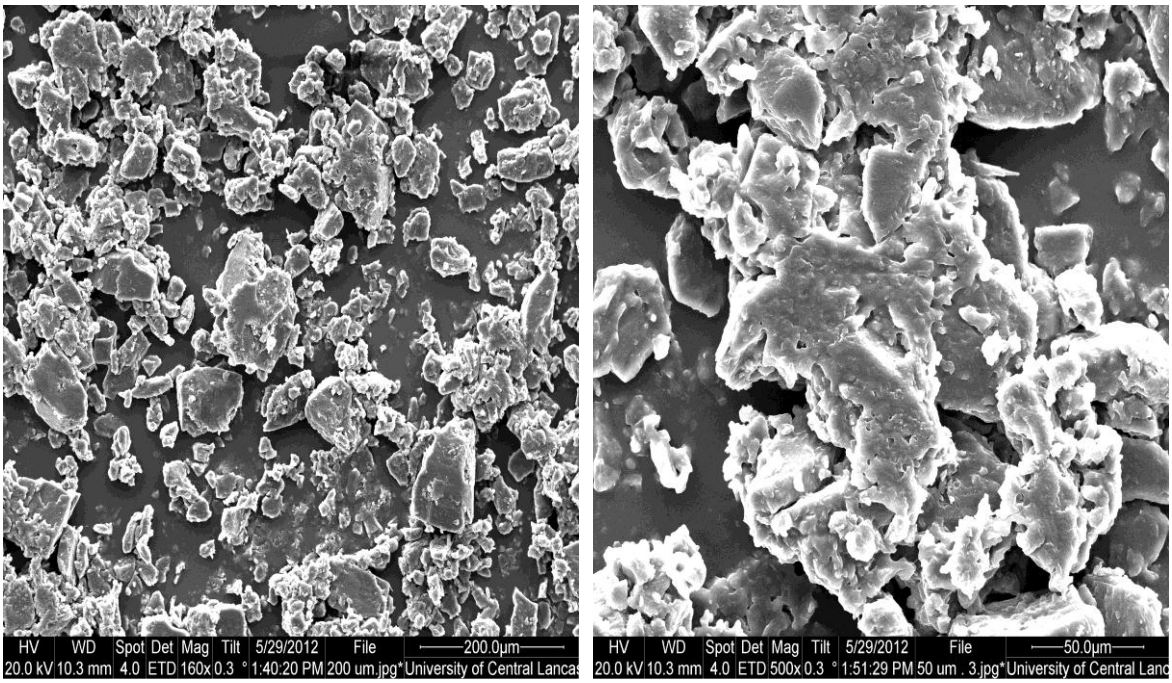
1:5 > 1:10 > 1:15

Both these carriers have been shown to exhibit almost similar patterns of coating; however, sucrose-based proliposomes have shown greater aggregation and formation of agglomerates, due to high lipid concentrations on their smooth surfaces; as ascertained by Stewart assay (Section 2.2.9 and 3.3.2). The trend of agglomerate formation associated with sucrose-based proliposomes compared to LMH-based proliposomes, has been previously shown using formulations traditionally manufactured via the lipid-injection method to prepare proliposomes (Elhissi et al., 2011). LMH particles (smaller than 250 µm) may provide a greater surface area for coating; possibly for this reason, LMH-based proliposome particles were less adherent. It was also observed that proliposomes prepared from sucrose were stickier and readily formed agglomerates (Figure 3.2 b).

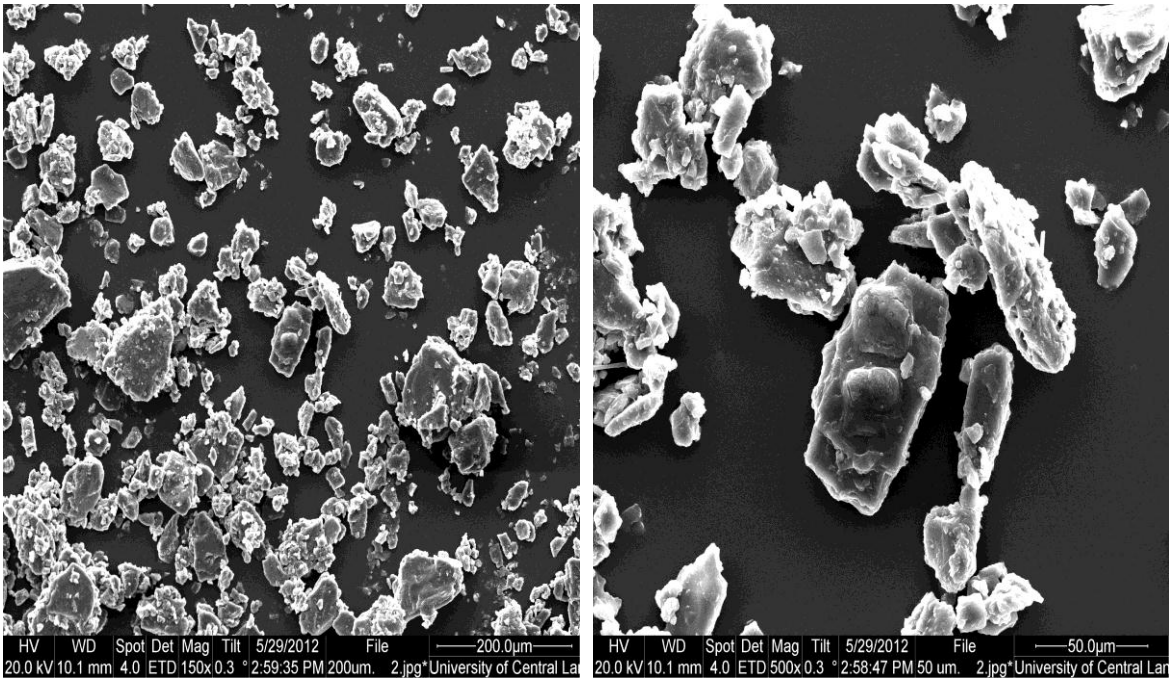
(a) *LMH particles (particle size < 250 µm) prior to loading with lipid phase*



(b) *Proliposomes (1:5 w/w, lipid to carrier) carrier size fraction less than 250 µm*



(c) *Proliposomes (1:10 w/w, lipid to carrier) carrier size fraction less than 250 µm*



(d) *Proliposomes (1:15 w/w, lipid to carrier) carrier size fraction less than 250 µm*

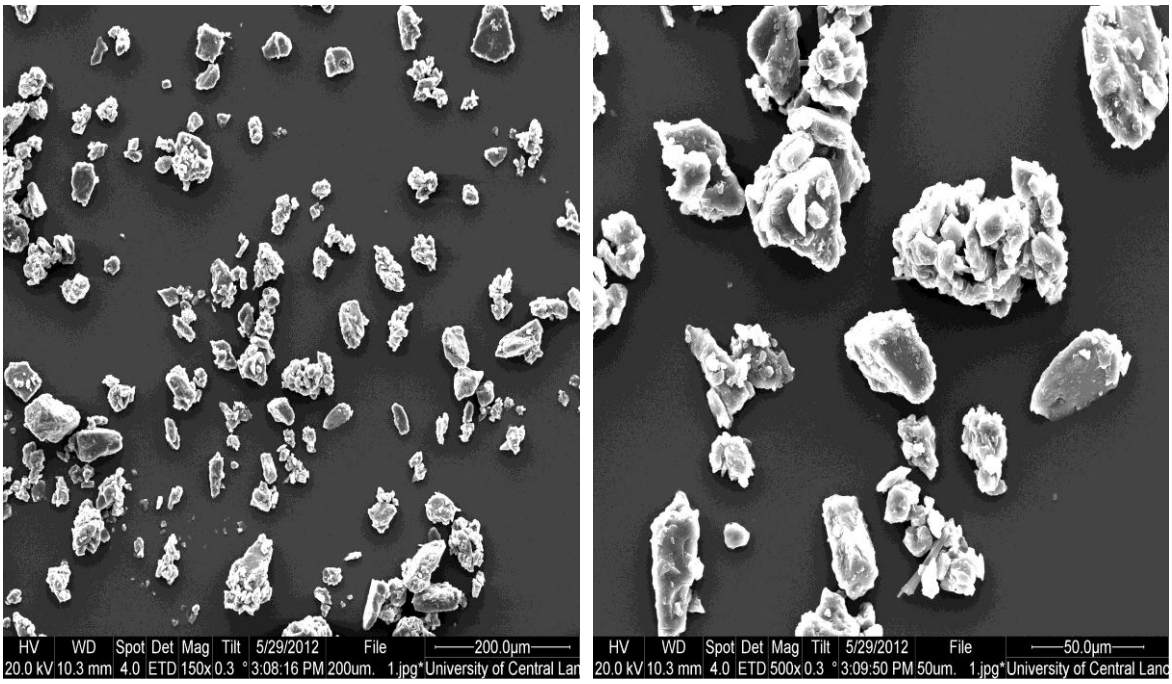
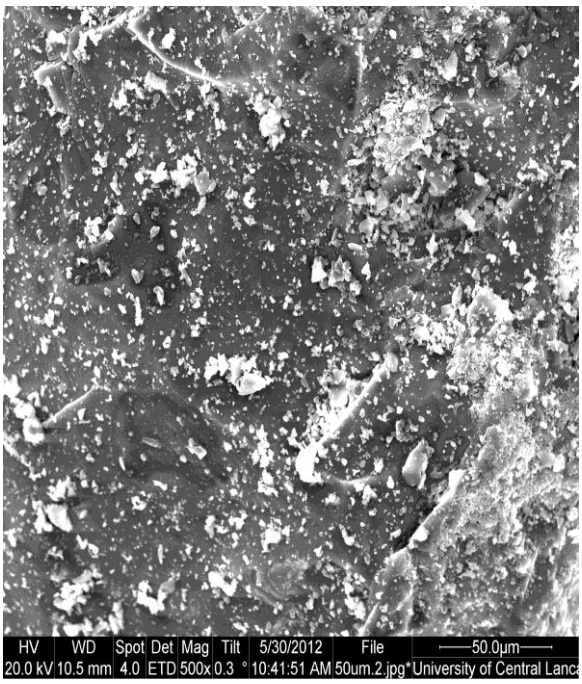
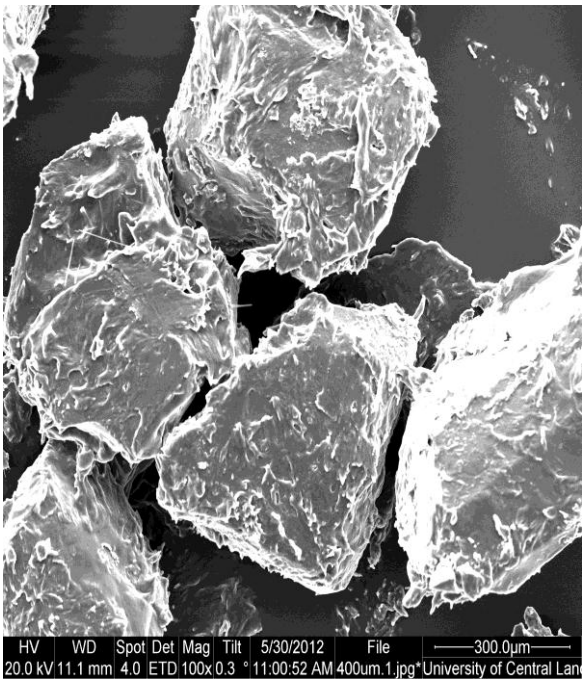


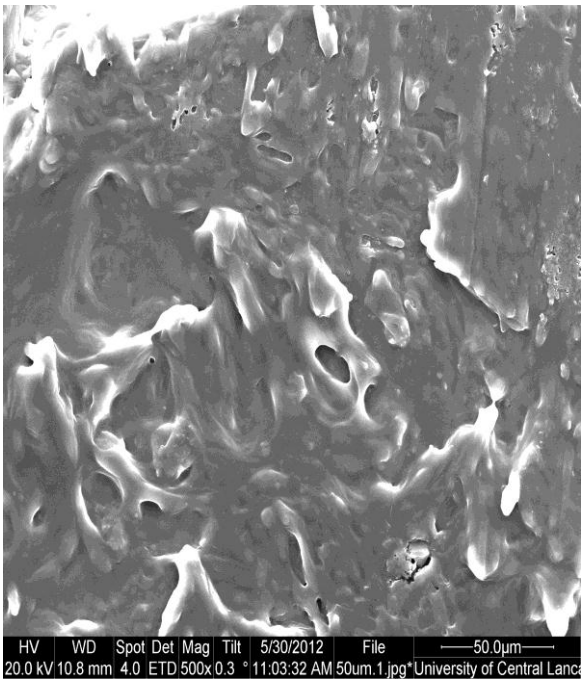
Figure 3.1: SEM images of; (a) coarse LMH prior to loading with lipid phase, (b) LMH loaded with lipid in a 1:5 w/w lipid to carrier ratio, (c) LMH loaded lipid in a 1:10 w/w lipid to carrier ratio, (d) LMH loaded lipid, in a 1:15 w/w lipid to carrier ratio. These images are typical of three such different experiments

(a) *Sucrose particles (particle size 300-500 µm) prior to loading with lipid phase*

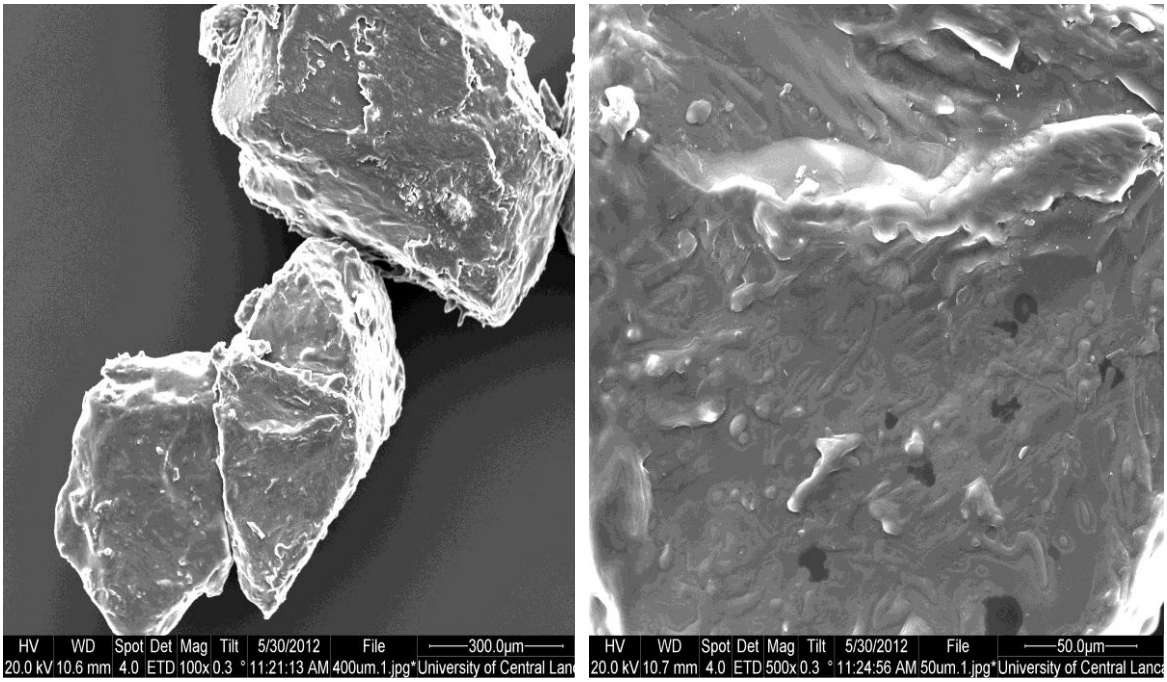


(b) *Proliposomes (1:5 w/w, lipid to carrier), carrier size fraction 300-500 µm*





(c) *Proliposomes (1:10 w/w, lipid to carrier) carrier size fraction 300-500 µm*



(d) *Proliposomes (1:15 w/w, lipid to carrier) carrier size fraction 300-500 µm*

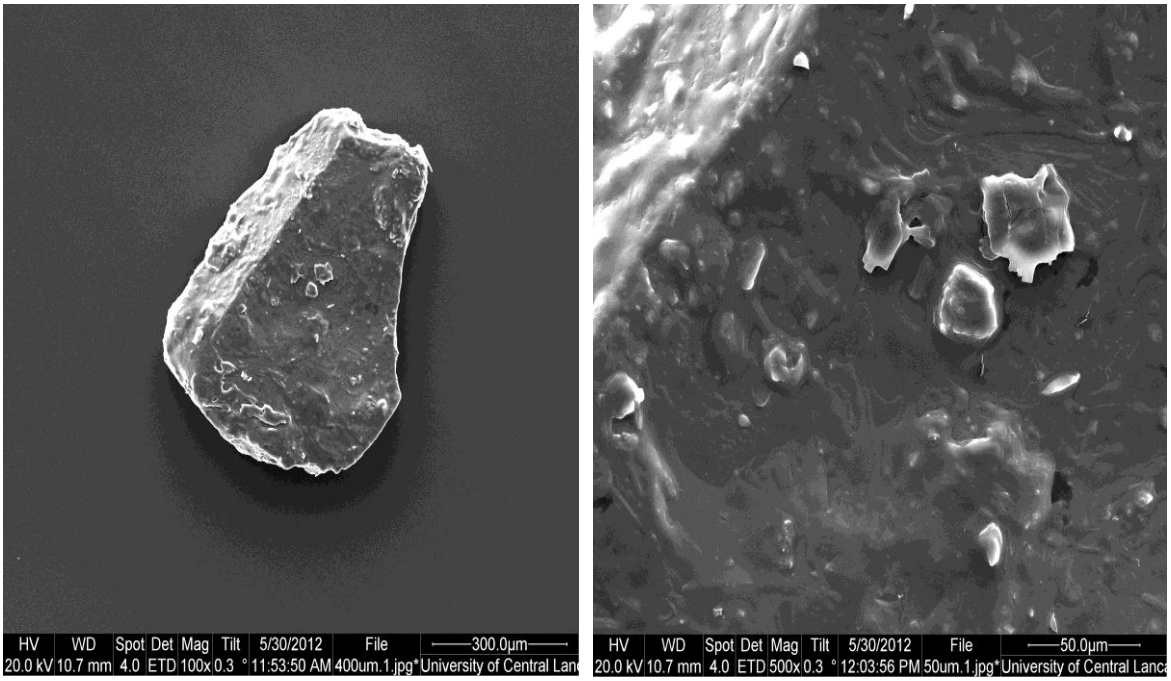
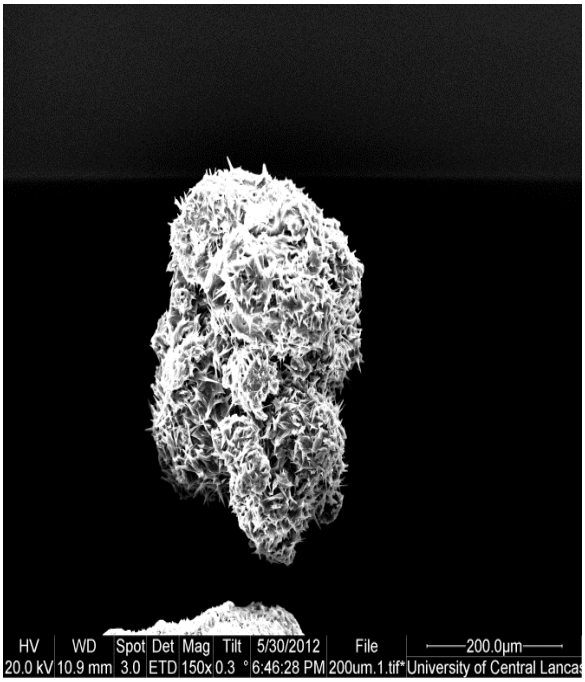


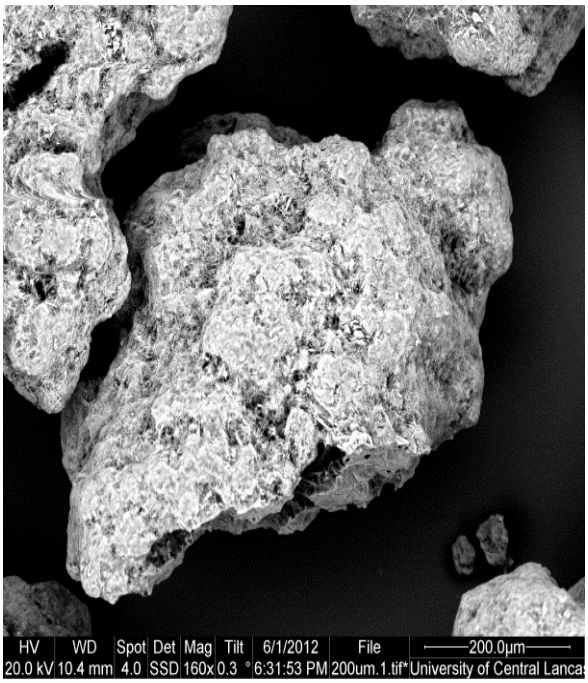
Figure 3.2: SEM images of; (a) coarse sucrose prior to loading with lipid, (b) sucrose loaded with lipid in a 1:5 w/w lipid to carrier ratio, (c) sucrose loaded lipid in a 1:10 w/w lipid to carrier ratio, (d) sucrose loaded lipid in a 1:15 w/w lipid to carrier ratio. These images are typical of three such different experiments

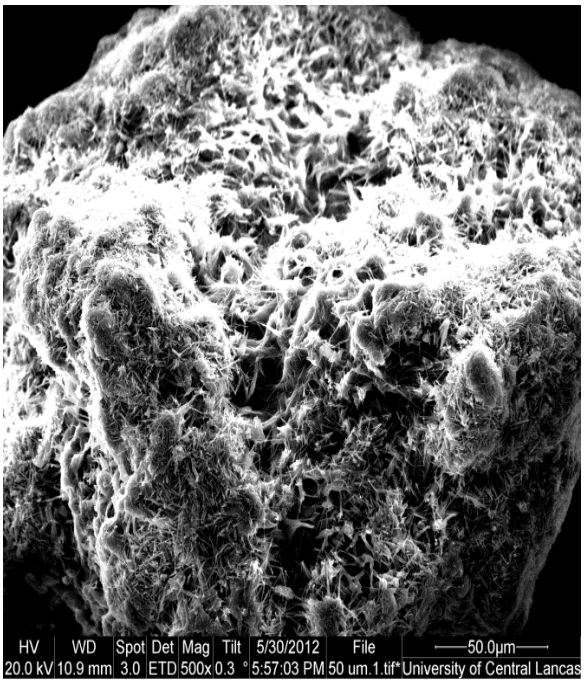
Upon examination of the surface morphology of sorbitol, particles were found to be irregular to spherical in shape, exhibiting a porous surface (Figure 3.3 a). Under higher magnification, it was noted that sorbitol particles possessed multiple micro-needles, protruding from the surface. These structures were believed to be responsible for the accommodation of the lipid phase upon the outer surface, and into the void spaces of the particles; negating agglomeration (Payne et al., 1986b, Newman et al., 1999, Elhissi et al., 2011). EPC when utilised in combination with sorbitol in a 1:10 w/w ratio, showed that even with a lipid coating on the surface of the particles, the resultant powder retained good flowability (Meyer, 2010). The ability of sorbitol to preserve its porous structure increases its desirability as a potential carrier in proliposomes, unlike other carriers which have a less porous surface (Meyer, 2010). At lower lipid concentrations (i.e. 1:10 and 1:15 w/w ratio), sorbitol's surface remained prominently porous. However at higher concentrations of lipid (i.e. 1:5 w/w), upon observation, sorbitol's porosity is less notable; this is in agreement with the findings described by Elhissi and co-workers (2011) (Elhissi et al., 2011) (Figure 3.3).

(a) *Sorbitol particles (particle size 250-700 µm) prior to loading with lipid phase*

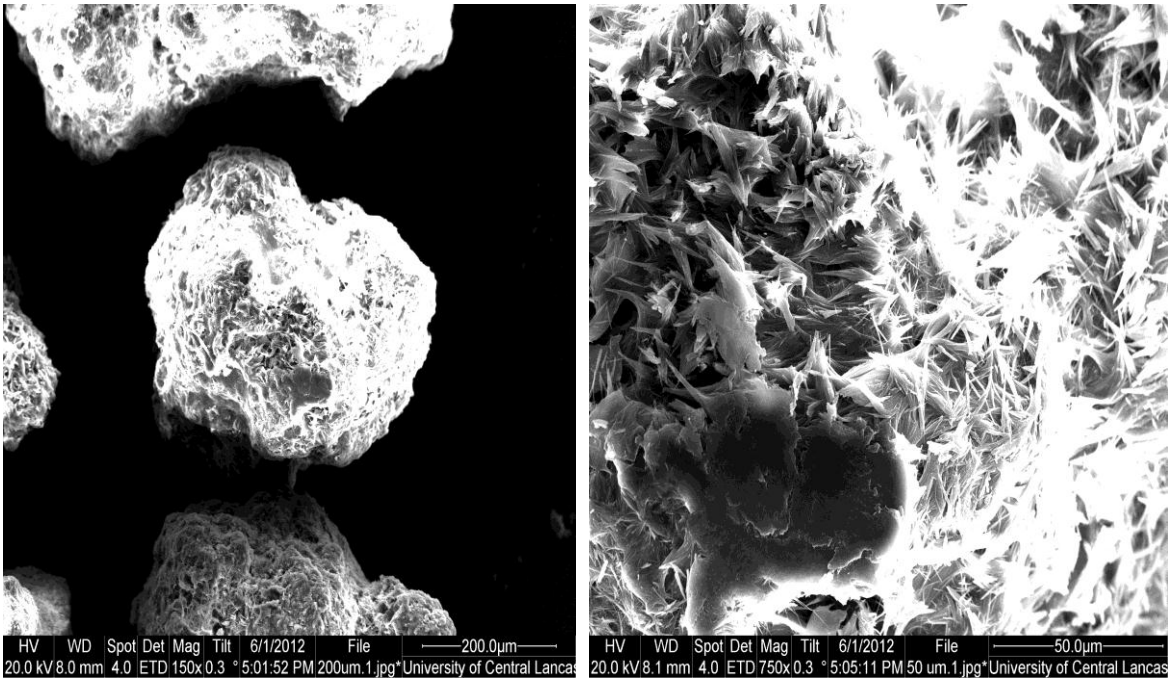


(b) *Proliposomes (1:5 w/w, lipid to carrier) carrier size fraction 250-700 µm*





(c) *Proliposomes (1:10 w/w, lipid to carrier) carrier size fraction 250-700 µm*



(d) *Proliposomes (1:15 w/w, lipid to carrier) carrier size fraction 250-700 µm*

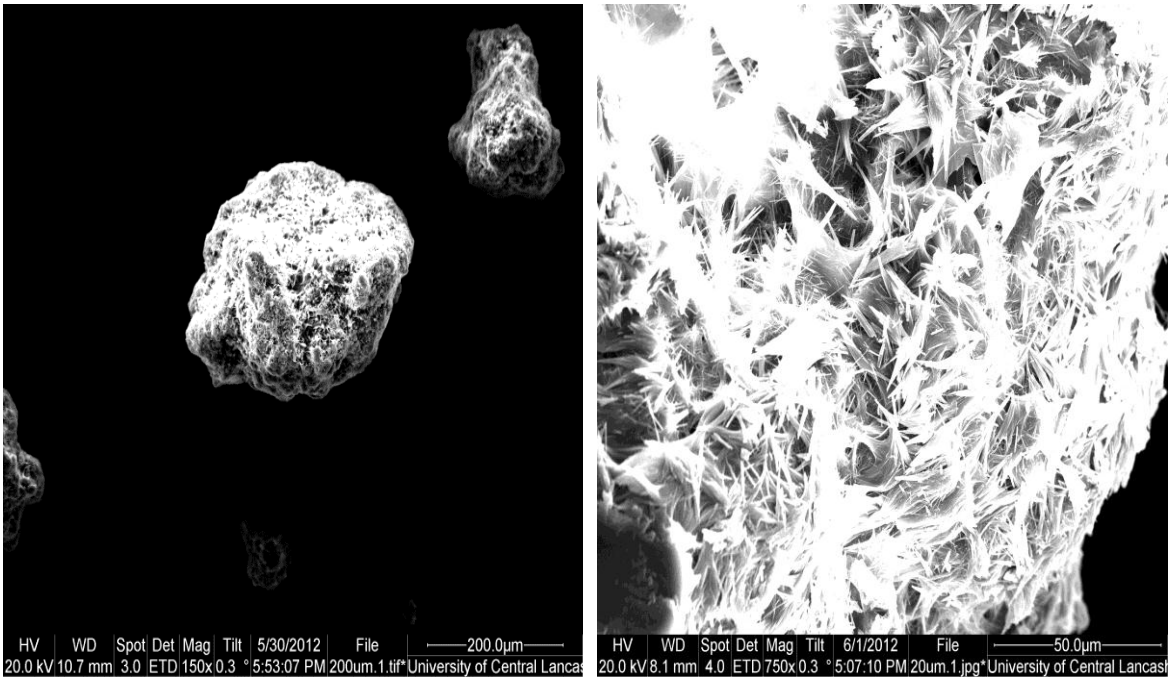
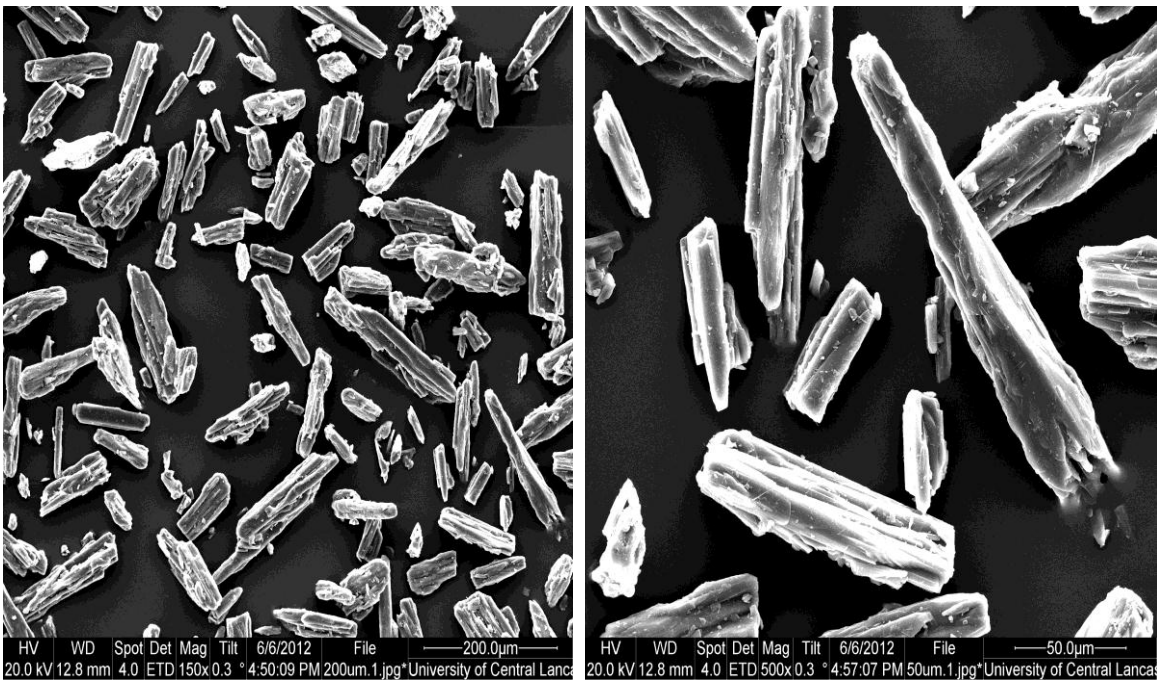


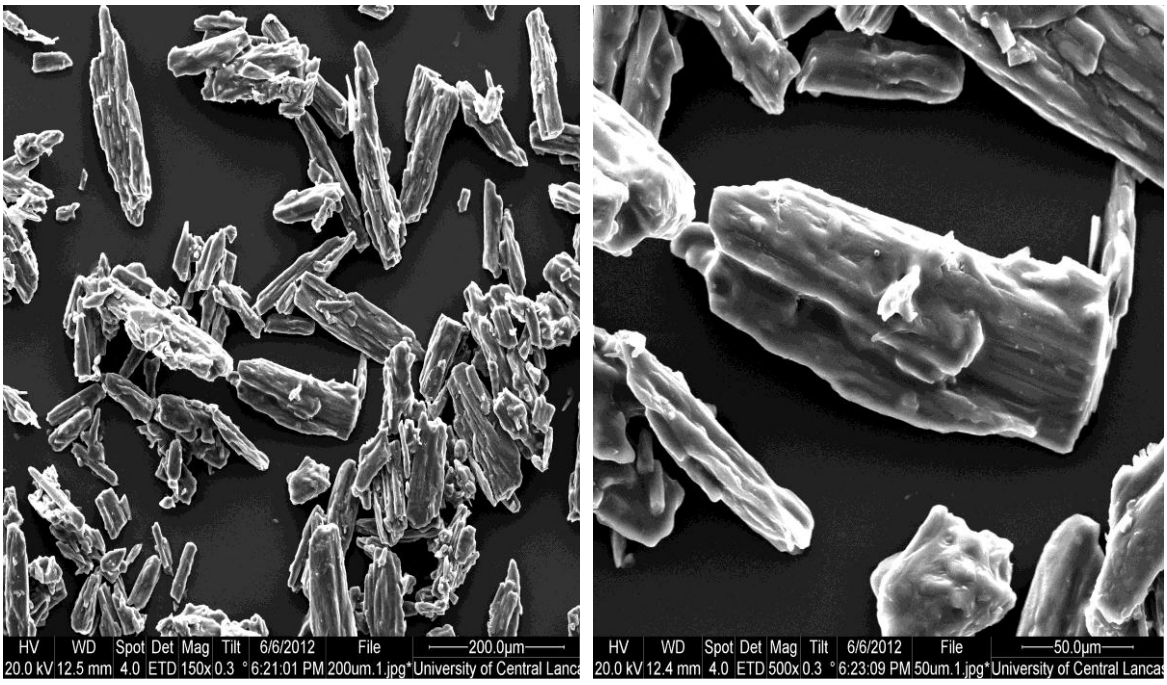
Figure 3.3: SEM images of (a) coarse sorbitol prior to loading with lipid phase, (b) sorbitol loaded with lipid in a 1:5 w/w lipid to carrier ratio, (c) sorbitol loaded lipid in a 1:10 w/w lipid to carrier ratio, (d) sorbitol loaded lipid in a 1:15 w/w lipid to carrier ratio. These images are typical of three such different experiments

D-mannitol coarse particles are free flowing and oblong ellipse in shape, with engraved linings on the surface (Figure 3.4 a) (Row et al., 2006). Being low in moisture content and non-hygroscopic, D-mannitol demonstrates good compatibility with both organic and inorganic excipients (Westermarck et al., 1998). Partial coating (of low lipid density) of the carrier with lipid phase was noted for proliposome formulations in a 1:15 w/w ratio (Figure 3.4 d), yet complete for 1:10 w/w formulation ratio (Figure 3.4 c). Comparatively, dense lipid layers were observed for the 1:5 w/w formulation ratio (Figure 3.4 b). Structurally different to alternate carriers (i.e. sorbitol), D-mannitol's engraved surface is known to accumulate lipid content upon coating in its various crevices. It is also known to exhibit poor agglomeration or cluster formation, even when formulated in a 1:5 w/w ratio; due to its small particle size (<250 μm).

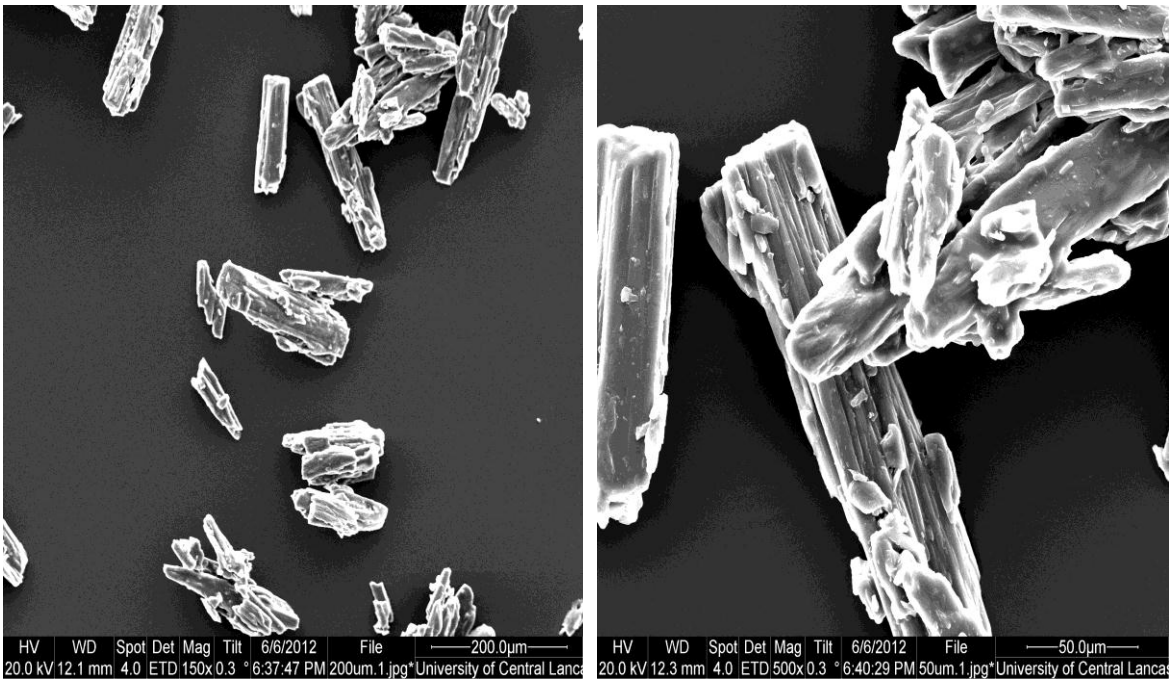
(a) *D-mannitol particles (particle size <250 µm) prior to loading with lipid phase*



(b) *Proliposomes (1:5 w/w, lipid to carrier) carrier size fraction less than 250 µm*



(c) *Proliposomes (1:10 w/w, lipid to carrier) carrier size fraction less than 250 µm*



(d) *Proliposomes (1:15 w/w, lipid to carrier) carrier size fraction less than 250 µm*

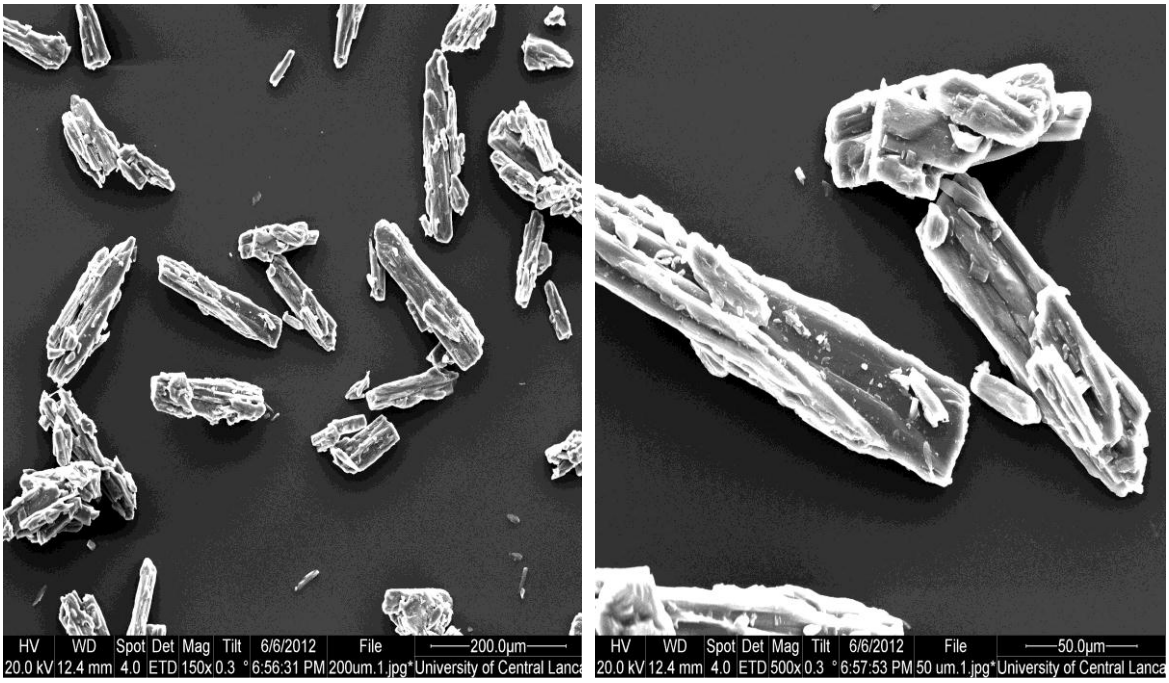


Figure 3.4: SEM images of (a**) coarse D-mannitol prior to loading with lipid phase, (**b**) D-mannitol loaded with lipid phase in a 1:5 w/w lipid to carrier ratio, (**c**) D-mannitol loaded lipid phase in a 1:10 w/w lipid to carrier ratio, (**d**) D-mannitol loaded lipid phase in a 1:15 w/w lipid to carrier ratio. These images are typical of three such different experiments**

3.3.2. Stewart assay for lipid quantification

In order to ascertain the lipid concentration for each carrier in the three respective formulation ratios (1:5, 1:10 or 1:15 w/w lipid to carrier ratio), Stewart assay (Section 2.2.9) was performed. It was concluded that proliposomes with a 1:5 w/w lipid to carrier ratio contained more lipid (as was expected), followed by the lipid quantity in the 1:10 w/w ratio; and finally the least amount in the 1:15 w/w lipid to carrier ratio. It is indicated by Table 3.1, that practical assessment is in agreement with the theoretical loading, showing that as the amount of carrier increased, lipid concentration decreased. This trend in decreasing lipid concentration is denoted by the following order:

1:5 > 1:10 > 1:15

In addition to ratio dependency, it was observed that proliposomes utilising sucrose, held a larger amount of lipid for all three formulations, followed successively by LMH, D-mannitol and sorbitol (Table 3.1). The amount of lipid quantified was found to be carrier dependant, showing decreasing lipid concentration in the following order:

Sucrose > LMH > D-mannitol > Sorbitol

Table 3.1: Stewart assay demonstrating the amount of lipid phase coated on carbohydrate carriers, including: LMH, sucrose, sorbitol or D-mannitol, with all three formulations ratios i.e. 1:5, 1:10 and 1:15 w/w lipid to carrier ratio. Data are mean ± SD, n=3; p<0.05

Carrier-based proliposome formulations	Percentage lipid concentration (%)		
	Lipid to carrier (1:5 w/w)	Lipid to carrier (1:10 w/w)	Lipid to carrier (1:15 w/w)
LMH	7.50 ± 1.42	4.27 ± 0.05	2.42 ± 0.16
Sucrose	18.99 ± 1.71	8.94 ± 1.68	5.93 ± 0.15
Sorbitol	3.04 ± 0.32	1.98 ± 0.12	1.39 ± 0.07
D-mannitol	3.58 ± 0.14	2.18 ± 0.62	1.64 ± 0.28

3.3.3. Size analysis of liposomes

Proliposome formulations based on each of the four carriers (i.e. LMH, sucrose, sorbitol or D-mannitol) were prepared using the "Slurry" method at three formulation ratios (i.e. 1:5, 1:10 and 1:15 w/w lipid to carrier). These preparations were manufactured in triplicate, with or without the incorporation of BDP to the lipid phase (also referred as empty liposomes).

Hydration and annealing of these formulations were conducted in DW in accordance to section 2.2.3, in order to generate liposomes. The size, also referred to as VMD, and the size distribution (SPAN) of liposomes were studied with the inclusion or omission of BDP. Size analysis was conducted using laser diffraction (Malvern Mastersizer 2000).

Results demonstrated that liposomes generated from LMH or D-mannitol-based proliposomes were sized in the range of 4.08 - 4.43 µm and 4.30 - 4.56 µm respectively. Moreover, empty liposomes and BDP containing liposomes, exhibited no statistically significant difference ($p>0.05$) in terms of liposome size (Figure 3.5 and Figure 3.6).

Liposomes prepared from sucrose-based proliposomes exhibited a VMD range of 4.72 - 5.79 µm. Differences in liposome size, between formulations with and without BDP were found to be statistically insignificant ($p>0.05$) for all three ratios investigated (i.e. 1:5, 1:10 and 1:15 w/w lipid to carrier) (Figure 3.5 and Figure 3.6). However, as documented by Elhissi et al., (2006), upon using sucrose as a carbohydrate carrier, liposome size and size distribution were found to decrease (Elhissi et al., 2006a). Though liposome size and size distribution are known to decrease with sucrose, it is noteworthy that liposomes prepared from this carrier are typically larger than those liposomes generated from LMH and D-mannitol-based proliposomes.

Significantly, liposomes produced from sorbitol-based proliposomes were the largest recorded out of the investigated carriers, as observed in Figure 3.5. Moreover, once more, no significant difference ($p>0.05$) in terms of size was found between empty and BDP containing liposomes. Additionally, upon direct comparison of the liposome size (VMD) generated, and size distribution (SPAN) from each carrier-based proliposome (at varied ratios), no statistically significant difference was observed ($p>0.05$) (Figure 3.5 and Figure 3.6).

According to Payne et al., (1986a), liposome size may be controlled by the appropriate selection of carrier type, size and porosity of carrier; and the degree of compatibility of the lipid phase with the carrier (Payne et al., 1986a). Upon observation of Figure 3.5 (a-b), liposomes prepared from LMH and D-mannitol were found to be smaller in size (i.e. statistically insignificant) than

liposomes generated from sucrose and sorbitol-based proliposome (enhancing their deposition in the lower pulmonary region).

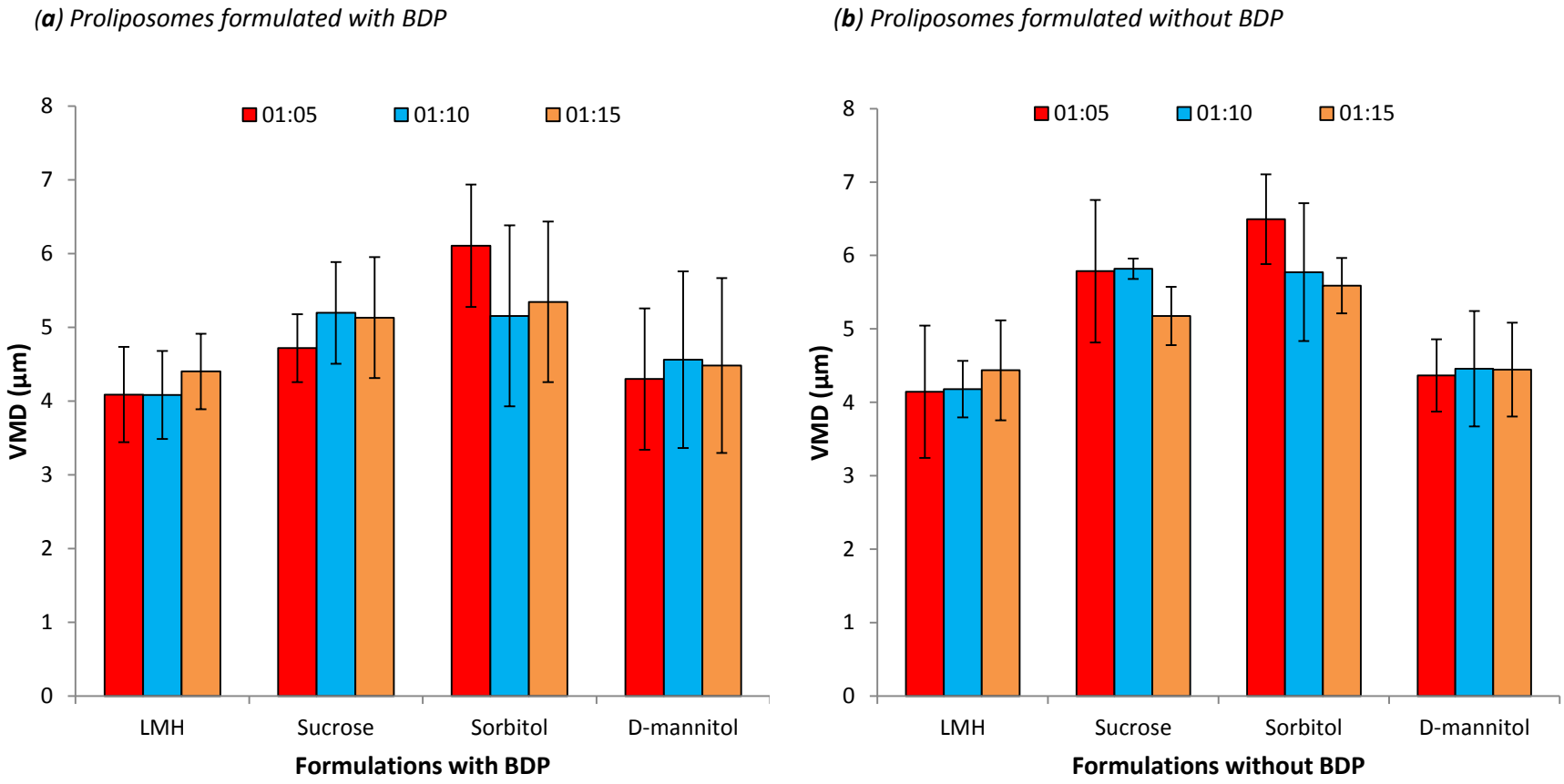


Figure 3.5: Graphical representation of liposome VMD; (a) BDP-entrapped in liposomes, (b) empty liposomes (containing no BDP). Each carbohydrate carrier (LMH, sucrose, sorbitol or D-mannitol) displays the three different lipid to carrier ratios utilised e.g. 1:05, 1:10 and 1:15 w/w. Data are mean ± STD, n=3; $p<0.05$

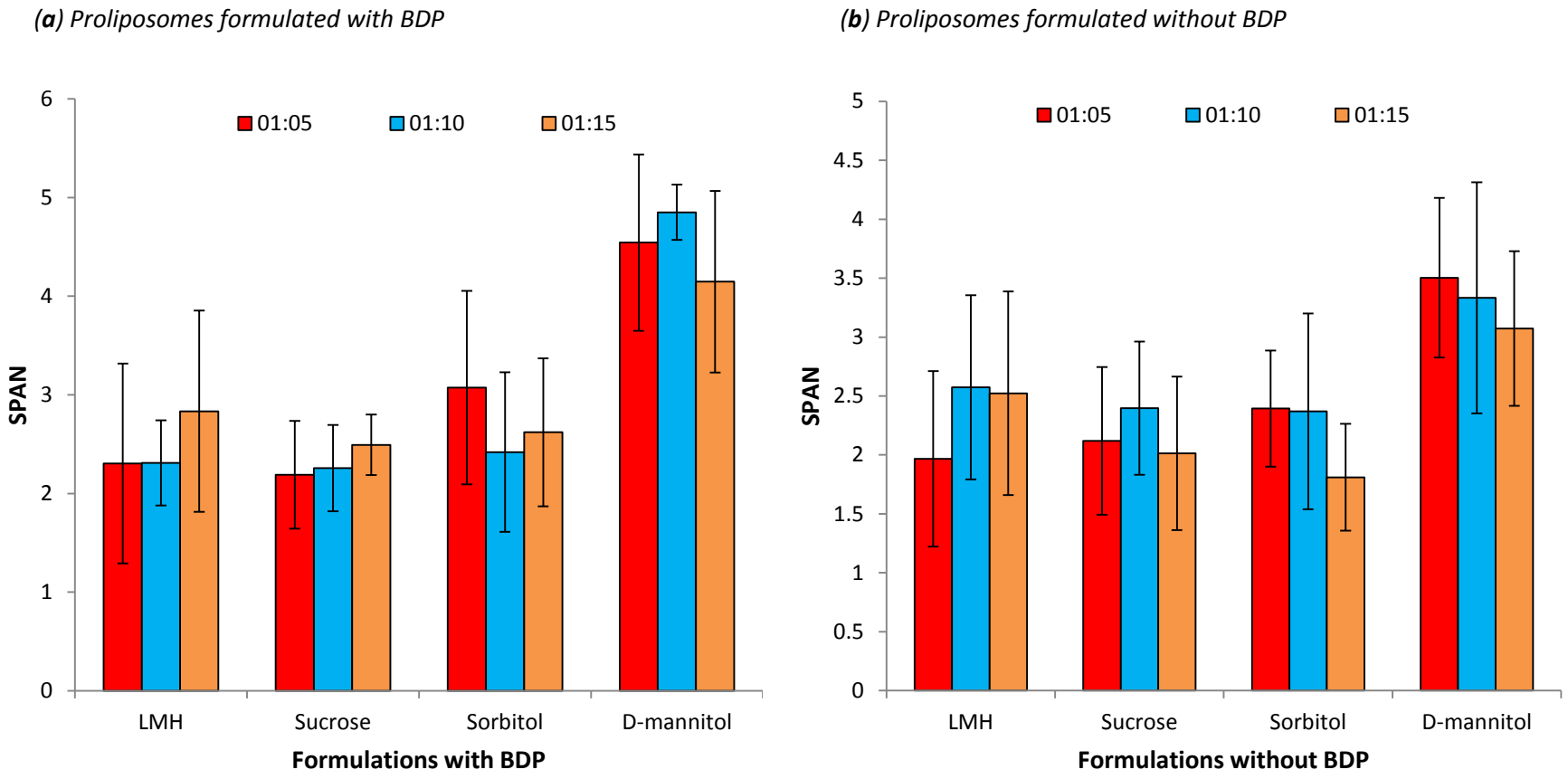


Figure 3.6: Graphical representation of liposome SPAN recorded; (a) BDP-entrapped in liposomes, (b) empty liposomes (containing no BDP). Each carbohydrate carrier (LMH, sucrose, sorbitol or D-mannitol) displays the three different lipid to carrier ratios utilised e.g. 1:05, 1:10 and 1:15 w/w. Data are mean ± STD, n=3; $p<0.05$

3.3.4. Zeta potential analysis of liposomes

Liposome zeta potential was analysed in accordance to section 2.2.8. All formulations investigated were found to be negatively charged. Furthermore, via statistical analysis, no significant difference (*p>0.05*) was detected between the empty and BDP incorporated liposomes; in all carrier-based formulations investigated (i.e. 1:5, 1:10 or 1:15 w/w lipid to carrier ratios) (Table 3.2). As SPC is a natural and neutral phospholipid, with a slight negative charge, this may account for the predisposition of the formulations (which incorporate this as an ingredient) to be negatively charged (Lipoid, 2014).

Table 3.2: Zeta potential of liposomes generated from proliposome formulations utilising LMH, sucrose, sorbitol and D-mannitol as carriers. Each preparation displays different lipid to carrier ratios utilised e.g. 1:5, 1:10 and 1:15 w/w. Data are mean ± STD, n=3; p<0.05

Carrier-based Proliposome formulations	BDP-incorporated formulations			Empty formulations		
	1:5 (w/w)	1:10 (w/w)	1:15 (w/w)	1:5 (w/w)	1:10 (w/w)	1:15 (w/w)
LMH	-1.49 ± 0.44	-1.43 ± 0.38	-1.49 ± 0.48	-1.74 ± 0.49	-2.11 ± 0.67	-1.13 ± 0.14
Sucrose	-1.29 ± 0.32	-2.13 ± 0.49	-2.02 ± 0.80	-1.25 ± 0.37	-1.58 ± 0.15	-1.76 ± 0.26
Sorbitol	-0.79 ± 0.39	-1.31 ± 0.83	-1.38 ± 0.62	-0.99 ± 0.39	-0.85 ± 0.50	-1.01 ± 0.75
D-mannitol	-2.51 ± 1.12	-2.44 ± 1.22	-2.44 ± 0.99	-1.80 ± 0.65	-2.18 ± 0.54	-1.97 ± 0.43

3.3.5. Optimisation of separation speed

Centrifugation speed plays a pivotal role in the separation of BDP-entrapped liposomes from the unentrapped (dissolved or crystalline) BDP. The purpose of this experiment was to optimise the

separation method, as conventional centrifugation methods (bench centrifugation) exhibited a high percentage of BDP-entrapment in liposomes. In order to validate the entrapment, both DW and D_2O were employed separately as dispersion media for proliposomes. Following proliposome hydration and annealing (Section 2.2.3), preparations were subjected to various centrifugation speeds, to aid in separation of the BDP-entrapped drug from the unentrapped BDP. Additionally, Stewart assay was employed in order to quantify lipid present in the sample (Section 2.2.9), in the process of optimising centrifugation speed.

Liposomes generated from proliposomes were dispersed in the media (DW or D_2O), forming suspensions (Figure 3.7 a). In DW, following bench centrifugation, two fractions/parts were noted in the centrifuge tube (Figure 3.7 b). The supernatant fraction was found to contain unentrapped BDP (dissolved or free BDP crystals with some lipid content). The lower fraction/part, was found to contain BDP-entrapped liposomes, sedimented in the form of a pellet at the bottom of the centrifuge tube (Figure 3.7 b) (Meisner et al., 1989, Taylor et al., 1990, Ma et al., 1991). Literature suggested that liposomes and BDP crystals are of similar size (Batavia et al., 2001). Based upon their size similarities, this would indicate that both BDP-entrapped liposomes and free BDP crystals may have sedimented in a similar fashion, following centrifugation in the DW medium.

The optimisation of the centrifugation process (i.e. addressing of liposome sedimentation in DW), was achieved by the use of an alternative solvent, namely D_2O, in order to separate BDP-entrapped liposomes from the unentrapped drug fraction, via the exploitation of the high density of D_2O, compared to DW.

D_2O was employed, as it possesses a higher density (1.053 g/ml at 20°C) (Weast, 1988a) than DW (0.9982 g/ml at 20°C) (Weast, 1988b). From literature, usage of a higher density medium has been identified as a potential tool to facilitate isolation of liposome entrapped drug from the free drug (Batavia et al., 2001, Vecellio et al., 2011).

On using D_2O as a dispersion medium, after centrifugation, three fractions/parts were isolated and identified (Figure 3.7 c). The lowermost (sedimented) part was found to contain free BDP crystals, which were in the form of a spot at the bottom of the centrifuge tube. Upon analysis of the middle fraction/part, this was identified as the unentrapped part (comprised of lipid with negligible detectable quantities of dissolved or free BDP (0.64 - 7.85%)). The uppermost part (supernatant) was noted to be creamy-white in appearance, and through examination was found to contain BDP-entrapped liposomes.

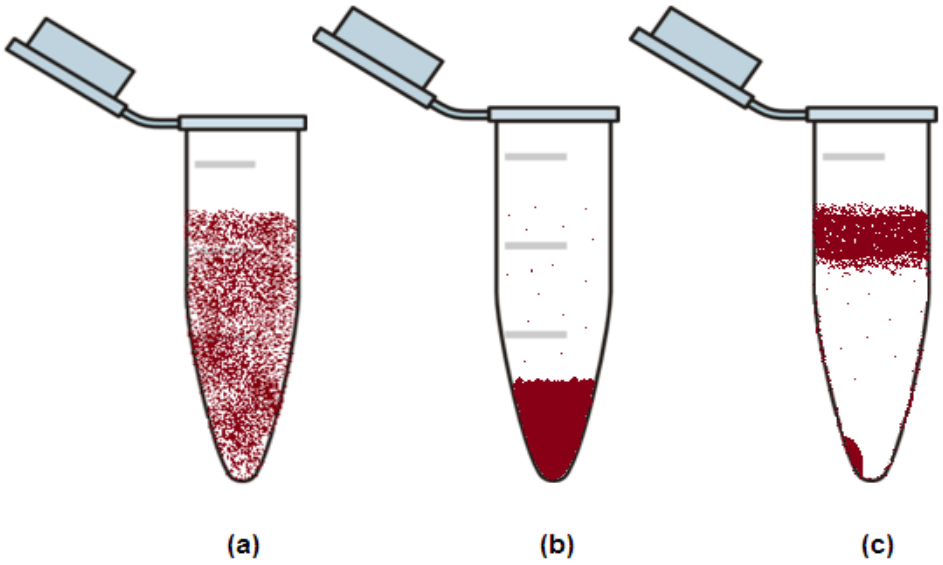


Figure 3.7: Effect of dispersion media upon the separation of BDP-entrapped liposomes from free BDP crystals; (a) normal distribution of liposomes in D_2O or DW prior to centrifugation, (b) dispersion of liposomes in DW post centrifugation, (c) dispersion of liposomes in D_2O post centrifugation

Method development entailed using speeds of 11,000, 12,000 and 13,000 revolutions per minute (RPM), employing a bench centrifuge for both DW and D_2O based suspensions. Triplicate samples of proliposomes (1:5 w/w lipid to carrier ratio) were hydrated and annealed in 5 ml of D_2O (by dispersing 150 mg of proliposome powder in 5 ml, giving the standard 30 mg/ml suspension utilised in this work) according to section 2.2.3. Following annealing, the liposomal suspension was centrifuged for 90 minutes at 11,000 RPM (11,100 g) (Section 2.2.6 and 3.3.6).

As mentioned earlier, three different fractions/parts were formed in the centrifuge tube. The middle fraction/part, identified as the unentrapped part (containing lipid with negligible detectable quantities of dissolved or free BDP) was isolated for Stewart assay, as outlined in section 2.2.5 (lower lipid concentration in the middle fraction/part, would indicate better separation of BDP-entrapped liposomes from free drug). This procedure was conducted for all samples (1:5 w/w lipid to carrier ratio formulations) using 12,000 RPM (13,200 g) and 13,000 RPM (15,500 g). In addition, 1 ml of liposomal suspension (prior to centrifugation, from the already prepared 5 ml in D_2O) was taken for Stewart assay to quantify the total amount of lipid. Total lipid or practical yield was measured to allow for comparison with the free lipid in the middle fraction/part and theoretical yield (Table 3.3). Theoretical yield may be described as the amount of lipid in each ml of liposomal dispersion, according to the weight taken for proliposome preparation i.e. expected yield based on constituents (100%). Practical yield or total lipid is the

actual lipid content per ml identified upon analysis of the liposome suspension. Practical yield was calculated using equation 3.1 based upon the theoretical yield;

$$Total\ or\ practical\ yield\ (\%) = \left(\frac{Practical\ yield}{Theoretical\ yield}\right) \text{X } 100 \qquad \ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 3.1)$$

Additionally, lipid content was calculated (equation 3.2) for the middle fraction/part;

$$Lipid\ content\ (\%) = \left(\frac{Lipid\ content}{Total\ or\ Practical\ yield}\right) \text{X } 100 \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 3.2)$$

Liposomes generated from sucrose-based proliposomes elicited comparatively higher free lipid (in the middle fraction/part), than the other carrier-based formulations investigated. However, in liposome suspensions generated from LMH-based proliposomes, lipid content was reduced significantly from 1.60 $\pm$ 0.31% to 1.56 $\pm$ 0.04%, through increasing of the centrifugation speed from 11,000 RMP to 13,000 RPM. Additionally, a higher practical yield was also noted (94.15 $\pm$ 5.49%) (Table 3.3). Similar trends i.e. decreasing lipid content were noted upon increasing the centrifugation speed (from 11,000 to 13,000 RPM) for both sorbitol and D-mannitol-based proliposomes. Here the lipid content was found to reduce from 4.77 $\pm$ 1.23% and 4.19 $\pm$ 0.49% to 3.35 $\pm$ 1.33% and 2.50 $\pm$ 0.46, respectively. Practical yields (the total lipid concentration in 1 ml of liposome suspension) were also higher for both liposomes generated from LMH and D-mannitol-based proliposomes, as oppose to sucrose or sorbitol-based liposomes (Table 3.3). This indicated that entrapment efficiency may be influenced by the type of carrier selected.

Overall, centrifugation speed was found to be an overriding factor (i.e. 13,000 RPM was found to offer much better separation than 11,000 and 12,000 RPM) in the degree of effective separation of the liposome constituents. Thus, a higher speed of 13,000 RPM in D_2O was implemented, in the separation process of liposome-entrapped drug from unentrapped BDP (dissolved or free BDP as well as lipid content) in this study (Table 3.3).

Table 3.3: Percentage lipid content, in the middle unentrapped fraction/part of the 1:5 w/w lipid to carrier ratio proliposome formulations, centrifuged at various speeds (based on theoretical yield i.e. 100%), with comparison to practical yield (liposome suspension prepared of 30 mg in 1 ml dispersion media) in D_2O, using bench centrifuge for 90 minutes. Data are mean ± STD, n=3; P<0.05

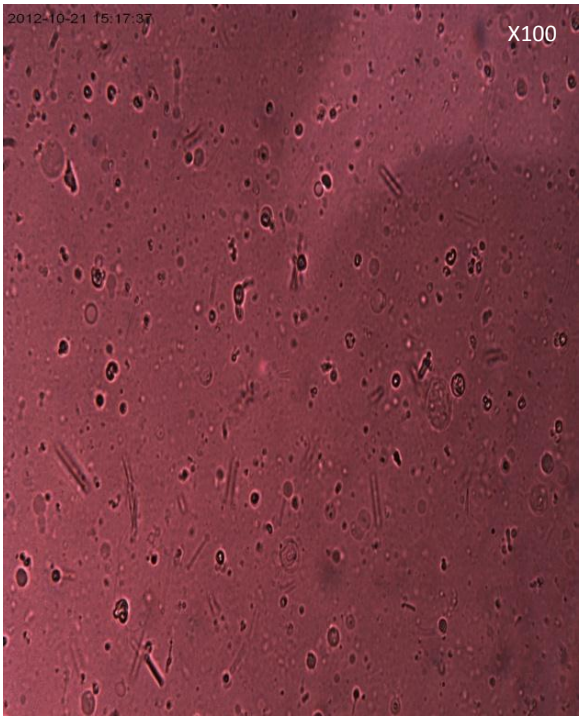
Carrier-based Proliposome formulation	Percentage lipid concentration (%)			
	Practical yield	11,000 RPM	12,000 RPM	13,000 RPM
LMH	94.15 ± 5.49	1.60 ± 0.31	1.56 ± 0.04	1.13 ± 0.24
Sucrose	84.21 ± 1.76	9.96 ± 1.27	7.72 ± 1.81	7.08 ± 2.02
Sorbitol	66.97 ± 5.06	4.77 ± 1.23	4.03 ± 1.50	3.35 ± 1.33
D-mannitol	89.50 ± 3.34	4.19 ± 0.49	3.08 ± 1.27	2.50 ± 0.46

3.3.6. Optimisation of separation time

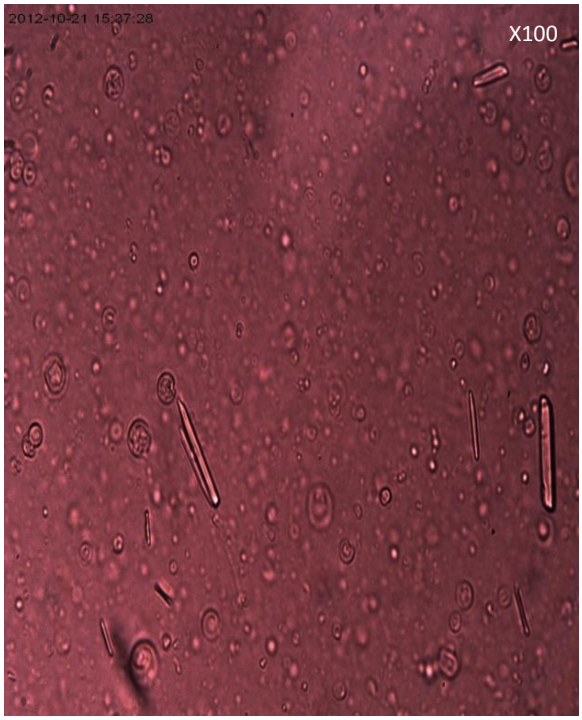
Proliposome powders formulated from LMH, sucrose, sorbitol or D-mannitol (1:5 w/w lipids to carrier ratios) were hydrated and annealed (Section 2.2.3) in D_2O or DW (forming a standard ratio of 30 mg dispersed in 1 ml aqueous medium). As separation speed (i.e. 13,000 RPM) was optimised for the separation of BDP-entrapped liposomes from the unentrapped BDP, separation time was also investigated and optimised.

Initially, liposomes were generated from proliposome powders (utilising carbohydrate carriers including LMH, sucrose, sorbitol or D-mannitol) in DW or D_2O. One to two drops of each liposomal suspension was then pipetted onto a glass slide before analysis with light microscopy; the remaining suspensions were centrifuged. Slides were adjusted under a light microscope at a multitude of magnifications. It was found that the "normal" liposomal suspension (in DW or D_2O) (i.e. prior to centrifugation) contained both liposomes and free BDP crystals (Figure 3.8).

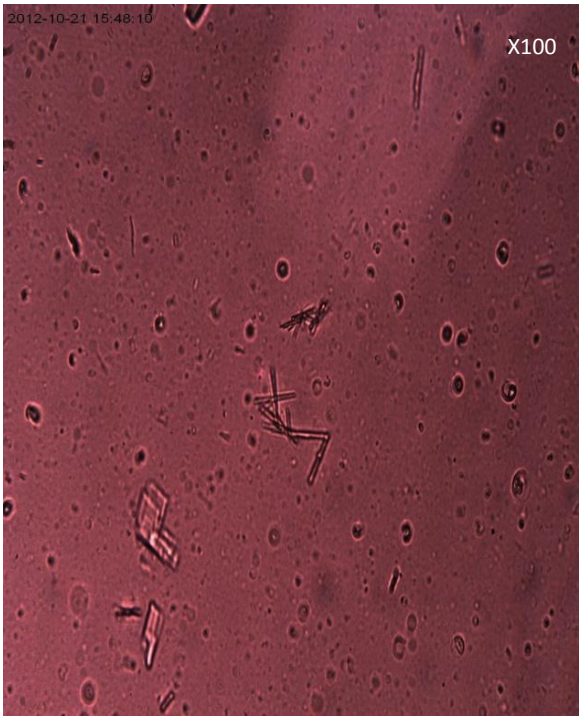
(***a***) *LMH (1:5 w/w, lipid to carrier ratio)*



(***b***) *Sucrose (1:5 w/w, lipid to carrier ratio)*



(***c***) *Sorbitol (1:5 w/w, lipid to carrier ratio)*



(***d***) *D-mannitol (1:5 w/w, lipid to carrier ratio)*

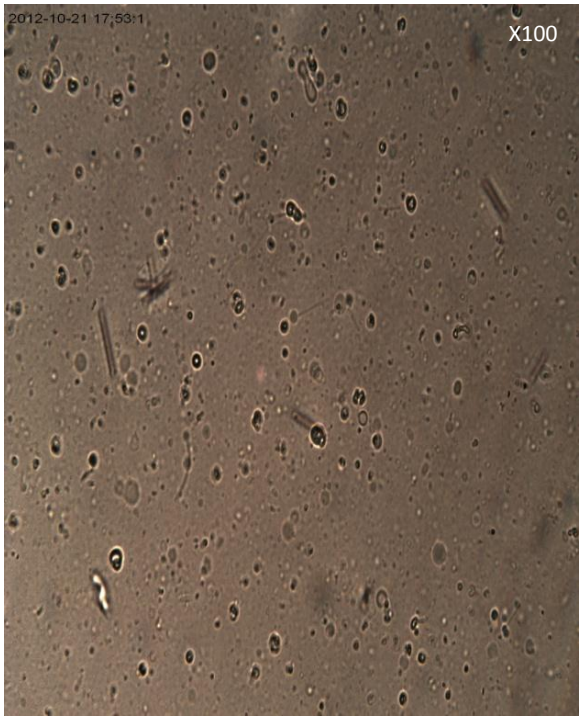
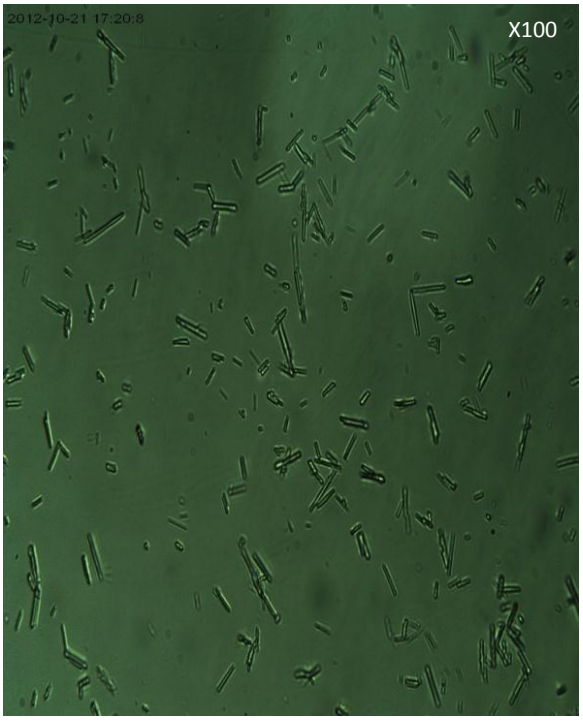


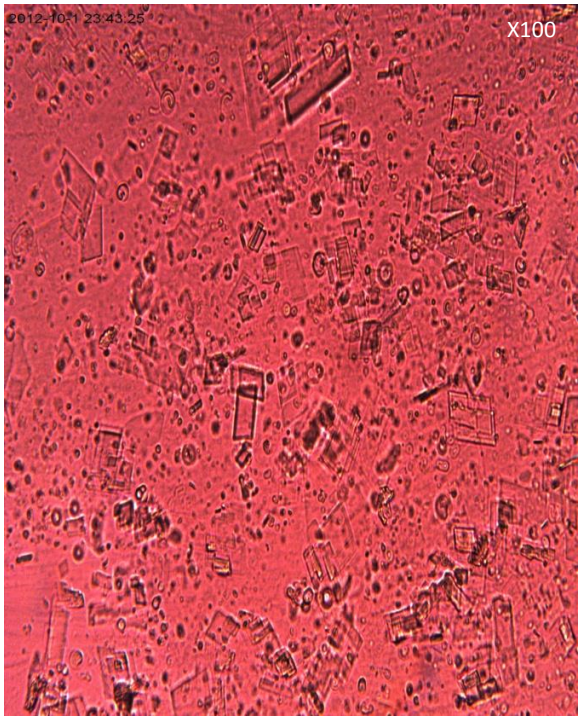
Figure 3.8: Light microscopy photographs; magnification X100 showing liposomes using alternate carriers after annealing in DW or D_2O; (a**) LMH in a 1:5 w/w lipid to carrier ratio, (**b**) sucrose in a 1:5 w/w lipid to carrier ratio, (**c**) sorbitol in a 1:5 w/w lipid to carrier ratio, (**d**) D-mannitol in a 1:5 w/w lipid to carrier ratio. These photographs are typical of three such different experiments**

Separation speed was optimised in section 3.3.5 (13,000 RPM or 15,500 g) for the separation of BDP-entrapped liposomes from the unentrapped (comprised of lipid and dissolved or free BDP). Here, a separation time of 60 minutes was employed for centrifugation of the liposomal suspension in D_2O. Yet again, the three fractions/parts were identified in the centrifuge tube and isolated. A creamy-white layer formed at the top, and a spot at the bottom of the tube containing free BDP crystals were collected, and carefully examined under a light microscope (Figure 3.9). This procedure was performed for all liposomal suspensions generated from the carriers-based proliposomes investigated; in a formulation ratio of 1:5 w/w lipid to carrier. Both the top layer and the bottom spot images were captured under different magnifications (Figure 3.9). It was observed that the upper creamy-white layer contained the majority of the liposomes, and a lower quantity of free BDP crystals for all formulations (Figure 3.9 b, d, f and h). However, the spot was noted to contain the majority of the free BDP crystals (Figure 3.9 a, c, e and g). According to Batavia et al., (2001), high amounts of BDP incorporation into liposome preparations may cause crystallization of BDP (Batavia et al., 2001), which was also found in the present investigation.

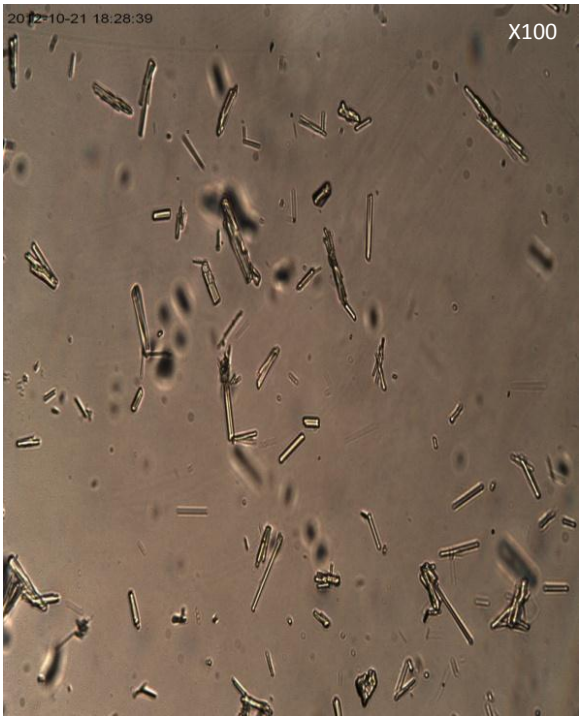
(***a***) *LMH (1:5 w/w, lipid to carrier ratio)*



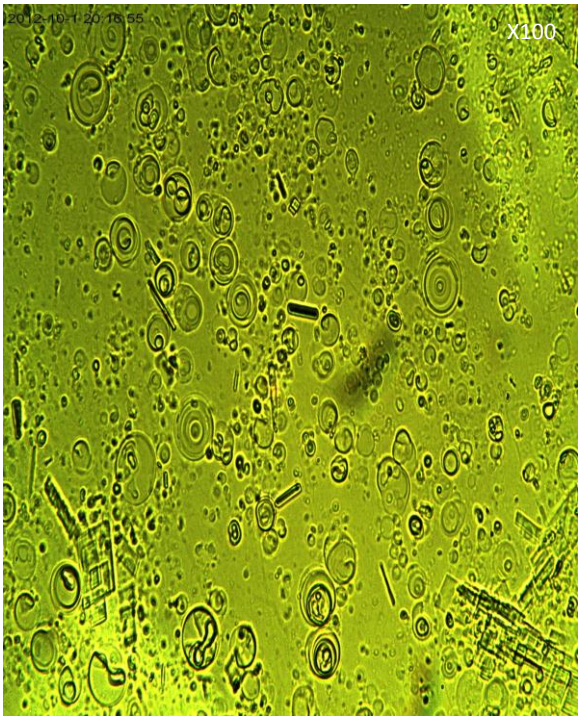
(***b***) *LMH (1:5 w/w, lipid to carrier ratio)*



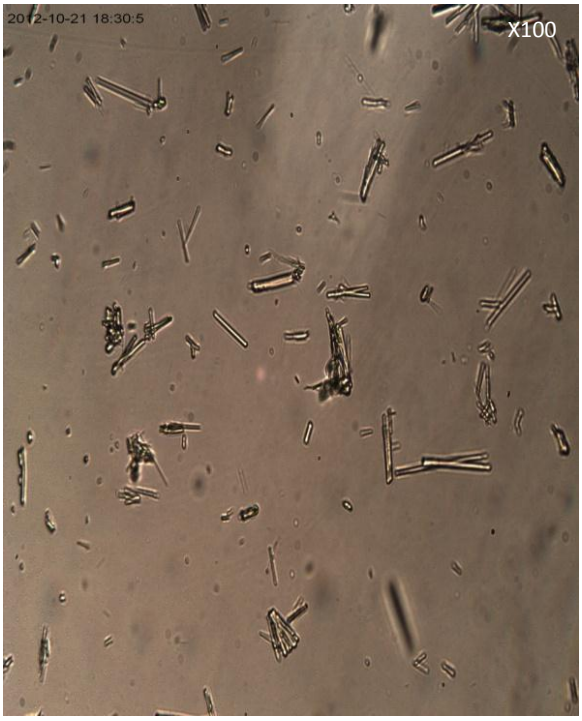
(***c***) *Sucrose (1:5 w/w, lipid to carrier ratio)*



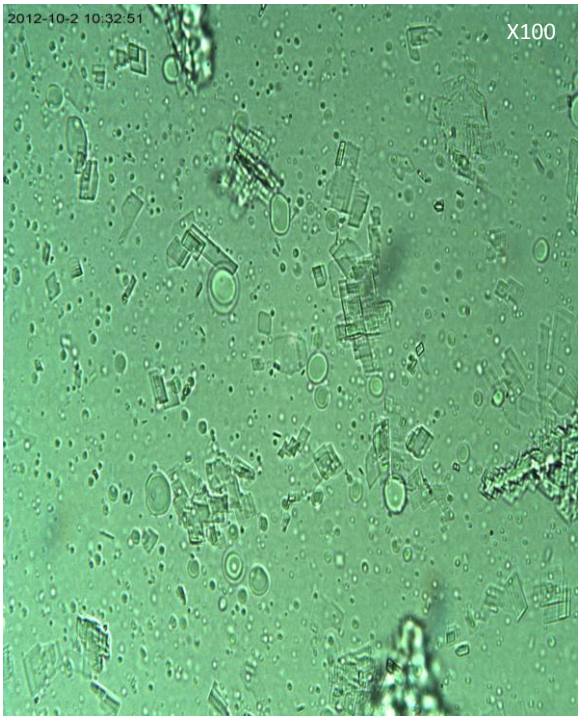
(***d***) *Sucrose (1:5 w/w, lipid to carrier ratio)*



*(**e**) Sorbitol (1:5 w/w, lipid to carrier ratio)*



*(**f**) Sorbitol (1:5 w/w, lipid to carrier ratio)*



*(**g**) D-mannitol (1:5 w/w, lipid to carrier ratio)*

*(**h**) D-mannitol (1:5 w/w, lipid to carrier ratio)*

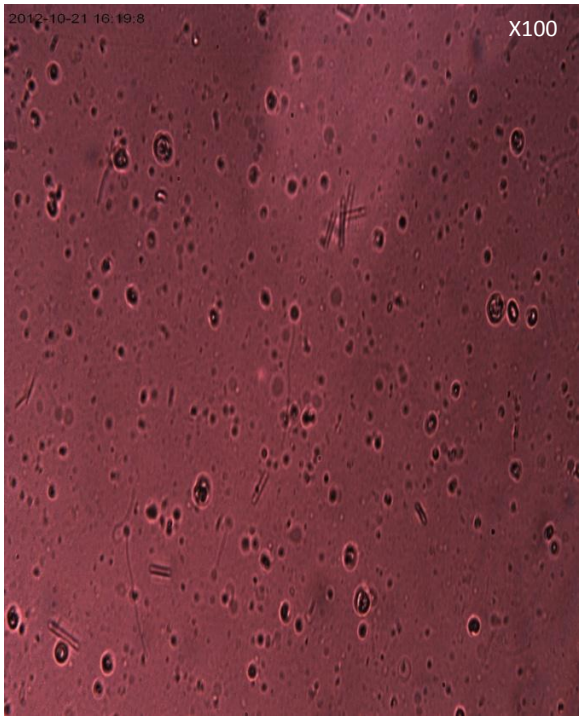
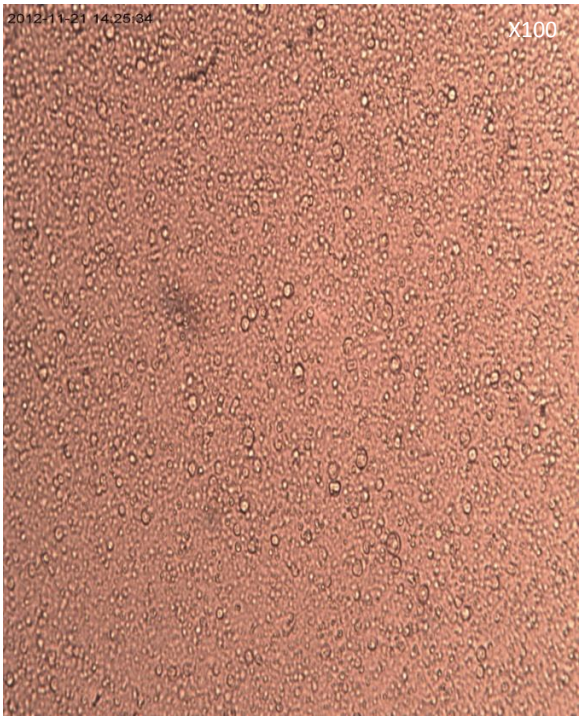


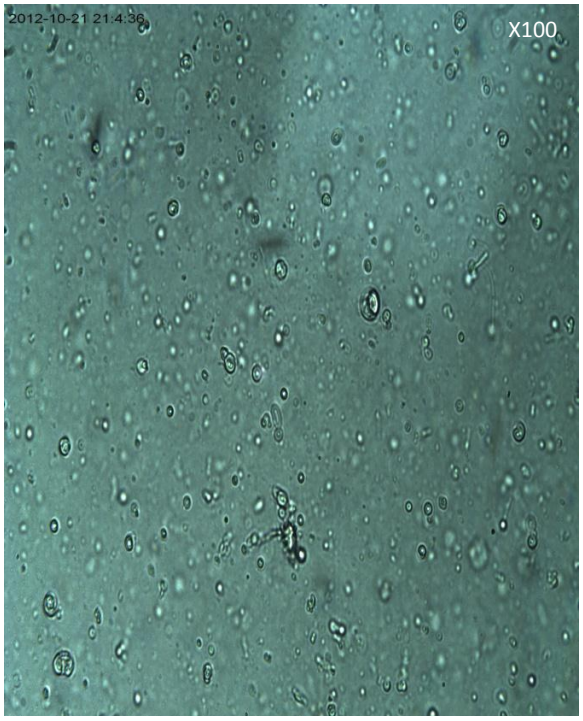
Figure 3.9: Light microscopy photographs; magnification X100 showing sedimented spot (at the bottom of the centrifuge tube) containing fewer liposomes and more BDP crystals (in various formulations of carriers in D_2O) post centrifugation (60 min at 13,000 RPM); (a) **LMH,** (c) **sucrose,** (e) **sorbitol,** (g) **D-mannitol. Supernatant containing more liposomes, and comparatively less BDP crystals post centrifugation** (b) **LMH** (d) **sucrose** (f) **sorbitol** (h) **D-mannitol. These photographs are typical of three such different experiments**

Light microscopy was employed for observation of the creamy-white liposomal layer (BDP-entrapped liposomes) for all samples (i.e. LMH, sucrose, sorbitol or D-mannitol proliposome powders with 1:5 w/w lipid to carrier ratio) after 90 minutes, using bench centrifugation at 13,000 RPM (15,500 g) in D_2O. From examination of Figure 3.10, the absence of BDP crystals in the upper fraction/part (supernatant creamy-white) is evident. Contrastingly, the majority of the free BDP crystals were accumulated at the bottom of the centrifuge tube (Figure 3.11). It is therefore suggested that 90 minutes of bench centrifugation is an appropriate duration to separate BDP-entrapped liposomes from the free BDP crystals in D_2O; as 60 minute duration was insufficient to offer complete separation.

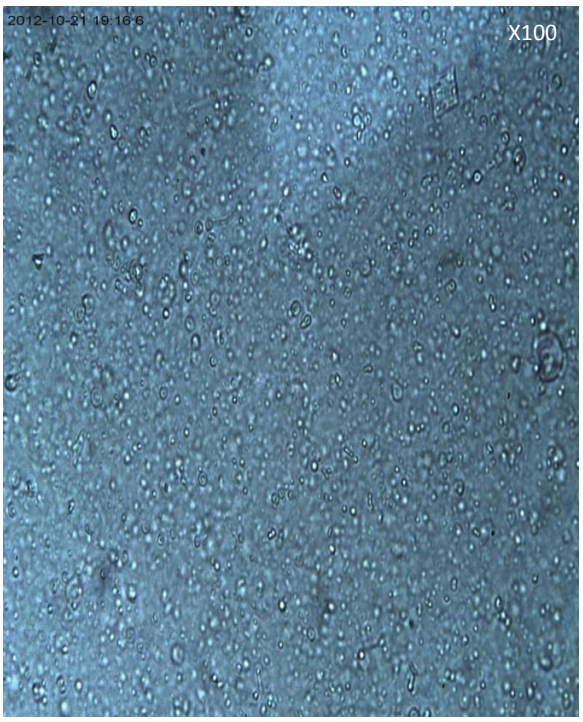
*(**a**) LMH (1:5 w/w, lipid to carrier ratio)*



*(**b**) Sucrose (1:5 w/w, lipid to carrier ratio)*



*(**c**) Sorbitol (1:5 w/w, lipid to carrier ratio)*



*(**d**) D-mannitol (1:5 w/w, lipid to carrier ratio)*

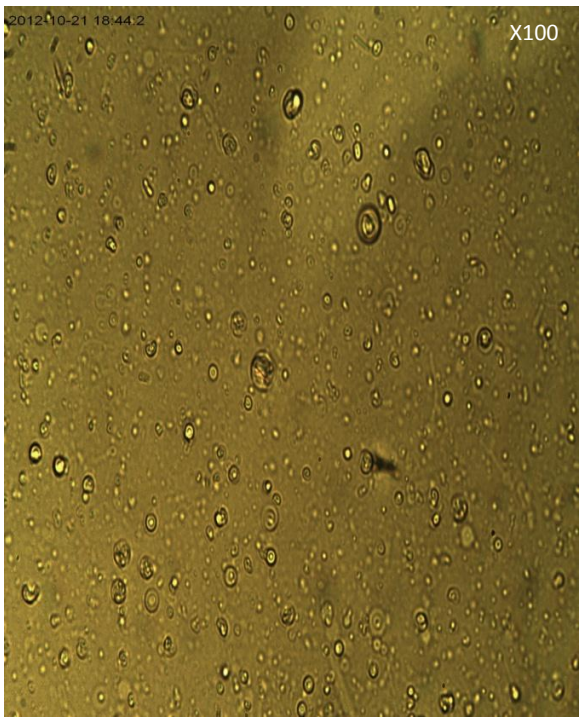


Figure 3.10: Light microscopy photographs; magnification X100 showing liposomes in the creamy-white layer (supernatant) of the centrifuge tube (liposome generated from LMH, sucrose, sorbitol or D-mannitol-based proliposomes in a 1:5 w/w lipid to carrier ratio in D_2O) following centrifugation for 90 minutes at 13,000 RPM; (a) LMH, (b) sucrose, (c) sorbitol, (d) D-mannitol. These photographs are typical of three such different experiments

*(**a**) BDP crystals under light microscope (Magnification x40),(**b**) BDP crystals under light microscope (Magnification x400)*

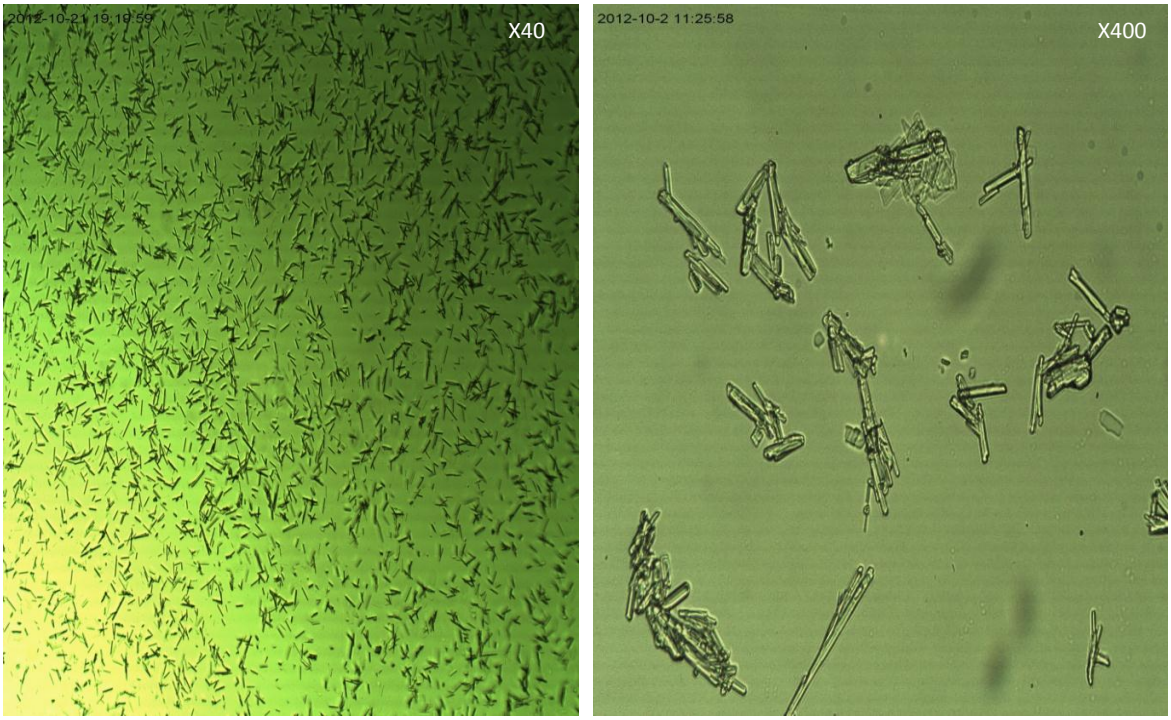


Figure 3.11: Light microscopy photographs; magnification X40 and X400 showing free BDP crystals sedimented (as spot) at the bottom of the centrifuge tube (when liposome generated from LMH, sucrose, sorbitol or D-mannitol-based proliposomes in a 1:5 w/w lipid to carrier ratio in D_2O) following centrifugation for 90 minutes at 13,000 RPM. These images are typical of three such different experiments

It was concluded from both light microscopy and Stewart assay (Section 2.2.9 and 3.3.5) that centrifugation at 13,000 RPM for 90 minutes were the most suitable parameters for separation of the BDP-entrapped liposomes (upper fraction) from the unentrapped BDP (middle fraction and spot containing crystals). The aforementioned separation speed and time was effectively used for all liposomes generated from LMH, sucrose, sorbitol or D-mannitol-based proliposome formulations in D_2O. BDP crystals as a spot at the bottom of centrifuge tube at varied magnifications were shown under light microscopy (Figure 3.11). HPLC was used to analyse the various fractions/parts separated post centrifugation.

3.3.7. Spot analysis via High performance liquid chromatography

To confirm the presence or absence of BDP in the isolated spot (as well as for quantification purposes), samples were subjected to HPLC and NMR analysis. For HPLC analysis, the spot (sedimented BDP crystals) was recovered from the centrifugation tube (following centrifugation in

D_2O), and dissolved in methanol. Spot composition analysis via HPLC was conducted to compare the retention time of the generated peaks with known standards of BDP. The peak retention time of the isolated spot was identified at 3.765 ± 0.4 minutes (Figure 3.12 b), which was almost identical to the standard peak generated for pure BDP (i.e. 3.711 ± 0.4 minutes) (Figure 3.12 a). Thus, HPLC as an analytical technique was used to identify and quantify the spot of BDP crystal (as indicated by light microscopy in Figure 3.11).

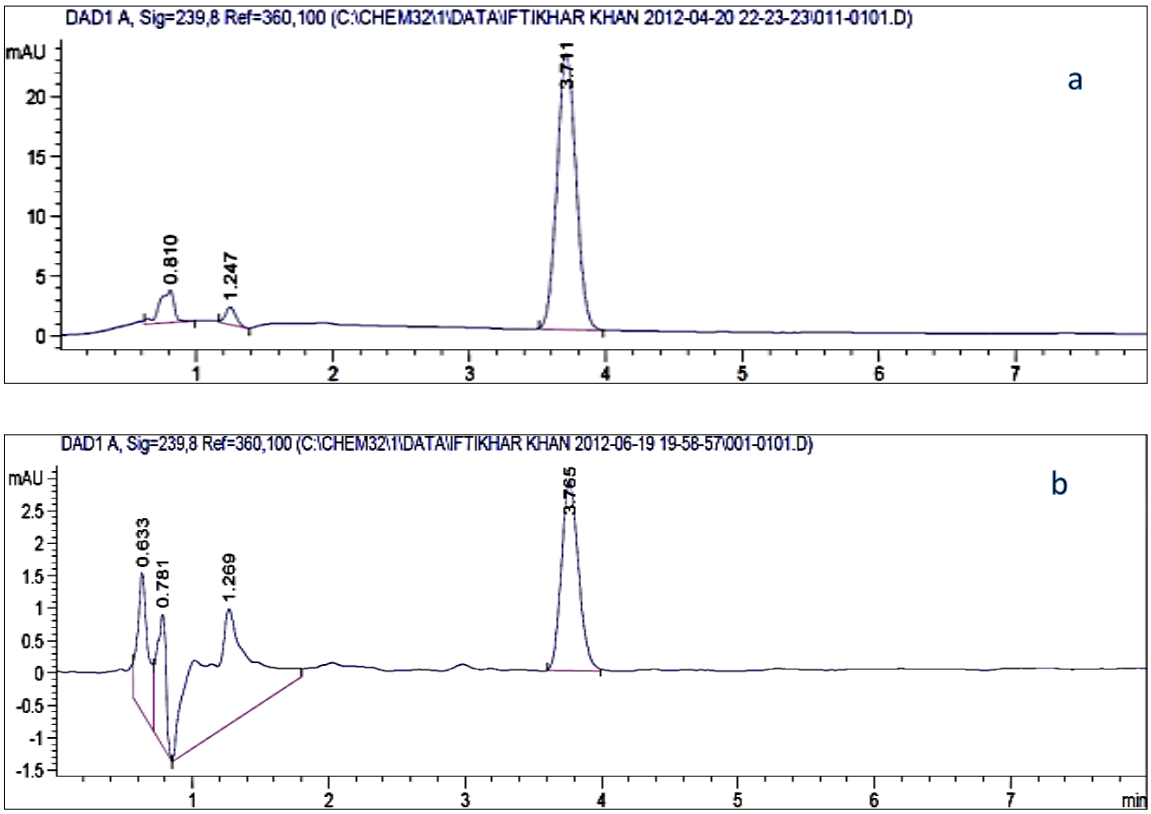


Figure 3.12: HPLC chromatogram, illustrating the peak of the sedimented spot following centrifugation at 13,000 RPM in D_2O. This was in comparison to pure BDP in methanol; (a) **retention time peak of pure BDP during calibration,** (b) **retention time peak of spot. Data are mean of three such different experiments**

3.3.8. Spot analysis via Nuclear Magnetic Resonance

In addition to HPLC, Nuclear Magnetic Resonance (NMR) was employed as a technique to further analyse and substantiate spot composition. Initially, spectra were generated for pure BDP, followed by the isolated spot, to confirm the presence or absence of BDP. Both samples were

analysed individually and compared directly. Both spectra showed common peaks for BDP, further confirming the presence of BDP in the isolated spot (Figure 3.13 a, b).

Therefore, the spot examined by HPLC and NMR chromatograms confirmed the presence of BDP. Thus, D_2O is clearly identified as a superior separation medium for BDP-entrapped liposomes from the unentrapped fraction (free BDP crystals).

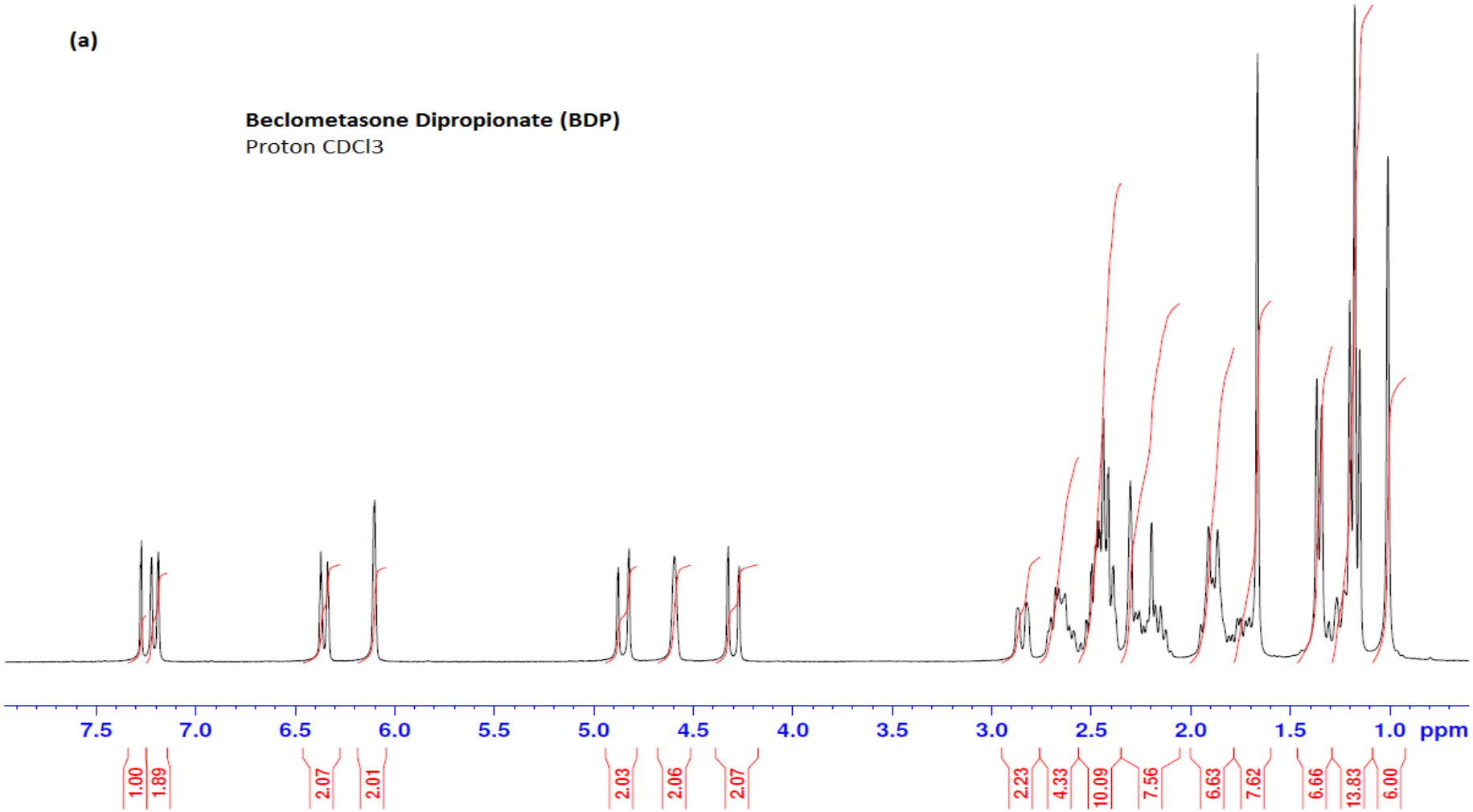


Figure 3.13 a: NMR chromatogram illustrating the molecular structure of pure BDP. Data is typical of three such different experiments

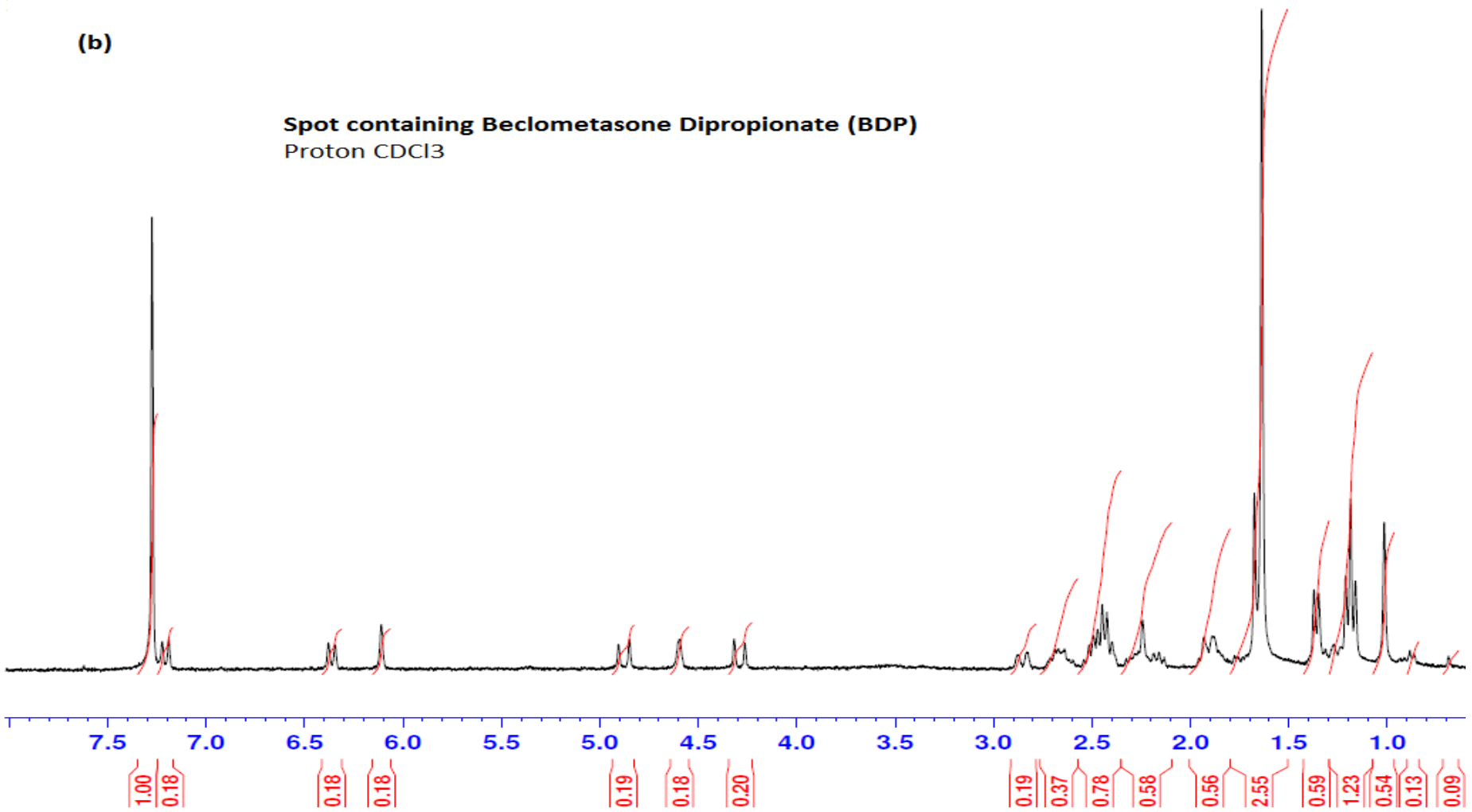


Figure 3.13 b: NMR chromatogram illustrating the molecular structure of spot isolated post centrifugation at the bottom of the centrifuge tube. Data is typical of three such different experiments

3.3.9. X-Ray diffraction

X-ray diffraction (XRD) is a technique which is used to investigate the crystallinity of a given sample (Sharma et al., 2012). In this research initially, coarse carbohydrate carriers i.e. LMH, sucrose, sorbitol, D-mannitol and BDP alone were analysed individually; all were identified as being crystalline in nature (Figure 3.14, Figure 3.15, Figure 3.16 and Figure 3.17). Having investigated coarse carbohydrate carriers, samples of coarse carrier mixed with absolute ethanol in a rotary evaporator (as in proliposome manufacture section 2.2.2 without lipid or drug) were investigated to establish the influence of ethanol on the crystallinity of the carrier. Samples treated with ethanol (without lipid phase and BDP as a control) were also found to be crystalline in appearance (Figure 3.14, Figure 3.15, Figure 3.16 and Figure 3.17).

Proliposome powders (2 mole% of BDP to the lipid phase) prepared from the given four carriers, in three lipid to carrier ratios (i.e. 1:5, 1:10 or 1:15 w/w lipid to carrier) were also investigated. All formulations exhibited a crystalline appearance via XRD, as the carbohydrate carriers generated peaks of high intensity. However, upon examination of proliposome formulations (2 mole% BDP to the lipid phase), no BDP peaks were detectable. This indicated that BDP may be present in amorphous form, irrespective to the carbohydrate carrier and lipid to carrier ratios. Upon examination of the physical mixture of proliposome powder (formulations without BDP) with added BDP crystals (in a 2 mole% concentration to the lipid phase), BDP was once again found not to exhibit a peak, indicating its amorphous structure (Figure 3.14, Figure 3.15, Figure 3.16 and Figure 3.17).

As in both the proliposomes formulated with BDP, and proliposomes with BDP as a physical mixture; BDP (2 mole% to the lipid phase) were found to exhibit no peak, amorphous form was therefore indicated in both preparations. For confirmatory purposes, the concentration of BDP in both mixtures (i.e. the proliposome formulated with BDP and proliposomes with BDP as a physical mixture) was increased to 100 mole% (i.e. 4.48 mg of BDP for 2 mole% and 244.05 mg of BDP for 100 mole%) with respect to the lipid phase (250 mg) in the 1:5 w/w lipid to carrier ratio proliposome formulation (utilising LMH, sucrose, sorbitol or D-mannitol as carbohydrate carriers). The physical mixture with 100 mole% BDP exhibited a peak upon examination, indicating the presence of crystalline BDP. Contrastingly, the 100 mole% BDP proliposome formulation did not generate comparable peaks, once again indicating the presence of amorphous BDP (Figure 3.14, Figure 3.15, Figure 3.16 and Figure 3.17).

Analysis of the findings suggested that although initially, BDP (in pure form) was present in crystalline form. It transmuted into amorphous form when formulated in proliposomes. However, recrystallisation occurred following hydration in aqueous media, as shown by examination of the lower fraction (spot), post centrifugation of the liposome suspension in D_2O (Section 2.2.4 and 2.2.5).

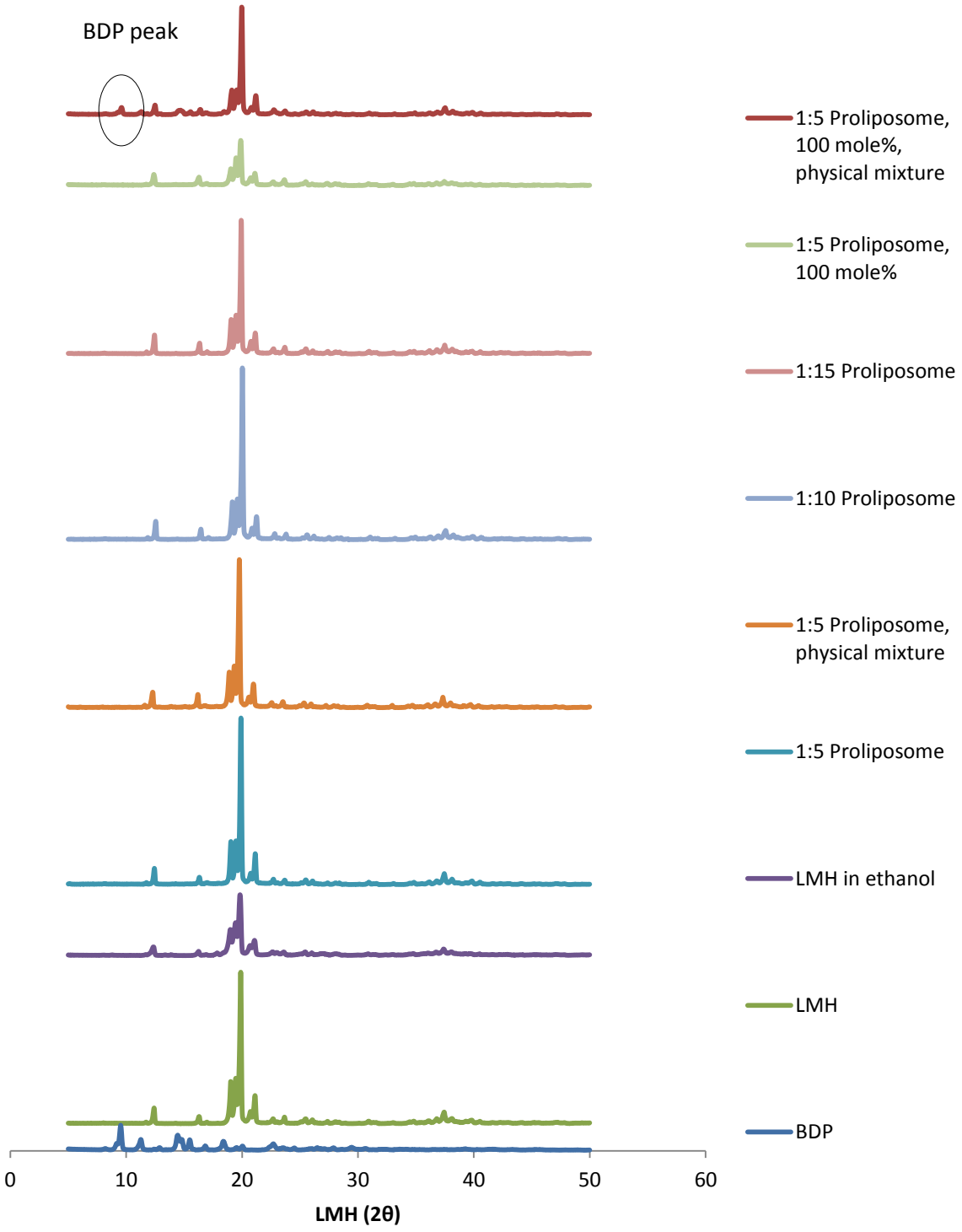


Figure 3.14: X-ray diffraction spectra for BDP, LMH, LMH treated with ethanol; and LMH-based proliposome formulations (1:5, 1:10 or 1:15 w/w lipid to carrier ratios) with BDP in a concentration of 2 mole% to the lipid phase. Additionally shown; is the spectra for the 100 mole% concentration of BDP to the lipid phase with the 1:5 w/w lipid to carrier ratio. LMH-based proliposomes (without BDP) in a 1:5 w/w lipid to carrier ratio were physically mixed individually with 2 mole% of BDP and alternatively, 100 mole% of BDP to the lipid phase. This data is typical of three such different experiments

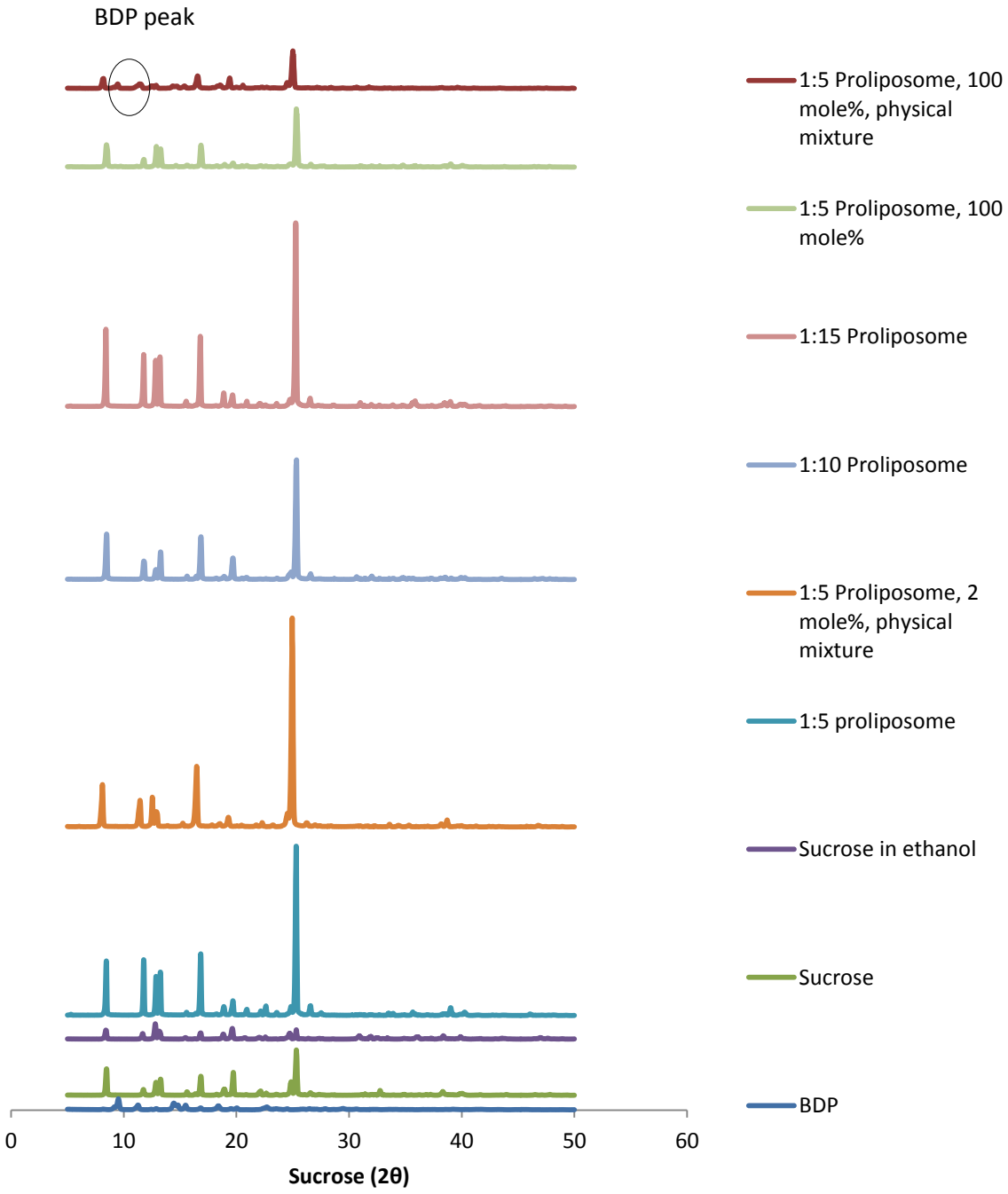


Figure 3.15: X-ray diffraction spectra for BDP, sucrose, sucrose treated with ethanol; and sucrose-based proliposome formulations (1:5, 1:10 or 1:15 w/w lipid to carrier ratios) with BDP in a concentration of 2 mole% to the lipid phase. Additionally shown; is the spectra for the 100 mole% concentration of BDP to the lipid phase with the 1:5 w/w lipid to carrier ratio. Sucrose-based proliposomes (without BDP) in a 1:5 w/w lipid to carrier ratio were physically mixed individually with 2 mole% of BDP and alternatively, 100 mole% of BDP to the lipid phase. This data is typical of three such different experiments

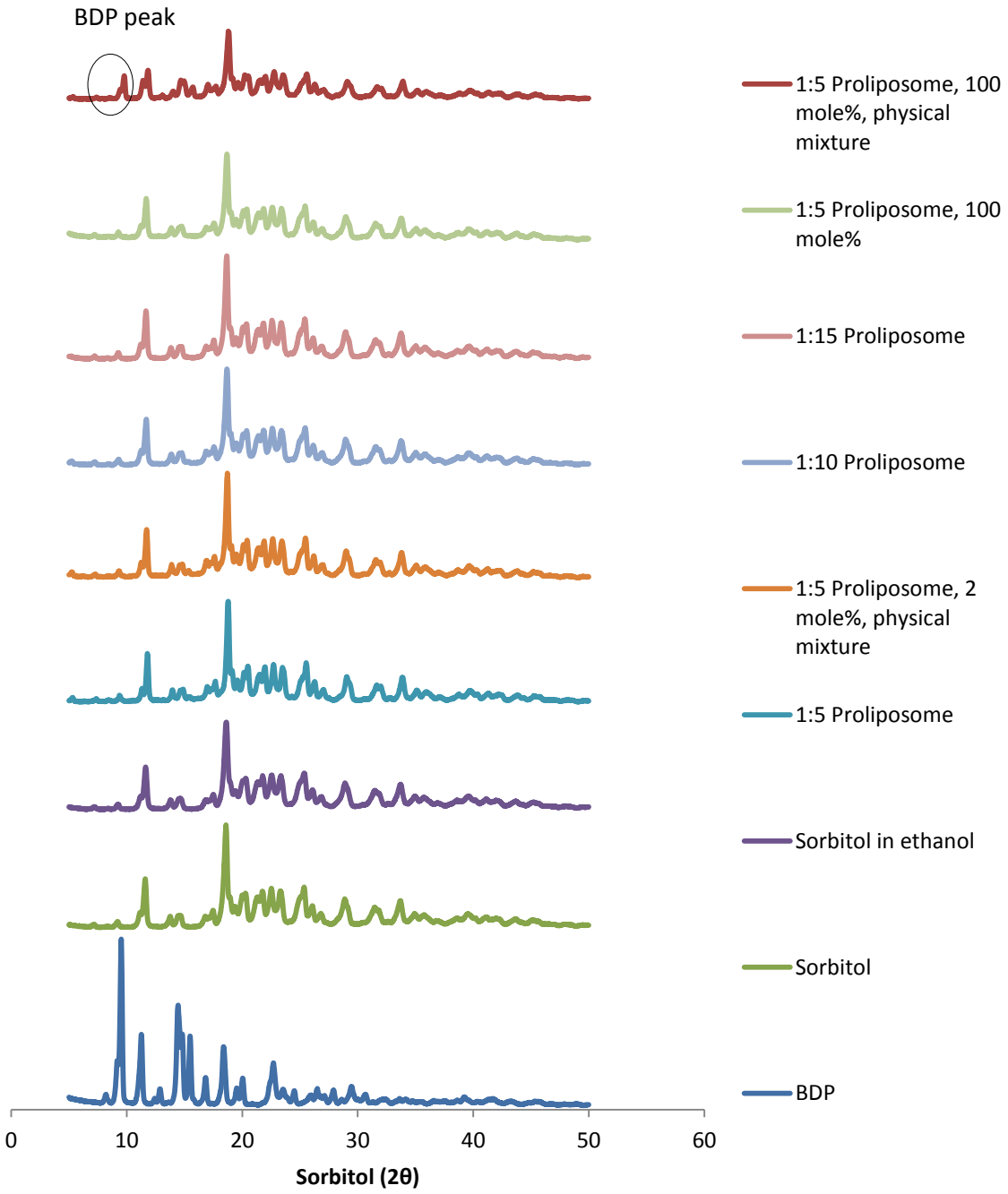


Figure 3.16: X-ray diffraction spectra for BDP, sorbitol, sorbitol treated with ethanol; and sorbitol-based proliposome formulations (1:5, 1:10 or 1:15 w/w lipid to carrier ratios) with BDP in a concentration of 2 mole% to the lipid phase. Additionally shown; is the spectra for the 100 mole% concentration of BDP to the lipid phase with the 1:5 w/w lipid to carrier ratio. Sucrose-based proliposomes (without BDP) in a 1:5 w/w lipid to carrier ratio were physically mixed individually with 2 mole% of BDP and alternatively, 100 mole% of BDP to the lipid phase. This data is typical of three such different experiments

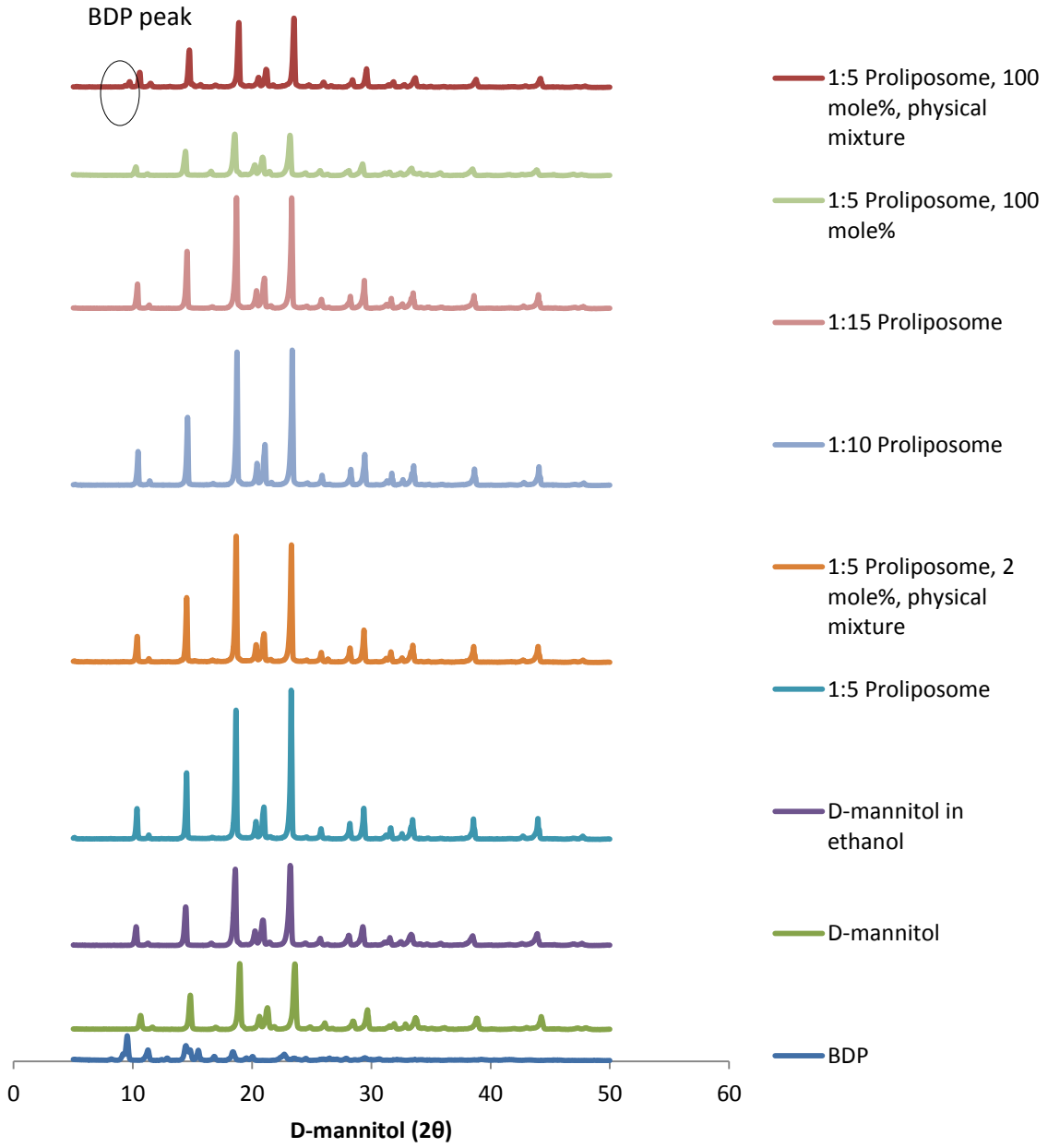


Figure 3.17: X-ray diffraction spectra for BDP, D-mannitol, D-mannitol treated with ethanol; and D-mannitol-based proliposome formulations (1:5, 1:10 or 1:15 w/w lipid to carrier ratios) with BDP in a concentration of 2 mole% to the lipid phase. Additionally shown; is the spectra for the 100 mole% concentration of BDP to the lipid phase with the 1:5 w/w lipid to carrier ratio. D-mannitol-based proliposomes (without BDP) in a 1:5 w/w lipid to carrier ratio were physically mixed individually with 2 mole% of BDP and alternatively, 100 mole% of BDP to the lipid phase. This data is typical of three such different experiments

3.3.10. Differential Scanning Calorimetry

As a further confirmatory technique to XRD, Differential Scanning Calorimetry (DSC) was implemented to assess the crystallinity of BDP in proliposome formulations. This was established by the individual peaks exhibited by the absorbance of heat (endothermic nature) of BDP at 104.59°C, cholesterol (95.96, 122.61 and 147.01°C), and sucrose at 186.60°C (Figure 3.18). Initially, 2 mole% of BDP to lipid phase was implemented for the proliposome preparation, however this was found to be an insufficient concentration for detection and analysis of BDP, as no peak was detected. Consequently, 100 mole% of BDP to the lipid phase (i.e. 4.48 mg of BDP for 2 mole% and 244.05 mg of BDP for 100 mole%) was employed in proliposome preparation, for the analysis of BDP presence in proliposome powders. Spectral data illustrated the melting point of sucrose to be 198.69°C, whereas three successive melting points were observed for cholesterol; 134.04, 141.23 and 172.46°C (Figure 3.18). Repeatedly, the BDP melting point was undetectable; this supports the data generated by XRD, which indicates that in proliposome formulations, BDP is present in amorphous form.

High quantity of BDP utilised (i.e. 100 mole% to the lipid phase) did not influence the intensity of the endothermic peak; however, sucrose and sorbitol peaks were identified for heat absorption. SPC was not analysed individually, as well as in the proliposome powder formulation, due to their low phase transition (below zero °C i.e. -20 ± 5°C) (Lipoid, 2014). The absence of a BDP peak further the supports the theory that BDP is present in amorphous form, in the proliposome powder formulation.

The shift in melting point may be due to the interaction of the proliposome constituents with each other, or also as a result of the elevation of the BDP concentration (i.e. 100 mole% of BDP to the lipid phase) in the proliposome formulation. The solubility of BDP and lipid phase (SPC and cholesterol) in ethanol alters the crystallinity of BDP, making it amorphous. Upon contact with aqueous media (hydration of proliposomes into liposomes), the BDP recrystallizes (indicated by spot formation post centrifugation of the liposome suspension in D_2O, confirmed with HPLC and NMR). Similar findings were noted for proliposomes prepared from the counterpart formulations i.e. proliposomes prepared from alternative carriers.

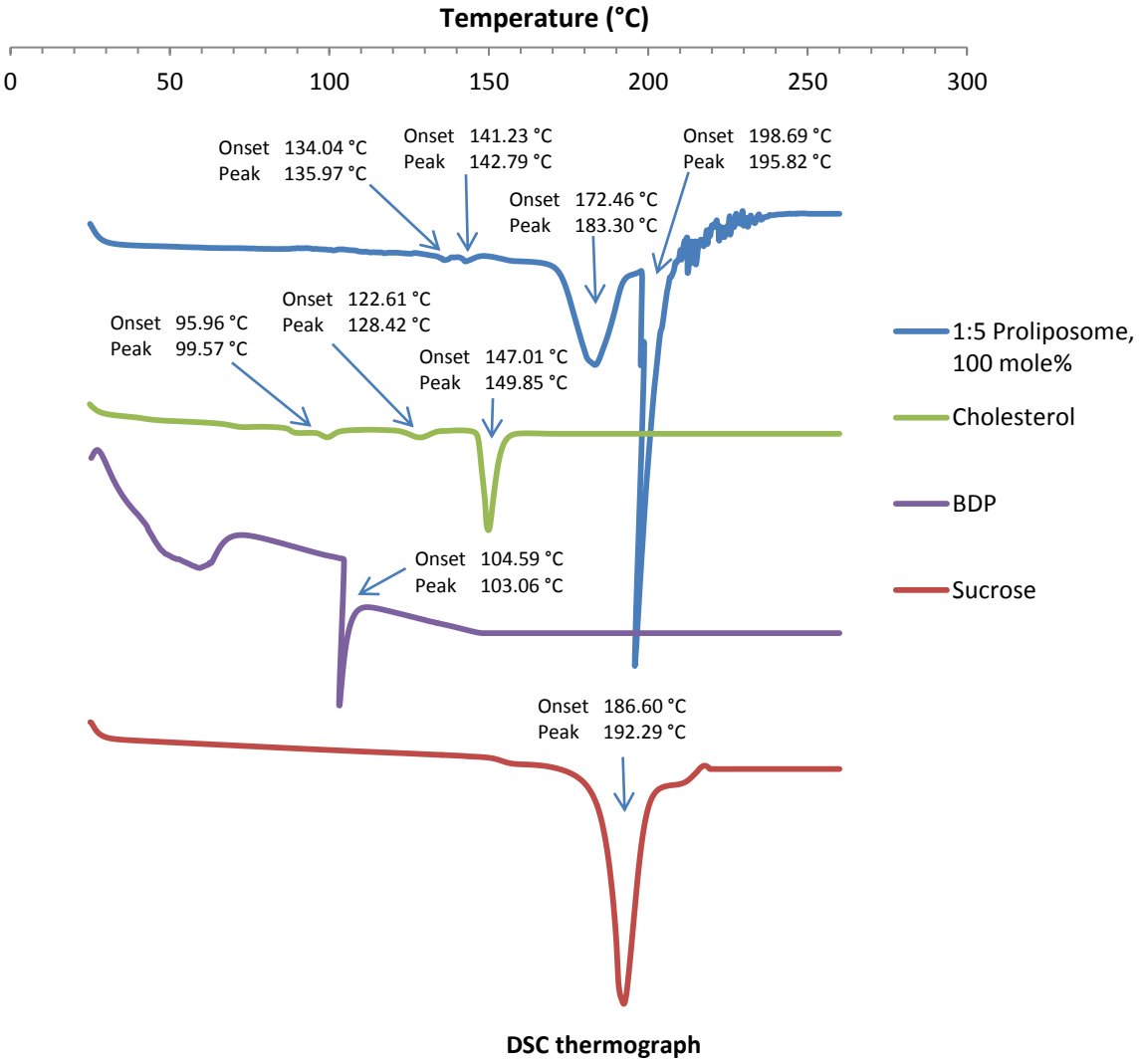


Figure 3.18: Superimposed DSC thermographs of individual BDP, sucrose, cholesterol for comparison with sucrose-based proliposomes in a 1:5 w/w lipid to carrier ratio; 100 mole% BDP to lipid phase. Data are mean ± STD, n=3; P<0.05

3.3.11. Entrapment efficiency analysis of BDP via HPLC using DW as a dispersion medium

Liposomes were generated in DW from LMH, sucrose, sorbitol or D-mannitol-based proliposome powders. It was observed that following centrifugation for 90 minutes at 13,000 RPM, unentrapped BDP and the majority of the liposomes with entrapped-BDP, sedimented to the bottom of the centrifuge tube (when utilising the traditional method of hydration in DW). This proposed notion was then substantiated by HPLC analyses; dramatically, in excess of 95% BDP-entrapment was derived (Figure 3.19) from the sedimented fraction/part. The unentrapped fraction i.e. supernatant (containing traces of lipid with dissolved or free BDP crystals), found

suspended in the centrifuge tube, exhibited circa 5% of the total BDP concentration. The unrealistically high entrapment (i.e. 95% when DW was used) prompted the change to alternative dispersion media; for comparison of entrapment of BDP in liposome bilayers. Although it is noteworthy that the entrapment values observed in DW during this research are in concordance with previous literature (Radhakrishnan, 1991, Jaafar-Maalej et al., 2011). In DW, the pellet formed, following centrifugation is comprised not only of BDP-entrapped liposomes, but also free BDP crystals. Thus, it was suggested that the liposome separation process was inefficient, as BDP crystals and BDP-entrapped liposomes sedimented collectively, giving rise to inaccurate estimation of entrapment efficiency. To overcome this limitation, a higher density medium (D_2O) was implemented in this research, resulting in enhanced separation of free drug crystals from the BDP-entrapped liposomes.

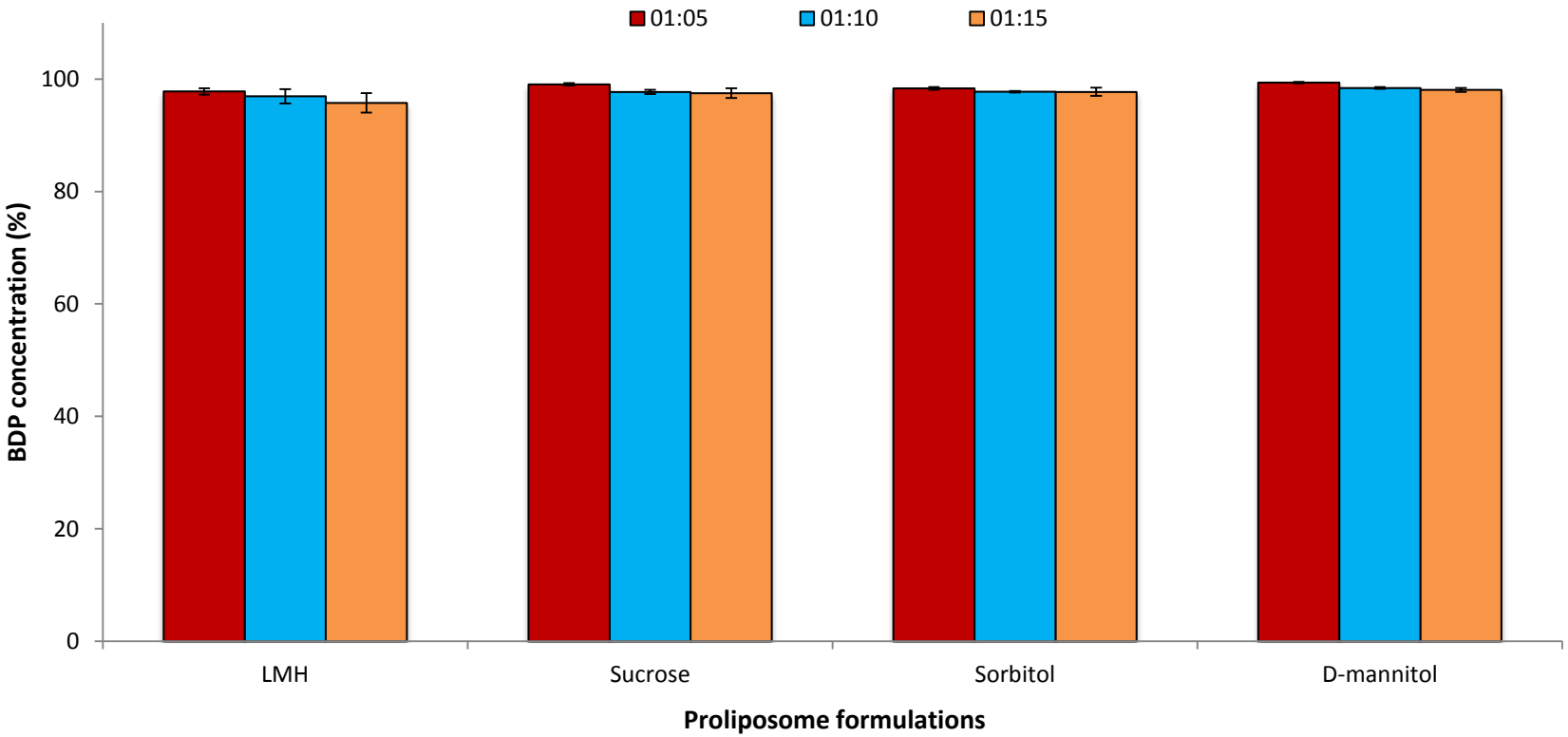


Figure 3.19: Entrapment efficiency of BDP in liposomes generated from proliposome powders prepared from carbohydrate carriers including; LMH, sucrose, sorbitol or D-mannitol, in formulation ratios of 1:5, 1:10 and 1:15 w/w lipid to carrier ratio in DW. Data are mean ± STD, n=3; P<0.05

3.3.12. Entrapment efficiency analysis of BDP via HPLC using D_2O as a dispersion medium

Following hydration of LMH, sucrose, sorbitol or D-mannitol-based proliposome powders in D_2O, liposomes were generated (Section 2.2.3). Subsequently, identical parameters, in terms of liposome separation (i.e. centrifugation for 90 minutes at 13,000 RPM) were used (Section 3.3.5 and 3.3.6). Upon centrifugation, due to the higher density of D_2O (1.105 g), BDP-entrapped liposomes were observed to float, forming a creamy-white layer at the top of the centrifuge tube. The middle fraction/part was identified as the unentrapped fraction; containing lipid traces with dissolved or free BDP crystals. The majority of free BDP crystals however, were found to sediment separately at the bottom of the centrifuge tube, as a spot. The claim, that the upper liposome layer was free from BDP crystals, was substantiated by light microscopy (Figure 3.10). Also, the presence of the majority of BDP crystals aggregating as a spot at the bottom of the centrifuge tube, was further supported by light microscopy (Figure 3.11). The suggestion made, that the upper liposome layer was free from BDP crystals and the majority of BDP crystals sedimented as a spot at the bottom of the tube were confirmed by light microscopy. Additionally BDP as a spot was also confirmed by NMR, and quantified by HPLC.

The current method therefore provides a means for reliable and accurate separation of BDP-entrapped liposomes from the unentrapped dissolved or free BDP crystals. The middle layer, which represents the unentrapped drug fraction, was found to contain less than 10% of the originally included BDP concentration (Figure 3.20 and Figure 3.21). By contrast, in the case of DW as dispersion medium, this was less than 5% (Figure 3.19).

It was found that the isolated spot contained the majority of the free BDP i.e. 79.18 $\pm$ 5.84, 74.16 $\pm$ 8.67, 69.30 $\pm$ 4.82 and 64.01 $\pm$ 3.55% for the 1:5 w/w lipid to carrier ratio, when using LMH, sucrose, sorbitol and D-mannitol as carriers in proliposome preparations, respectively. As may be seen in Figure 3.10, all formulations in a 1:5 w/w lipid to carrier ratio exhibited a high concentration of free BDP in the form of a spot. Upon statistical analysis, no significant difference ($p>0.05$) was observed when carbohydrate carrier type was varied. The presence of BDP crystals (in the spot), may be accounted for by the incompatible stearic fit of the crystals in the concentric bilayers of the liposomes, preventing their entrapment (Radhakrishnan, 1991).

In LMH-based liposome suspensions (Figure 3.20 a), the spot containing the maximum amount of BDP in a 1:5 w/w ratio (79.18 $\pm$ 5.84%) was observed. Free BDP concentration was found to decrease upon decreasing lipid content, i.e. the 1:10 and 1:15 w/w ratios respectively dropped to

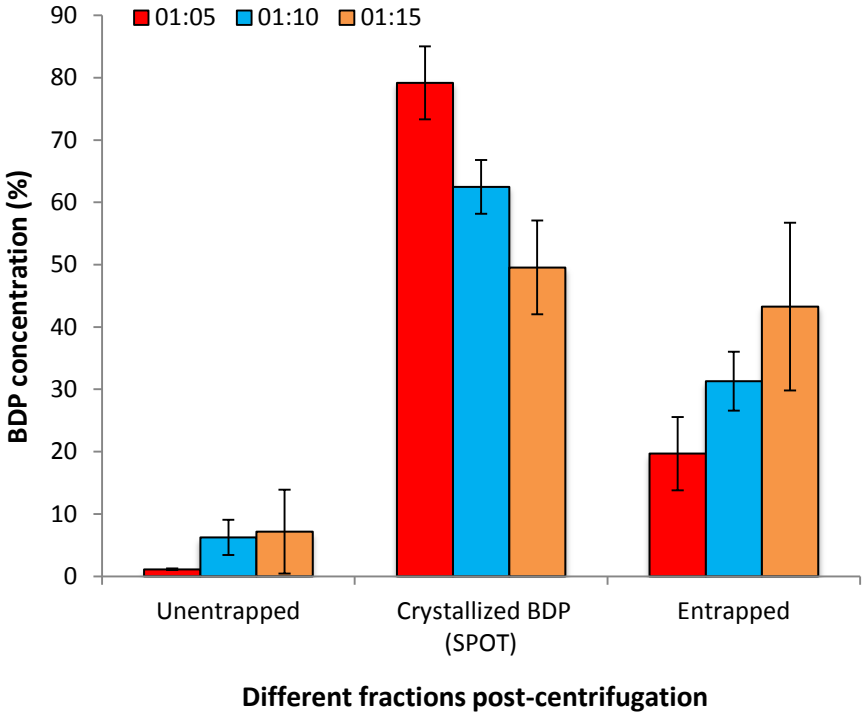
62.47 ± 4.32% and 49.55 ± 7.53%, as the concentration of lipid was notably lower in these formulations compared to the 1:5 w/w (lipid to carrier ratio) formulation. Moreover, increasing BDP entrapment was noted upon increasing the carbohydrate carrier concentration, whilst maintaining the lipid concentration (Figure 3.20 a). For LMH-based liposomes in D_2O, no significant difference (*p>0.05*) was observed between the unentrapped parts of all three formulation ratios (i.e. 1:5, 1:10 and 1:15 w/w). However, upon examination of the isolated spot, a statistically significant difference (*p<0.05*) was detected, between the 1:5 and 1:15 w/w lipid to carrier ratios, containing high free BDP concentration in the 1:5 w/w formulations, and a lesser concentration in 1:15.

Liposomes generated from sucrose-based proliposomes in D_2O exhibited a high concentration of free BDP crystals in the form of a spot, in the 1:5 w/w ratio (74.16 ± 8.67%) (Figure 3.20 b). This ratio however, exhibited no significant difference (*p>0.05*) when compared to the 1:10 and 1:15 w/w lipid to carrier ratios, containing free BDP crystals (50.75 ± 5.28 and 51.59 ± 2.78% respectively). Additionally, liposome entrapped-BDP analysed (derived from sucrose-based proliposomes), contained similar concentrations of BDP i.e. 47.85 ± 4.96 and 47.05 ± 2.70% for the 1:10 and 1:15 w/w lipid to carrier ratios, respectively. Liposomes generated from sucrose-based proliposome formulations in 1:10 and 1:15 w/w ratios, exhibited the second highest drug entrapment of all the carbohydrate carriers investigated.

The highest degree of entrapment of BDP was displayed by liposomes generated from sorbitol-based proliposomes in D_2O. The percentage entrapment in the 1:10 and 1:15 w/w ratios was 54.34 ± 7.16 and 61.91 ± 9.92% correspondingly, identifying sorbitol as an ideal carbohydrate carrier in terms of drug entrapment (Figure 3.21 a). This may be attributed to the porous nature of the sorbitol carrier, which may enhance the loading of lipid phase containing BDP onto the carrier surface. Moreover, both 1:10 and 1:15 w/w lipid to carrier ratios, exhibited a statistically significance difference (*p<0.05*) giving higher entrapment values, when compared to the 1:5 w/w lipid to carrier ratio.

Liposomes (generated from D-mannitol-based proliposomes) presented no significant difference (*p>0.05*) amongst all three formulation ratios (i.e. 1:5, 1:10 and 1:15 w/w) for the unentrapped part, free BDP crystals and the BDP-entrapped in liposomes (Figure 3.21 b). Comparatively, liposomes in a 1:10 w/w lipid to carrier ratio entrapped 46.05 ± 9.95% of the utilised BDP, making it the third most suitable carrier in terms of BDP entrapment.

*(**a**) LMH at varied lipid to carrier ratios.*



*(**b**) Sucrose at varied lipid to carrier ratios.*

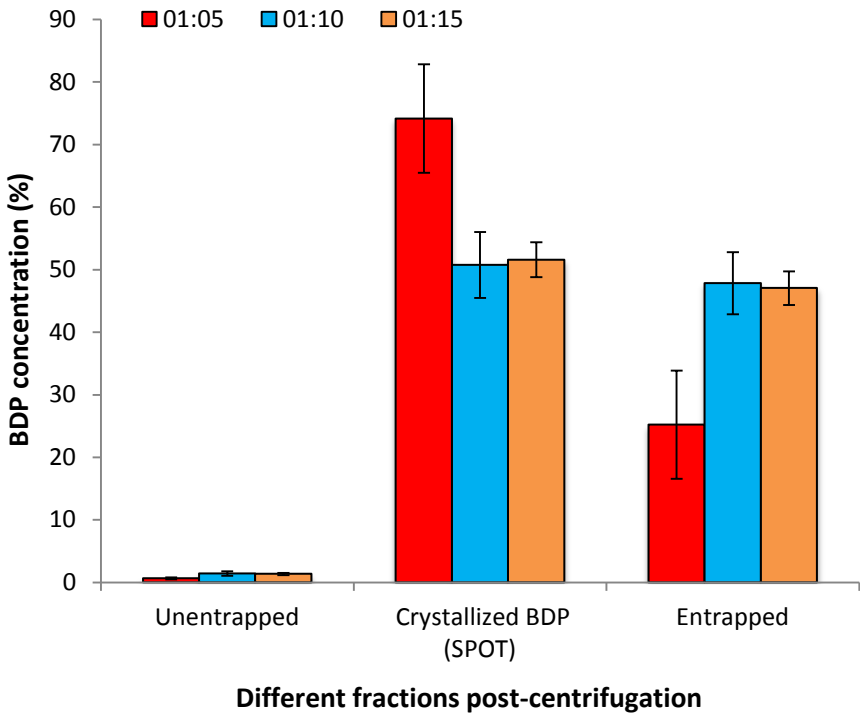
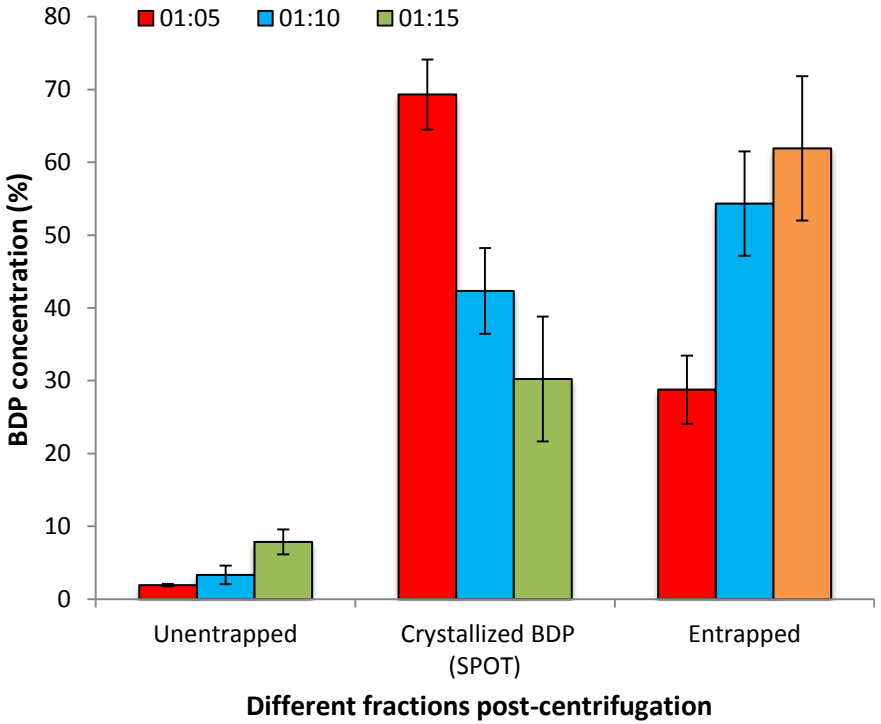


Figure 3.20: Percentage of entrapped and unentrapped BDP in three different fractions/parts of the tube, including: unentrapped, spot and entrapped fraction, using various carriers and lipid to carrier ratios i.e. 1:5, 1:10 and 1:15 w/w in D_2O; (a) LMH, (b) sucrose. Data are mean ± STD, n=3; P<0.05

*(**c**) Sorbitol at varied lipid to carrier ratios.*



*(**d**) D-mannitol at varied lipid to carrier ratios.*

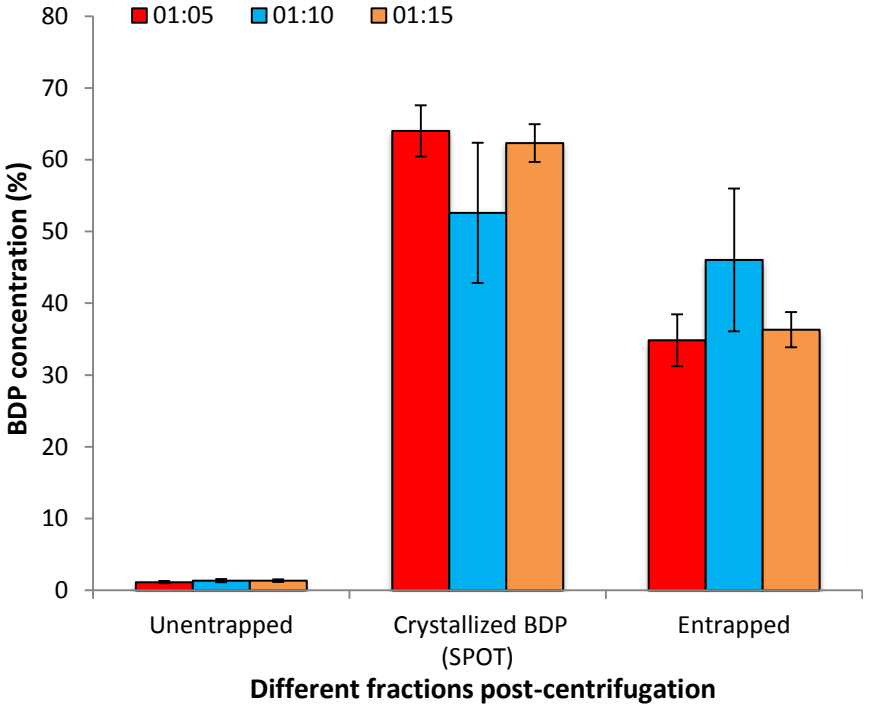


Figure 3.21: Percentage of entrapped and unentrapped BDP in three different fractions/parts of the tube, including: unentrapped, spot and entrapped fraction, using various carriers and lipid to carrier ratios i.e. 1:5, 1:10 and 1:15 w/w in D_2O; (a) sorbitol and (b) D-mannitol. Data are mean ± STD, n=3; P<0.05

3.4. Conclusions

A novel "Slurry" method was used to prepare proliposome powders, using LMH, sucrose, sorbitol or D-mannitol as carbohydrate carriers, to produce liposomes on the addition of aqueous phase (DW or D_2O), by vortex mixing. Surface morphology of carbohydrate carriers was examined by SEM, and it was observed that LMH and sucrose were non-porous, whilst D-mannitol had an engraved lined surface. Sorbitol was noted to be spherical to irregular in shape and porous; with needle-like structures protruding from its surface.

Liposomes generated from proliposome formulations did not differ in size (i.e. VMD), as well as size distribution (i.e. SPAN), with or without BDP incorporation; indicating stability in the formulation, in terms of drug incorporation. Bench centrifugation was used to optimise the separation, in addition to the implementation of Stewart assay. D_2O was used as the aqueous dispersion medium in order to generate liposomes, and a multitude of speeds were used to effectively separate the entrapped and unentrapped fractions. Finally, a speed of 13,000 RPM was selected as optimum for complete separation of the suspension constituents. LMH and D-mannitol were selected as the best carriers in terms of liposome generation, displaying the lowest concentration of free lipid in the unentrapped middle fraction/part. In addition to separation speed, separation time was also optimised to 90 minutes; following confirmation of complete separation using light microscopy.

In DW, the entrapment efficiency was in excess of 95% for all formulations in agreement with literature. It was observed that DW was not the best medium for the separation of the entrapped and unentrapped fractions/parts. However, the slightly higher density medium D_2O was demonstrated to be superior, as this was capable of separating the unentrapped BDP from the liposomes containing entrapped-BDP. LMH was selected as the better proliposome performing carrier, as this yielded a smaller VMD than comparative formulations. Finally, the entrapment efficiency was highest for sorbitol-based proliposomes as compared to all other formulations in D_2O.

Sorbitol-based proliposomes elicited the greatest degree of BDP-entrapment in liposomes generated; as a consequence, this carrier alongside D-mannitol was carried forward for further research (particularly the 1:10 and 1:15 w/w ratios for both formulations).

Interestingly, the differences in entrapment values of BDP in liposomes, when using DW or D_2O are perplexing. The disparity in entrapment values, suggests that previous BDP entrapment values in similar research (i.e. when using DW as the dispersion medium) are disputable, owing to the

presence of BDP crystals in centrifuged suspensions; which may negatively influence or mask the actual BDP entrapment values. D_2O has been shown to be a superior medium, which is capable of separating unentrapped BDP from the BDP-entrapped in liposomes. As discussed, this discovery may have implications on previous, on-going and future research.

CHAPTER 4: PREPARATION AND CHARACTERISATION OF PROLIPOSOME-BASED TABLETS

"With faith, discipline and selfless devotion to duty, there is nothing worthwhile that you cannot achieve"

- Muhammad Ali Jinnah -

4.1. Introduction

Liposomes are commonly used as carriers for many drugs which are entrapped into vesicles bilayers if hydrophobic, or, into the central core of liposomes if hydrophilic. Although liposomes are established drug carriers, they exhibit instability during storage (Hunt and Tsang, 1981). These stability issues pertain to drug leakage from the liposomes as well as aggregation and fusion of these vesicles (Wong and Thompson, 1982). To overcome these issues, liposomes have been generated from dry formulations called proliposomes (Payne et al., 1986a, Payne et al., 1986b). Proliposomes may be described as consisting of a lipid component (phospholipid and cholesterol and/or BDP), coated as a film/layer over carbohydrates carrier particles. These proliposomes are then transformed into liposomes through hydration via the addition of an aqueous solvent. Proliposomes may be stored at low temperatures, in order to minimise the rate of phospholipid degradation.

Proliposome powders were prepared using lipid and carbohydrate carriers (LMH, sucrose, sorbitol or D-mannitol), in various ratios (1:5, 1:10 and 1:15 w/w). The properties of the liposomes generated upon addition of aqueous phase were optimised in terms of separation of the entrapped drug from the unentrapped (detectable dissolved or free BDP crystals with additional traces of free lipid), as detailed in Chapter 3. This was then followed by entrapment efficiency studies in the same chapter, utilising D_2O as a dispersion medium.

Many formulations are available in powder form due to their mechanical feasibility and stability. However, powder formulations are typically difficult to manage in terms of manufacture on a large scale, owing to their bulkiness, and also because ultrafine solids may potentially cause health and environment related problems (EPA, 2013). For these reasons, powders are commonly tableted offering multiple benefits, such as; easy and uniform dosing and improved handling capability, making them much more convenient as dosage forms in terms of manufacture and transport. Generally, the excipients used in powder and tablet manufacture are comprised of carbohydrates, with a wide range of choices being available (Rojas et al., 2014). Carbohydrates also significantly provide bulk for the formulation, facilitating compression into tablets, which are uniform in size, shape and weight.

Tablet compression is with no doubt a complicated and challenging engineering conundrum, where powdered materials are transmuted into densely compacted tablets. The strength exhibited by tablets is heavily dependent upon the composition of the powder (Sinka et al., 2009). Though tableting is associated with a number of difficulties, comparatively to alternative dosage

forms (which are comprised of semi-solid or liquid preparations), they are still considered to be easy and economical to produce (Jivraj et al., 2000, Sastry et al., 2000). In addition to their comparative ease in production, in terms of stability, accuracy in dosing and ease in handling and transportation, tablets retain superiority in comparison to other dosage forms due to high patient acceptability.

Owing to the high popularity and stability of solid dosage forms, in this work, liposomes were initially manufactured as dry proliposome powders. These proliposome powders were prepared utilising carbohydrate carriers into various formulation ratios via the "Slurry" method (Chapter 3). These proliposome powders were then tableted, in a bid to overcome the pitfalls of working with powder formulations. The lack of requirement of additional excipients may be explained by the presence of large quantities of carbohydrates in the proliposome powders, which provide bulk and high aqueous solubility, offering formulations that are readily dispersible in aqueous phase (Fu et al., 2004).

This chapter aims to identify, assess and evaluate the best formulation for tablet manufacture. For each of the four carbohydrate carriers, three individual proliposome formulations, with varying lipid to carrier ratio were formulated for the purpose of investigation. A series of studies were conducted to analyse the flow properties of the proliposome powders, to investigate the influence of powder flowability on the compressibility of the powders to form proliposome tablets. Post-manufacturing, Quality Control (QC) tests were implemented, including; weight variation, disintegration, hardness and friability tests to analyse tablet quality; in concordance to BP and USP guidelines (BP., 2010d, USP., 2012). Finally, the effect of compression on proliposome powders was evaluated via entrapment studies, utilising D_2O as a dispersion medium for the generation of liposomes from proliposomes.

4.2. Methodology

4.2.1. Manufacture of proliposomes using the "Slurry" method

Proliposomes were manufactured in accordance with the aforementioned "Slurry" method (Section 2.2.2). LMH, sucrose, sorbitol or D-mannitol were chosen as carbohydrate carriers in a 1:5, 1:10 and 1:15 w/w lipid to carrier ratios, for the preparation of proliposome tablets in this

study. SPC and cholesterol were incorporated in a 1:1 mole ratio, and BDP was included at 2 mole% based upon the lipid phase.

4.2.2. Tapped Density and Angle of Repose

Tapped density and Angle of Repose (AOR) are key parameters; which are used for the determination of the flowability of powder formulations. These parameters were implemented to investigate the proliposome powder formulations prepared as in section 2.2.2 for the aforementioned property. Tapped density was used to record the bulk and tapped densities of the powder, in order to measure the compressibility index and Hausner ratio; which subsequently assesses the flowability of the tested powder, as outlined in section 2.2.12.

4.2.3. Direct compression

Direct compression, was conducted in this study, as stated in section 2.2.14. The weight of sorbitol and D-mannitol-based proliposome tablets was maintained in the range of 82 - 91.18 mg and 62.66 - 65.65 mg, for both 1:10 and 1:15 w/w lipid to carrier ratios; for both BDP containing proliposome powders and drug-free proliposome powders.

4.2.4. Characterisation of Proliposome-based tablets

Proliposome-based tablets were characterised using a number of recognised official tests, including: Weight variation (Section 2.2.16), disintegration testing (Section 2.2.17), hardness (Section 2.2.18) and friability (Section 2.2.19) were implemented in accordance to the BP and US Pharmacopeia. The aforementioned tests were implemented as markers for Quality Control (QC), in order to determine the mechanical strength of the tablets (Riippi et al., 1998).

4.2.5. Scanning Electron Microscopy

SEM was employed as an analytical tool for the investigation of proliposome tablet surface morphology. Images were taken at various magnifications in order to study the surface, in addition to images of tablet cross-sections to investigate potential structural changes within the tablets.

4.2.6. Drug Entrapment in liposomes using D_2O as dispersion medium

Proliposome tablets were hydrated to generate liposomes (in a 30 mg/ml suspension) (Section 2.2.3); the resultant entrapment efficiency of BDP in the liposomes was then determined using the separation and HPLC analysis protocols outlined in section 2.2.10 and 2.2.11.

4.3 Results and Discussion

4.3.1. Preliminary investigations and general observations

Preliminary investigations and general observations simply refer to the general physical appearance of the manufactured tablets, their ability to withstand manual handling (i.e. physical touch such as picking up and squeezing), in addition to testing using a hardness tester.

4.3.1.1. Proliposome powder (1:5 w/w lipid to carrier)

Irrespective of carrier type, proliposome powder formulations at this ratio were found to exhibit a glossy and shiny appearance; as previously shown in section 3.3.1. The observed appearance may be attributed to the high level of lipid content within the formulations. Subsequently, Stewart assay was conducted in triplicate as a confirmatory technique to quantify the lipid content in each formulation (Stewart, 1980).

Whilst the high lipid content was found to exert an effect upon the appearance of the proliposomes, differences in proliposome powder properties were also observed depending upon carrier type. For example, sucrose-based proliposomes at the 1:5 w/w lipid to carrier ratio were observed to be fluffy in terms of appearance and upon manual handling. Through tableting, proliposome powders prepared using sucrose as carrier were deformed with ease, under low compressive force to form tablets (when tableting machine was used manually, compression force is not observable). However, upon manual handling the tablets prepared from sucrose-based proliposomes (i.e. 1:5 w/w ratio) were found to crumble, indicating their inability to withstand pressure.

High amounts of lipid in the formulation were deemed to be responsible for the weakness of the tablets, potentially preventing sufficient compression of the powder into tablets. As a counteractive action, a high compression force was applied manually, aiming to increase the tablet hardness. Upon increasing compression force, tablet hardness increased dramatically (more than 200 kN by performing hardness/crushing test), indicating lack of control over hardness. Three main issues were identified upon increasing compressive force; firstly, the occurrence of sticking or picking (separation of the upper part of the tablet) and capping (partial or complete separation of the top or the bottom layer of the tablet), which was found to increase with increasing the compression force.

Secondly, the produced tablets, though harder, were found to disintegrate well out of the required time (i.e. in excess of the 15 minutes as required per BP standards; and in excess of 30 minutes as per USP standards), indicating their lack of suitability for use (BP., 2010b, USP., 2010). Thirdly, the use of excessively high compressive force was deemed to be detrimental to the longevity of the punches used within the machine; as these are delicate, when taking into account the width of the die (6 mm) utilised in this study. Consequently, due to potential risk of machine damage, high compression force was avoided. For these reasons, further experiments using sucrose as carrier in proliposome tablets were discontinued.

The poor compressive properties of the 1:5 w/w lipid to carrier ratio formulations may not be exclusively attributed to the high lipid content. The low compressibility elicited by sucrose may be attributed to the fact that it is a low moulded sugar, which is resistive to force (Sastry et al., 2000). In terms of morphology, sucrose is known to be crystalline or irregularly/cubically shaped, making its compression highly challenging (Row et al., 2006). Moreover, the simplicity of the formulation i.e. sucrose and lipid component (SPC and cholesterol and/or BDP), being the sole constituent, without any excipients to aid in tableting, may also be partially responsible for the poor compressibility of the proliposome formulation.

Similar issues were found with alternative carriers at the same lipid to carrier ratios. LMH, sorbitol and D-mannitol-based proliposomes were subjected to the compressive force associated with tableting. Due to the high lipid content, yet again poor compression was observed. Notably however, proliposome powders formed from sorbitol, were found to compress well into tablets. This may be explained by the carrier's porous structure, which responded well to direct compression. LMH and D-mannitol-based proliposome powders were also found to show adequate compressive properties when compared to sucrose-based proliposomes. However, LMH, sorbitol and D-mannitol were all discontinued at the 1:5 w/w lipid to carrier ratio, due to poor entrapment of the model drug (BDP) in liposomes making them preferential to pursue for research (Section 3.3.12).

Whilst issues were identified with each carrier, the overriding factor in the poor tableting was attributed to the high lipid content in the investigated ratio (1:5 w/w). Due to the identified unsuitability of the 1:5 w/w lipid to carrier ratio, tableting of proliposomes with high lipid content was discontinued. In contrast, higher entrapment efficiencies were observed in carriers formulated in the 1:10 and 1:15 w/w lipid to carrier ratios (Section 3.3.12).

4.3.1.2. Proliposome powders (1:10 and 1:15 w/w lipid to carrier)

Using the 1:10 and 1:15 w/w lipid to carrier ratios, sucrose-based proliposomes were found to exhibit the same compressive properties as observed at 1:5 w/w lipid to carrier ratio. This suggested that the crystalline and brittle nature of the sucrose carrier was detrimental in terms of tablet manufacture, regardless of the lipid concentration in the proliposome formulation. Whilst sucrose-based proliposomes were found to be unsuitable for tableting, they were however theorised to be appropriate for sachet dosing, as oppose to compaction into a solid dosage form; this merits further investigations.

LMH-based proliposome powders at a ratio of 1:5 w/w lipid to carrier showed similar results to sucrose-based proliposome powder at the same ratio; in terms of manual handling. Compressibility remained poor, producing tablets which collapsed during manual handling. This suggested poor compressibility and binding properties between lactose and the remaining proliposome constituents (Mattsson, 2000). High compression forces once again were found to produce harder tablets however this entailed longer disintegration times for the formulation (outside of BP and USP standards i.e. in excess 15 and 30 minutes) for the aforementioned reasons, LMH-based proliposomes were not used further in this study.

Contrastingly, sorbitol and D-mannitol (1:10 and 1:15 w/w lipid to carrier ratios) were observed to be reliable carriers for formulation into proliposome tablets, retaining their particle morphology (Section 3.3.1) following compaction. D-mannitol was used as a carrier in the proliposome formulations due to its non-hygroscopic nature, deemed to be a beneficial quality for formulation stability against degradation, which may occur due to moisture (Anh and Kathleen, 2001). On examination, D-mannitol-based proliposome tablets were not sufficiently strong to withstand the compression force during crushing (using a hardness tester). Lipid concentration was found not to exert an effect upon the compaction of the sorbitol crystals. Sorbitol-based proliposomes were compressed with ease into tablets for both ratios (1:10 and 1:15 w/w lipid to carrier) investigated. Tablets manufactured were visibly shiny, possessed no fractures or breaks, and were hard enough to withstanding manual handing.

For the aforementioned reasons, D-mannitol and sorbitol-based proliposome powders (i.e. 1:10 and 1:15 w/w lipid to carrier ratios) were carried forward for research into tablet dosage forms, whereas sucrose and LMH-based proliposomes powders were discontinued, due to their poor tableting properties. These include; high lipid content resulting in poor compressibility and hardness, longer disintegration time and low entrapment efficiencies. The aforementioned issues, resulted in sucrose and LMH-based proliposome powders to be withdrawn from further investigations.

4.3.2. Tapped density for coarse carbohydrates and proliposome powders

Tapped density is used as a rapid test to provide information with regards to powder flowability, packing arrangement and compaction properties during tablet compression (Lowell et al., 2004). Both compressibility index and Hausner ratio are derived using values from bulk and tapped density, as discussed previously (Section 2.2.12).

Out of the coarse carriers investigated, sucrose elicited the best compressibility index (4.52 $\pm$ 2.36%) and Hausner ratio (1.05 $\pm$ 0.03), with respect to the remaining coarse carriers ($p<0.05$) (Sorbitol, LMH and D-mannitol); identifying coarse sucrose as the most ideal carrier based on Carr's scale of Flowability (showing *excellent* range values) (Table 4.1,Table 4.2 and Table 4.3) (Carr, 1965). Second in line to sucrose, sorbitol showed *fair* flowability (based on Carr's scale of flowability) (Table 4.1) (Carr, 1965), with a compressibility index of 17.06 $\pm$ 0.14 and a Hausner ratio of 1.21 $\pm$ 0.01 (Table 4.2 and Table 4.3) ($p>0.05$), when compared to the remaining carriers (LMH and D-mannitol), which comparatively were found to *show very, very poor flow* properties

(Table 4.1). Coarse carriers were utilised as a control, in order to study the effect of lipid content on the carbohydrate carriers; and subsequently to investigate their influence on proliposome powder flowability for the purpose of tableting. Furthermore, all carbohydrate carrier proliposome formulations showed a statistically insignificant difference (*p>0.05*) between BDP loaded formulations and drug free formulations; for the 1:10 and 1:15 w/w lipid to carrier ratios, in terms of Carr's index of flowability. Therefore as a consequence, proliposome formulations with drug were generally used for comparison in the forthcoming discussions.

Differences in terms of Carr's scale of Flowability were also observed between the various proliposome formulation ratios investigated. A highly significant difference (*p<0.05*) was noted when comparing the 1:10 with the 1:15 w/w lipid to carrier ratio, when LMH was utilised as a carrier. *Good* flowability, as indicated by compressibility index (13.01 $\pm$ 1.11%) for the 1:10 w/w lipid to carrier ratio formulation; and *passable* flowability (23.72 $\pm$ 1.11%) for the 1:15 w/w lipid to carrier ratio was found for BDP incorporated proliposome formulations (Table 4.1 and Table 4.2). Better flow properties of proliposome powder, may enhance die filling; resulting in better tableting outcome. Consequently flow properties considered to be highly important.

Sorbitol and D-mannitol are isomeric forms of one another (Anh and Kathleen, 2001, Yoshinari et al., 2003). This may offer an explanation perhaps, as to why they did not show a significant difference (*p>0.05*), when compared to one another at the 1:10 and 1:15 w/w lipid to carrier ratios.

Overall, the compressibility index and Hausner ratio both indicated that the 1:10 w/w lipid to carrier formulations possessed marginally better (*p>0.05*) flow properties for all carriers in comparison to formulations at the 1:15 w/w lipid to carrier ratios. Consequently, formulations at a 1:10 w/w ratio were identified as appropriate for tablet manufacturing. This was attributed to their superior flowability, which would allow for improved filling of the tablet machine die; and hence improve tablet weight uniformity.

Table 4.1: Carr's; scale of flowability (Carr, 1965)

Compressibility index (%)	Flow character	Hausner ratio
1-10	Excellent	1.00-1.11
11-15	Good	1.12-1.18
16-20	Fair	1.19-1.25
21-25	Passable	1.26-1.34
26-31	Poor	1.35-1.45
32-37	Very poor	1.46-1.59
> 38	Very, very poor	> 1.60

Table 4.2: Percentage (%) Compressibility index via tapped density of coarse carbohydrate carriers or proliposome powders (1:10 and 1:15 w/w lipid to carrier ratios), using LMH, sucrose, sorbitol or D-mannitol as carriers. Data are mean ± SD, n=3, p<0.05

Formulations	Coarse powder	1:10 (w/w) with BDP	1:10 (w/w) without BDP	1:15 (w/w) with BDP	1:15 (w/w) without BDP
LMH	40.55 ± 1.43	13.01 ± 1.11	13.60 ± 1.96	23.72 ± 1.11	24.53 ± 0.46
Sucrose	4.52 ± 2.36	22.94 ± 1.77	22.35 ± 1.93	19.11 ± 0.21	19.62 ± 1.00
Sorbitol	17.06 ± 0.14	14.13 ± 2.75	11.99 ± 3.65	17.81 ± 4.46	20.39 ± 1.84
D-mannitol	34.78 ± 2.03	17.57 ± 1.96	17.05 ± 0.17	18.85 ± 1.55	18.86 ± 1.89

Table 4.3: Hausner ratio via tapped density of coarse carbohydrate carriers and proliposome powders; utilising carbohydrate carriers: LMH, sucrose, sorbitol or D-mannitol in a 1:10 or 1:15 w/w lipid to carrier ratios, with and without BDP. Data are mean ± SD, n=3; p<0.05

Formulations	Coarse powder	1:10 (w/w) with BDP	1:10 (w/w) without BDP	1:15 (w/w) with BDP	1:15 (w/w) without BDP
LMH	1.68 ± 0.01	1.15 ± 0.01	1.16 ± 0.01	1.31 ± 0.01	1.33 ± 0.01
Sucrose	1.05 ± 0.03	1.30 ± 0.03	1.29 ± 0.03	1.24 ± 0.01	1.24 ± 0.02
Sorbitol	1.21 ± 0.01	1.17 ± 0.04	1.14 ± 0.05	1.22 ± 0.07	1.26 ± 0.03
D-mannitol	1.53 ± 0.05	1.21 ± 0.03	1.21 ± 0.01	1.23 ± 0.02	1.23 ± 0.01

As in proliposome formulations, the lipid phase was used to form a film over the carbohydrate carrier particles; lipid content was subsequently analysed by Stewart assay (Stewart, 1980). The assay results demonstrated that the 1:10 w/w ratio formulations had a higher lipid concentration than the 1:15 w/w ratio (Section 2.2.9 and 3.3.2). According to Yang et al., (2011), tapped density is influenced by a number of factors such as; particle surface morphology and shape, as well as inter-particulate forces (Yang et al., 2011). Inter-particulate interactions are perceived to be less significant in free flowing powders, hence, bulk and tapped densities differ slightly (World Health Organization, 2012). Contrastingly, materials possessing poor flowability are known to have a greater degree of inter-particle interaction, as a consequence, the resultant tapped and bulk density of such powders are notably different (World Health Organization, 2012). The extent of difference in bulk and tapped density values are evaluated using Carr's scale of Flowability, in accordance to both United State Pharmacopeia., 2012 and British Pharmacopeia., 2010 (BP., 2010d, USP., 2012). The degree to which particles made from the same excipients in different ratios can affect powder flow properties has been illustrated in Table 4.2 and Table 4.3.

The higher lipid content was deemed to be responsible for causing powder coherence, preventing adequate compaction during tapping of the powders. This would explain the lower tapped density values observed for the 1:10 w/w formulation ratio, when compared to those prepared using 1:15

w/w lipid to carrier ratio (Table 4.2). The reduction in lipid content in the latter formulation is associated with a reduction in void spaces between the particles, when compared to the 1:10 w/w ratio. The only exceptions observed in this trend are the sucrose-based proliposome formulations, where no significant difference (*p>0.05*) was found between the 1:10 and 1:15 w/w lipid to carrier ratios. This may be due to the high lipid content on the surface of carriers (due to sucrose's smooth surface and lack of porosity), negating particle flow; and hence resulting in equally high compressibility index values, irrespective of lipid concentration.

Finally, it is noteworthy that formulations made from sorbitol and D-mannitol powders in a 1:10 w/w (lipid to carrier) ratio, showed greater adherence to the punches in the machine during tablet manufacture. However, it was found that the automated function of the Minipress single punch tablet machine could be used for sorbitol-based proliposomes; formulated in a 1:15 w/w lipid to carrier ratio, with a compression force of 3.00 - 4.50 kN to make 20 $\pm$ 4 tablets per minute.

4.3.3. Angle of repose

The principal aim of this test was to determine the flow properties of the formulated proliposome powders (using all carriers at the 1:10 and 1:15 w/w lipid to carrier ratios).

On comparison of the investigated formulations, a number of notable differences were observed in terms of AOR. Firstly, it was noted that the incorporation of BDP was found to insignificantly affect the AOR of the proliposome formulations (*p>0.05*); consequently, only BDP containing formulations were discussed in detail.

The AOR values of sorbitol-based BDP incorporated formulations in 1:10 and 1:15 w/w lipid to carrier ratios (25.62 $\pm$ 1.08° and 26.61 $\pm$ 0.08°), were found to be significantly lower (*p<0.05*) than coarse sorbitol (28.33 $\pm$ 0.38°). All values for sorbitol-based formulations were found within the range of 25 - 30° (Table 4.4 and Table 4.5), indicating *excellent* flowability compared to the corresponding carbohydrate-based proliposome powders. Anh and Kathleen (2001) have reported that particle size of sorbitol is responsible for its good flowability, and hence suitability for direct compression (Anh and Kathleen, 2001).

The excellent flowability observed using sorbitol may be therefore attributed to the morphology of the particles. Sorbitol is well noted for being irregular to spherical in shape, identifying it as a carrier with exceptional flowability (Section 3.3.1). In addition to its spherical shape, sorbitol particle surfaces are noted to possess protruding needles (Newman et al., 1999), which may

prevent particle agglomeration and provide void spaces. This is potentially due to the ability of these porous structures to accommodate the lipid phase during proliposome formulation.

As a consequence lipid on the exposed surface of the carrier may be reduced (or better accommodated), hence flowability is maintained (Ahn et al., 1995, Elhissi et al., 2011). Sorbitol at a 1:15 w/w lipid to carrier ratio demonstrated high flowability. Literature indicates that powder flowability may be highly influenced by the size, shape and bulk density of the particles (Liu et al., 2012, Saw et al., 2013, Szalay et al., 2014). This potentially has been demonstrated in the case of sorbitol-based formulations (Table 4.5).

Table 4.4: Flow properties and corresponding angle of repose (Carr, 1965)

Flow property	Angle of repose (degree)
Excellent	25-30
Good	31-35
Fair (aid not needed)	36-40
Passable (may hang up)	41-45
Poor (must agitate, vibrate)	46-55
Very poor	56-65
Very, very poor	> 66

Upon examination of the alternative carbohydrate carriers, it may be concluded that the AOR trend demonstrated by sorbitol is exclusive to that particular carrier. By comparison, out of the other formulated carriers investigated, D-mannitol-based proliposomes exhibited the highest AOR values, ranging from 40.88 $\pm$ 0.56 and 40.02 $\pm$ 0.51° for 1:10 and 1:15 w/w lipid to carrier ratios: to 43.78 $\pm$ 1.30° for coarse D-mannitol. On categorisation of these values using Carr's indicator for powder flow properties, D-mannitol elicited *passable or adequate* flowability (Table 4.4 and Table 4.5) (Carr, 1965). Again, morphology of the carbohydrate carrier particles was found to be an influential factor in powder flow (i.e. the crystalline, oblong shape of D-mannitol is undesirable for good flow of powder), as confirmed by Yan-yu et al., (2006) (Yan-yu et al., 2006) (Section 3.3.1).

Aside from morphology, which was agreed to a major factor in powder flowability, the lipid content of the proliposome powder formulations was believed to be highly influential in terms of powder flow, exhibiting its own identifiable trend. The reduction in lipid content may result in a

decrease in AOR (Table 4.4 and Table 4.5). As carrier concentration is increased; the added lipid is essentially spread more thinly amongst the particles, negating the adhering properties associated with the lipid content; offering an explanation for the reduction in AOR. As a consequence, powders with less lipid content (i.e. the 1:15 w/w lipid to carrier ratio) are less sticky, and hence exhibit improved flowability in comparison to the other carriers, at ratios with higher lipid content (i.e. the 1:10 w/w lipid to carrier ratio).

Table 4.5: Angle of repose of coarse carbohydrate carriers and proliposome powders utilising 1:10 and 1:15 w/w lipid to carrier ratios; for LMH, sucrose, sorbitol or D-mannitol carbohydrate carriers. Data are mean ± SD, n=3; $p<0.05$

Formulations	Coarse Powder	1:10 w/w with BDP	1:10 w/w without BDP	1:15 w/w with BDP	1:15 w/w without BDP
LMH	41.61 ± 3.71	39.64 ± 0.78	39.46 ± 0.47	35.99 ± 0.78	36.12 ± 0.71
Sucrose	21.47 ± 1.21	42.30 ± 1.08	41.59 ± 0.59	37.17 ± 1.25	36.86 ± 1.36
Sorbitol	28.33 ± 0.38	26.61 ± 0.08	26.08 ± 0.84	25.62 ± 1.08	25.86 ± 0.70
D-mannitol	43.78 ± 1.30	40.88 ± 0.56	41.14 ± 1.38	40.02 ± 0.51	40.56 ± 0.89

Sucrose-based proliposome formulations were found to exhibit significantly higher AOR values than proliposome formulations based on the other carriers ($p<0.05$) (Table 4.5). A massive increase was observed in AOR between coarse sucrose powder (21.47 ± 1.21°) and formulated proliposomes in a 1:10 and 1:15 w/w lipid to carrier ratio (42.30 ± 1.08 and 37.17° ± 1.25°) (Table 4.5), this indicated their *passable* flowability; and therefore reduced suitability compared to sorbitol-based proliposome powders (Table 4.4). Sucrose is noted for having a relatively smooth surface, with irregular or cubic shapes, permitting high concentrations of lipid to adhere to the particle surface. The high lipid content which was found to adhere to the particle surface resulted in a visibly fluffy surface appearance, which was observed to be excessively sticky (Payne et al., 1986b). This theory was further substantiated by results derived from Stewart assay (Stewart,

1980), confirming the highest concentration of lipid in sucrose-based proliposome formulations, when compared to alternative carbohydrate-based proliposome powders (Section 2.2.9 and 3.3.2). By comparison to sucrose and sorbitol, LMH coarse carriers elicited an AOR of 41.61 ± 3.71°, indicating *passable or adequate* flowability. LMH-based proliposome powders (i.e. BDP incorporated) in a 1:10 and 1:15 w/w lipid to carrier ratios showed *fair* flowability (39.64 ± 0.78° and 35.99 ± 0.78°, respectively) (Table 4.5).

Significant differences ($p<0.05$) in AOR values were observed between sorbitol-based proliposome formulations and all the other carrier-based formulations, demonstrating a much lower AOR (Table 4.5). Sorbitol was identified as a more flowable proliposome powder (when compared to the other carrier-based formulations), which may ultimately produce tablets with more favourable characteristics; in terms of appearance, hardness and disintegration time. Analysis of the remaining formulations (LMH, sucrose and D-mannitol formulations) with each other proved to be statistically insignificant ($p>0.05$), supporting the trend that there was little difference; suggesting or indicating unsuitability for use.

4.3.4. Selection of proliposome formulations for tablet manufacturing

The manufacture of tablets is dependent upon a number of variables, pertaining to the powder characteristics. The flow property of the powder is one such characteristic, as well as particle shape, size and morphology (Sinka et al., 2009).

Sucrose-based proliposomes were found to hold a large amount of lipid on their surfaces (as indicated by Stewart assay) (Section 2.2.9 and 3.3.2). The high level of lipid on carrier surfaces may account for agglomeration of the powder (Payne et al., 1986b). This may explain the poor flow properties of sucrose-based proliposomes, as indicated by AOR and tapped density values (Section 4.3.2 and 4.3.3). Good flowability is especially important during tablet manufacture, where the diameter of the die (i.e. 6 mm) is small, offering short filling time (resulting in uniform tablet weight); hence, good powder flow is highly advantageous (Szalay et al., 2014).

LMH-based proliposomes exhibited a high degree of cohesiveness or inter-particulate resistance, resulting in poor powder flow. A high level of particle resistance induces cohesion between the particles, and consequently inhibits the flow of powder (Szalay et al., 2014); which ultimately would dramatically influence the filling of Minipress tablet die during the manufacturing process. Differences in die filling are known to be a major cause of significant variation in tablet weight, in

a given batch (Mateo-Ortiz et al., 2014). The effects of such differences are deleterious to say the least; change in weight of tablet will change the quantity of drug administered to the patient, a matter of great concern for drugs with narrow therapeutic index ranges.

Surface morphology (as discussed in section 3.3.1) recognised sorbitol to possess a shape ideal for tablet manufacture. D-mannitol-based proliposomes were observed to be the second most preferable carrier for tableting. The aforementioned sections (Section 4.3.2 and 4.3.3) of tapped density and AOR were used as key indicators of powder flow properties. Both these key characteristics of powder flow have identified sorbitol and D-mannitol proliposomes possessing a superior flowability for the formulation in 1:10 and 1:15 w/w lipid to carrier ratios. As a consequence, these proliposome formulations at these ratios were carried forward for tableting of proliposomes.

During tablet manufacture, sorbitol-based tablets at the ratio of 1:10 w/w were prepared manually, whilst those at a 1:15 w/w lipid to carrier ratio were prepared automatically (i.e. 20 $\pm$ 4 tablets per minute). This was necessary, since at the 1:10 w/w ratio (where the lipid content was higher) tablets were found to adhere to the lower punch, thus automation of tablet manufacture was not possible. Whilst at lower lipid content (i.e. 1:15 w/w lipid to carrier ratio), the "stickiness" of the powder did not preclude the automated manufacture of proliposome tablets.

4.3.5. Surface morphology characterisation of proliposome tablets using SEM

SEM was implemented for qualitative analysis of shape, size, roughness and porosity of the tablets; comparing with the surface morphology of the proliposome powder prior (Section 3.3.1) and post compaction. Distributions of the drug, in addition to matrix element analysis are deemed important factors in formulation performance. Comparisons of BDP containing and BDP free formulations (tablets) were found to show no difference in surface morphology, indicating that BDP did not significantly affect surface morphology.

Whole tablet, in addition to tablet cross-sections were imaged at high resolution, to allow for examination of the structure and morphology, as well as comparison with other carried-based formulations investigated.

Upon SEM examination of sorbitol-based proliposomes (1:10 w/w lipid to carrier), the surface morphology of the tablets (Figure 4.1 a) imaged at 1 mm, exhibited a smooth appearance with presence of small pores or cracks. At a 100 and 20 µm scale magnification (Figure 4.1 b, c),

needles were found to protrude from the surface, minimising space present in the cracks. Full tablets were found to contain needles upon the tablet surface in cracks, indicating that a proportion of the carrier particles partially retained their shape (Figure 4.1 b, c). The presence of these cracks or pores may shorten the disintegration time, by facilitating the penetration of water into tablets with subsequent rupture of the tablet by disintegration. Cross-sections of the sorbitol tablets were suggestive that complete compression of the proliposome powder had occurred, the porous structure of the sorbitol particles may be accountable for the complete compaction observed in the sorbitol-based proliposome tablets (Figure 4.1 d, e, f). The effect of compression force on the proliposome powder is observable in the tablet morphology (Figure 4.1 c). This trend was observable upon examination of drug-free sorbitol-based proliposomes (1:10 w/w lipid to carrier) (Figure 4.1 and Figure 4.2). Moreover, only 2 mole% BDP to lipid phase was employed in the formulations; hence, the drug's effect on powder and tablet morphology was undetectable.

At the 1:15 w/w lipid to carrier ratio, sorbitol-based proliposome tablets (with or without BDP) elicited less roughness in terms of open cracks on the tablet surface. The low proportion of lipid to sorbitol in the formulation was confirmed using Stewart assay (Section 2.2.9 and 3.3.2, Table 3.1), moreover cracks on the tablet surface did not contain protruding needles in the tablets prepared from the 1:15 w/w lipid to carrier ratio. The reduced lipid content was sufficient to facilitate compaction of the proliposomes, without causing excessive stickiness of the proliposome powder to the punch surfaces; or causing weakness of the tablet. Regardless of BDP inclusion, proliposome tablets made using sorbitol as carrier with low lipid content (1:15 w/w lipid to carrier ratio), tended to elicit fewer "cracks" on the proliposome surface (Figure 4.3 b, c, e, f), compared to the formulation with higher lipid content (i.e. 1:10 w/w lipid to carrier) (Figure 4.4 b, c, e, f).

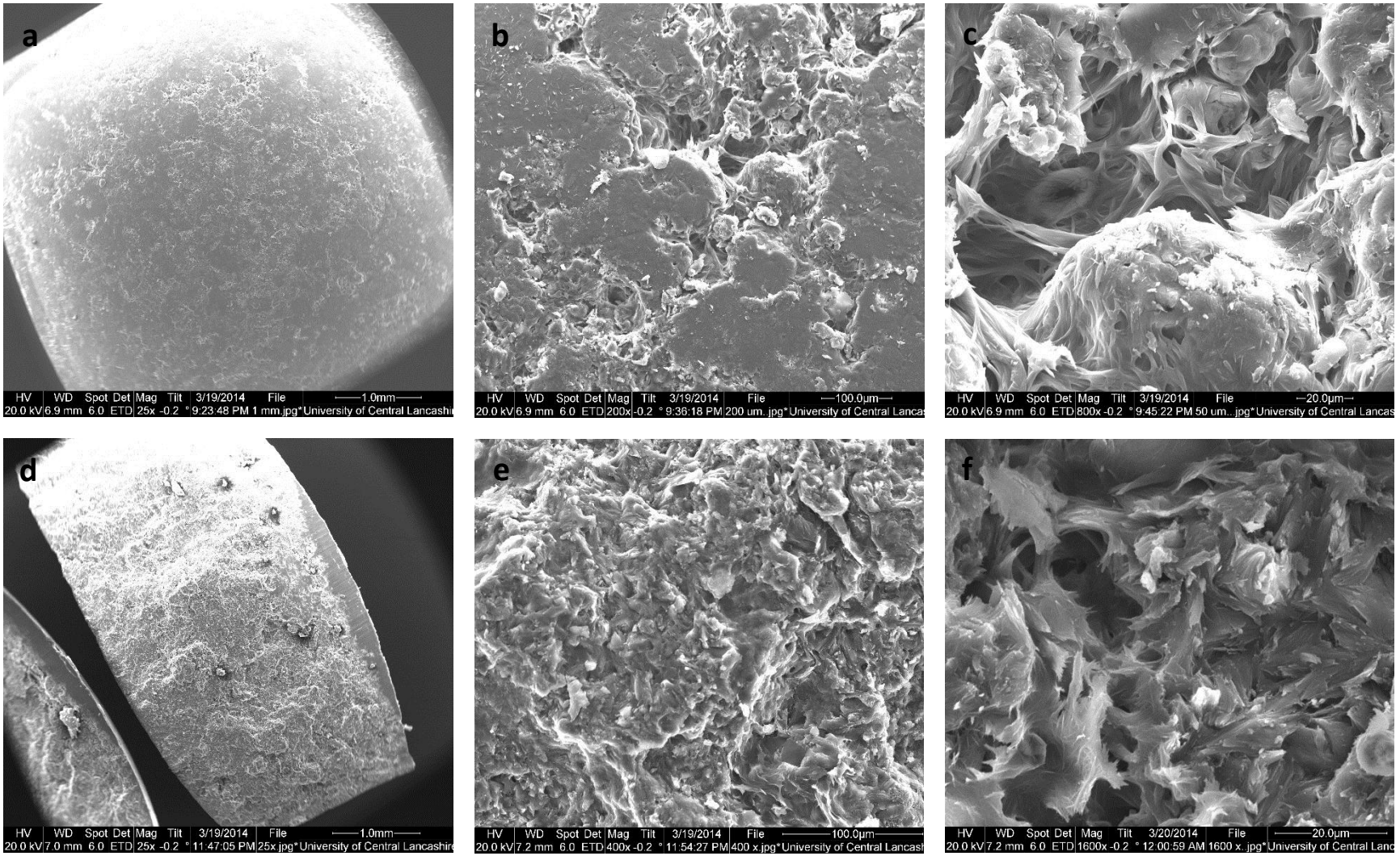


Figure 4.1: Sorbitol-based proliposome tablets in a 1:10 w/w lipid to carrier ratio with BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

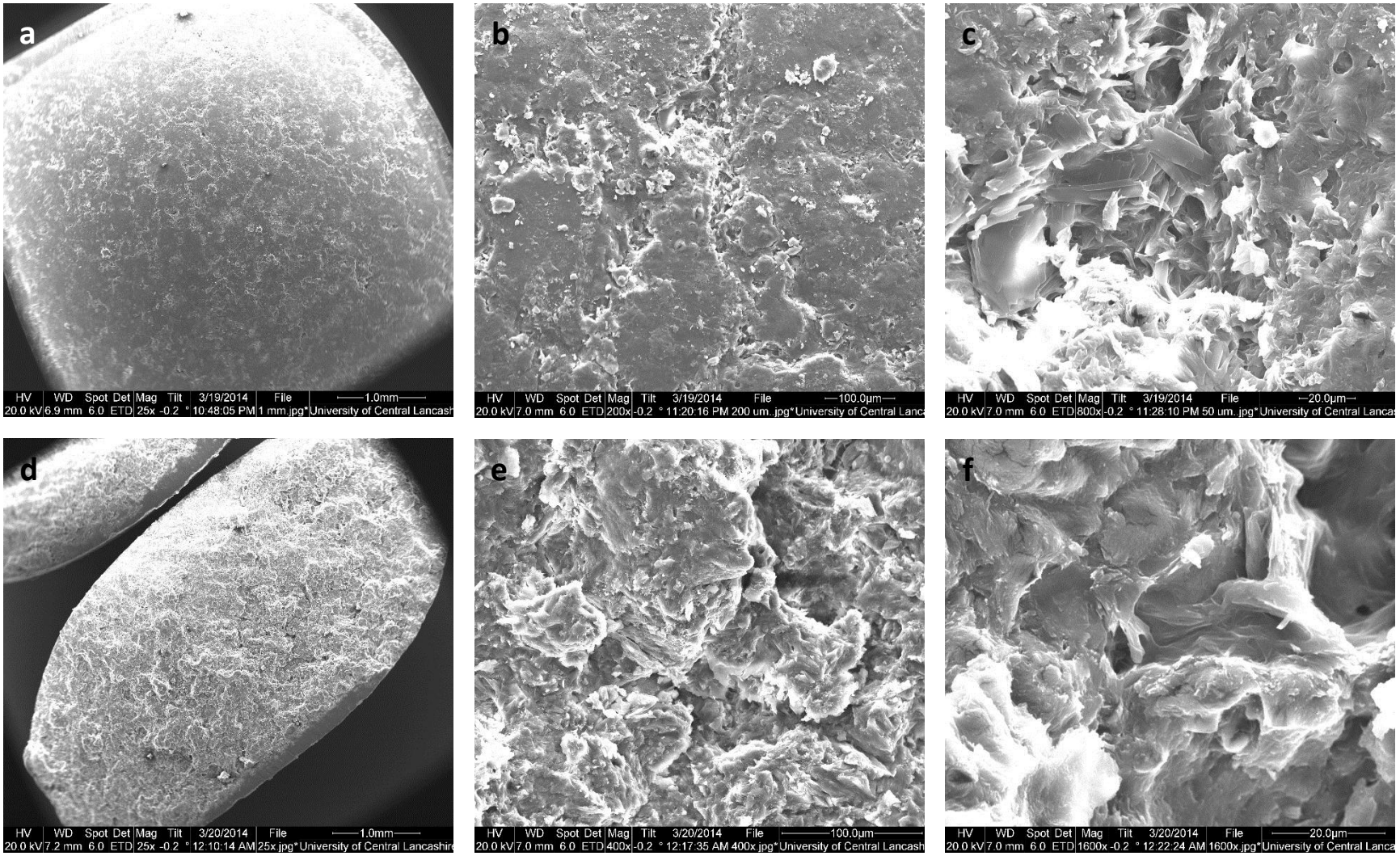


Figure 4.2: Sorbitol-based proliposome tablets in a 1:10 w/w lipid to carrier ratio without BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

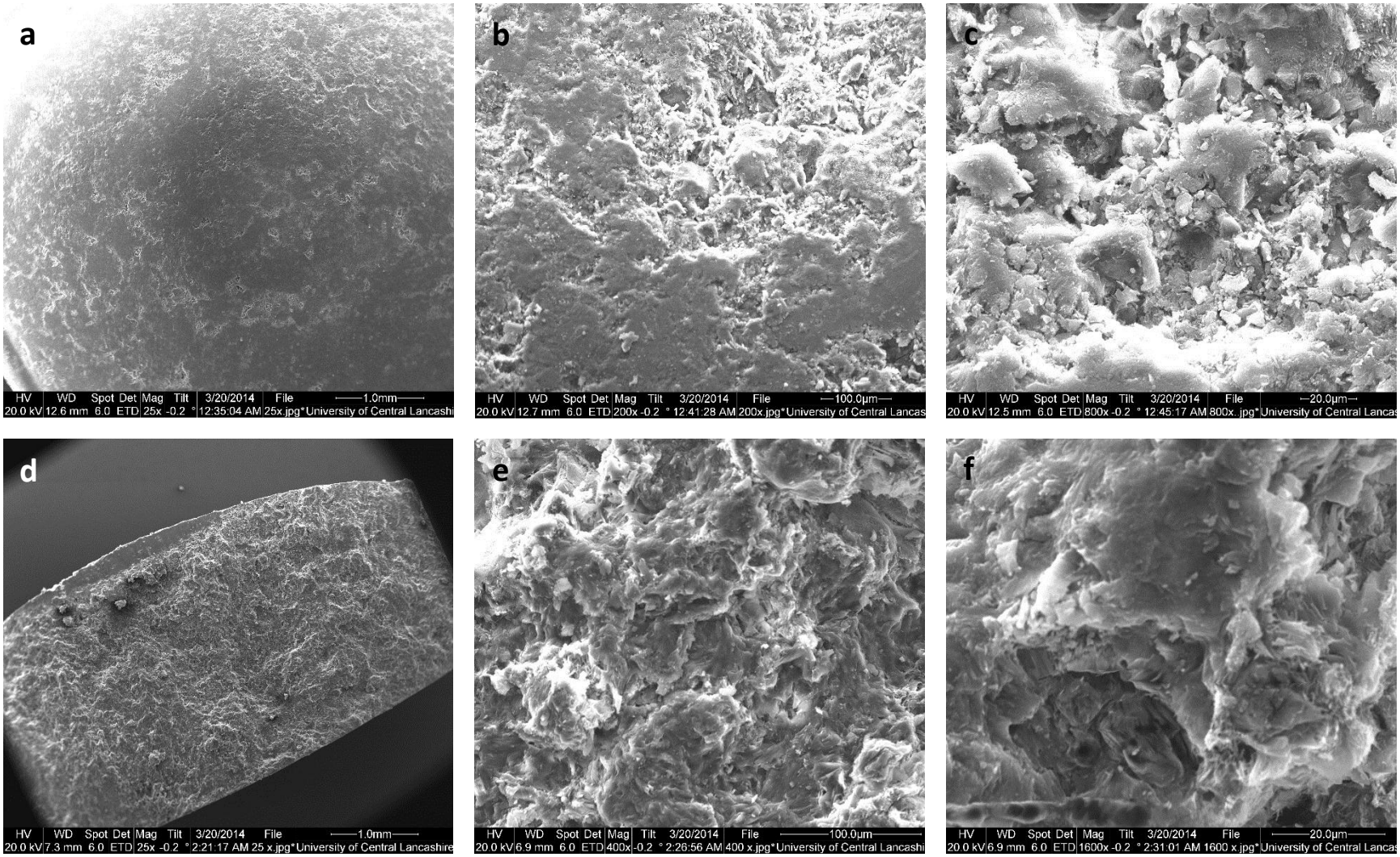


Figure 4.3: Sorbitol-based proliposome tablets in a 1:15 w/w lipid to carrier ratio with BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

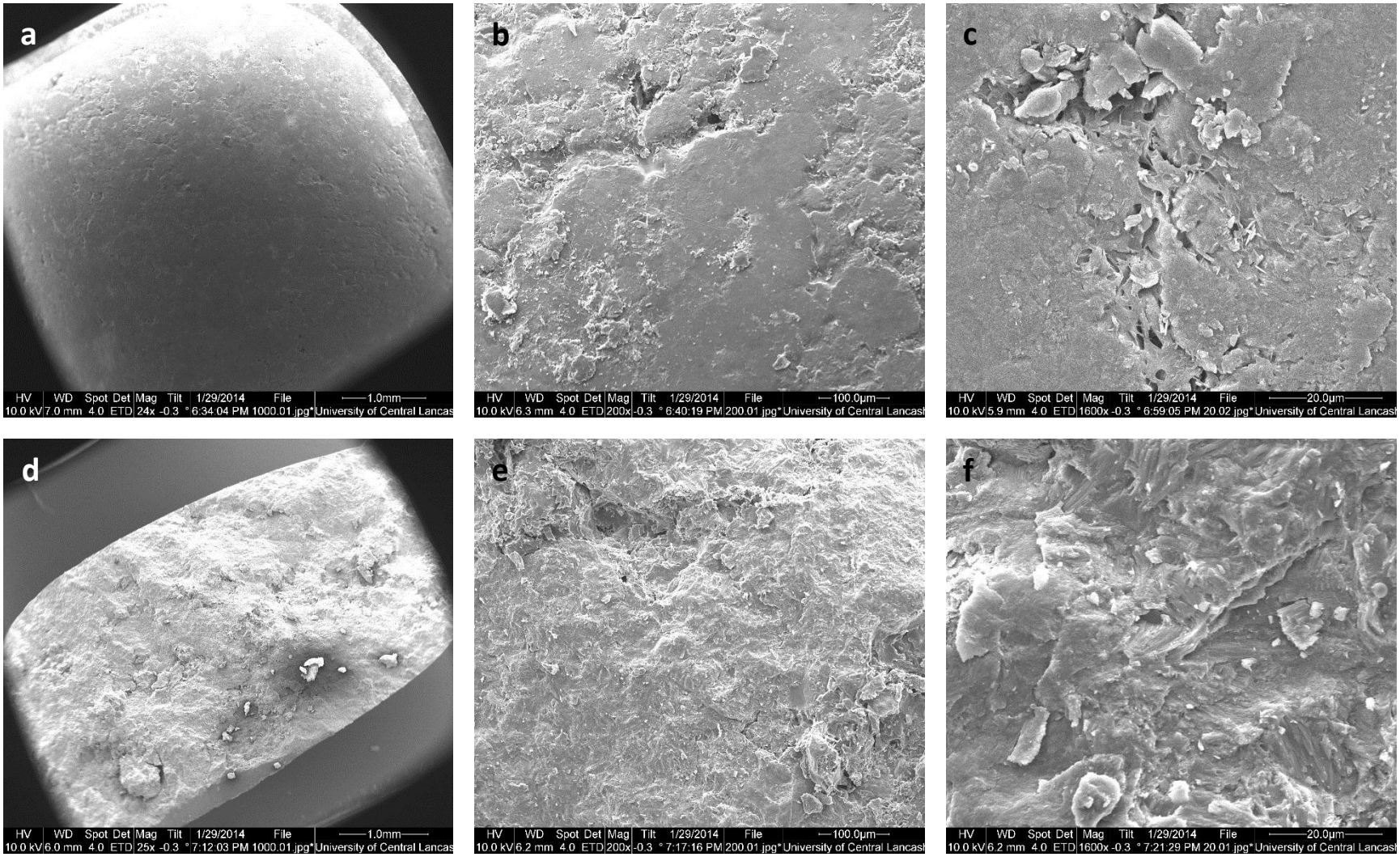


Figure 4.4: Sorbitol-based proliposome tablets in a 1:15 w/w lipid to carrier ratio without BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

When D-mannitol was used as carrier in proliposome formulations, differences in the appearance of tablets at 1:10 and 1:15 w/w lipid to carrier ratios were evident from the SEM images (Figure 4.5 and Figure 4.7). The high amount of lipid was visible on the surface of the entire tablet (Figure 4.5 b and 4.6 b). Additionally, since the same compression force was applied on both the (1:10 and 1:15 w/w ratio) D-mannitol-based proliposome powders. The difference in the characteristics of tablets is attributed to the concentration of lipid and carrier type in the formulations. D-mannitol-based tablets in a 1:10 w/w lipid to carrier ratio were found to adhere to the punches, and as SEM revealed, crystalline D-mannitol on various sections of the tablet surfaces was observed. XRD results in section 3.3.9 (Figure 3.17) also showed D-mannitol to be present in crystalline form in the formulated proliposome powders (i.e. 1:10 and 1:15 w/w lipid to carrier ratio), thus explaining the presence of crystals on the tablet surface.

Additionally, as a general rule in normal tablet (i.e. not proliposome tablets) manufacture, high compression force may break the crystals, hence decreasing the crystallinity of the sugar (Riippi et al., 2000). Moreover, surface smoothness is considered to be directly proportion to compression force (Matsumoto et al., 2007). Whilst it may promote the smoothness of tablet surfaces, high compression forces may also prolong the time needed for the tablet to disintegrate.

Regardless of drug incorporation in the formulation, D-mannitol-based proliposome tablets using the 1:15 w/w lipid to carrier ratio were found to have smoother surfaces and no cracks (Figure 4.7 a and Figure 4.8 a), compared to tablets manufactured using a 1:10 lipid to carrier (Figure 4.5 a and Figure 4.6 a). D-mannitol crystals in proliposome tablets formulated in a 1:15 w/w lipid to carrier ratio were less visible on the surface of tablet (Figure 4.7 b, c and Figure 4.8 b, c). In addition to the smoother tablet surface at the lower lipid to carrier ratio (i.e. 1:15 w/w), cross-sections of the tablets suggested improved compaction of the powder (Figure 4.7 d, e, f and Figure 4.8 d ,e, f).

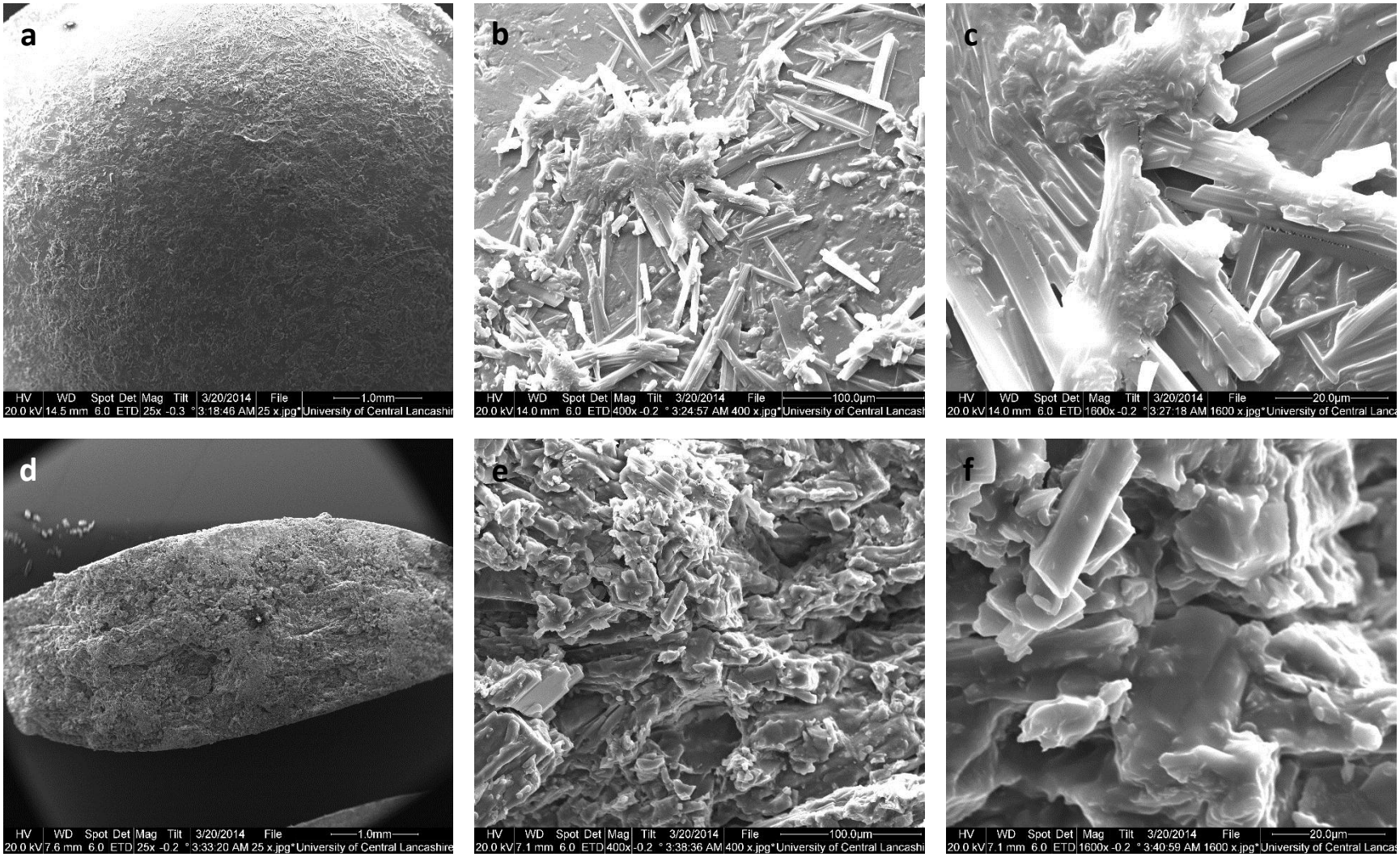


Figure 4.5: D-mannitol-based proliposome tablets in a 1:10 w/w lipid to carrier ratio with BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

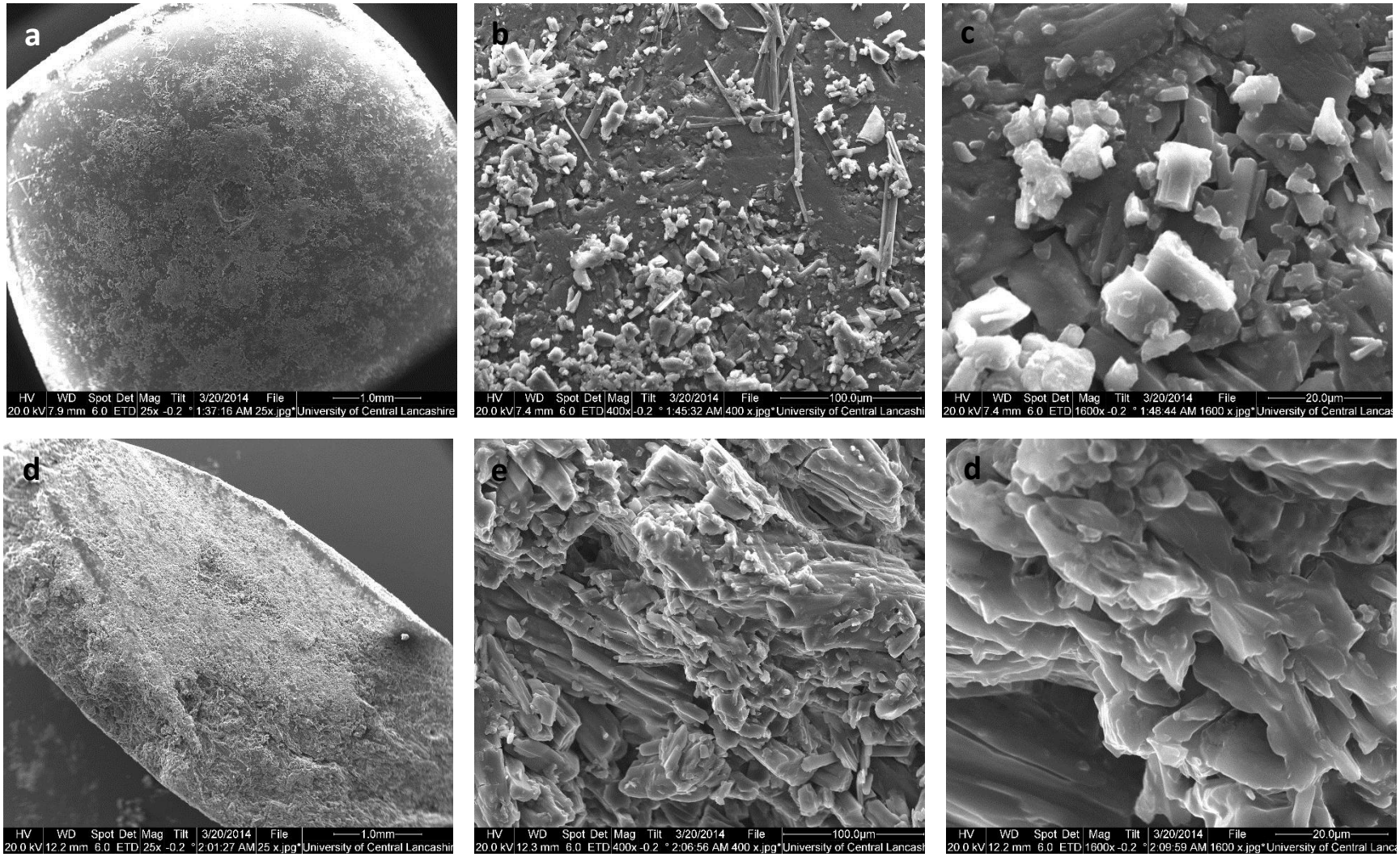


Figure 4.6: D-mannitol-based proliposome tablets in a 1:10 w/w lipid to carrier ratio without BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

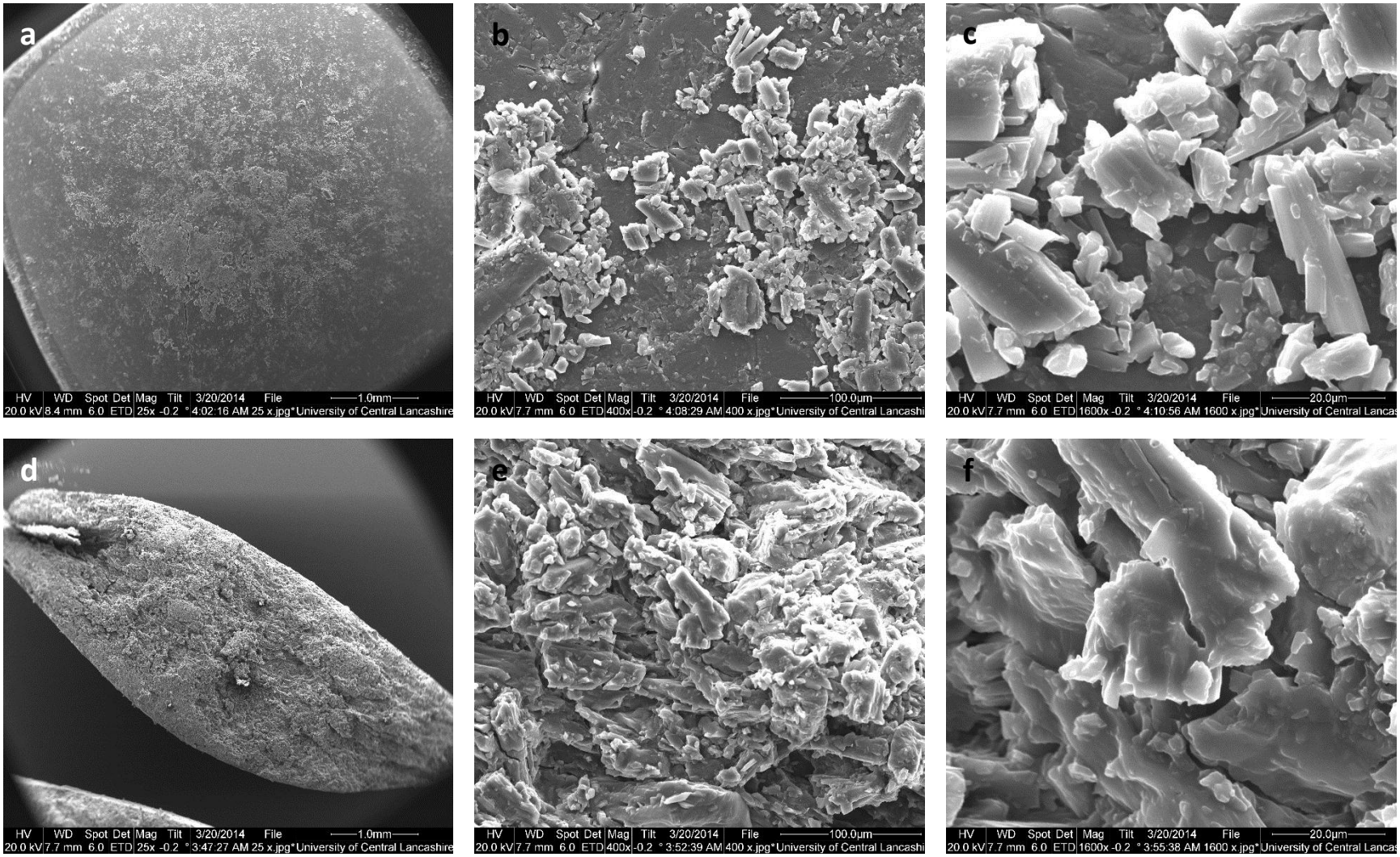


Figure 4.7: D-mannitol-based proliposome tablets in a 1:15 w/w lipid to carrier ratio with BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

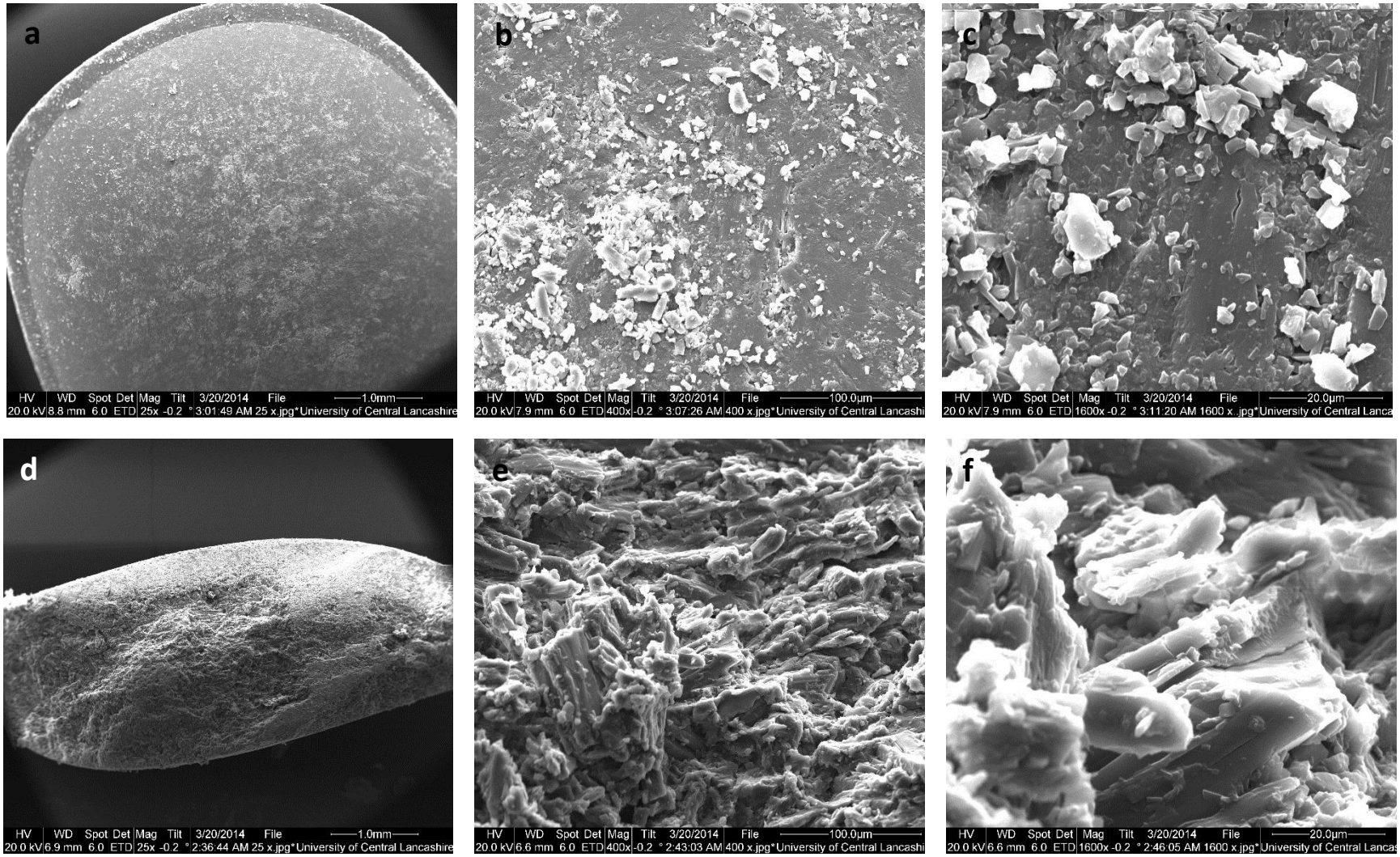


Figure 4.8: D-mannitol-based proliposome tablets in a 1:15 w/w lipid to carrier ratio without BDP, showing SEM images of the whole tablet at various magnifications; (a) 1 mm, (b) 100 µm, (c) 20 µm. Cross-section images of the same tablets with varied magnification; (d) 1 mm, (e) 100 µm, (f) 20 µm

Conclusively, general trends observed through SEM illustrated a high surface roughness for both sorbitol and D-mannitol formulations at the 1:10 w/w lipid to carrier ratio; compared to the 1:15 w/w formulation. In terms of compaction, sorbitol showed improved compaction compared to D-mannitol. Additionally, XRD results in section 3.3.9 (Figure 3.16 and Figure 3.17) demonstrated that both sorbitol and D-mannitol carriers on their own (i.e. in coarse form) and in proliposome formulations were in crystalline form. Examination of cross-sections of tablets indicated that sorbitol particles had higher deformability upon compaction, possibly due to their porous nature. Whereas, D-mannitol was deemed to retain its crystalline structure to a stronger degree upon compaction on surface. A reduction in lipid content (i.e. 1:15 w/w ratio formulations) improved powder flow for both carriers (sorbitol and D-mannitol), as evidenced by the AOR and tapped density experiments (Table 4.2,Table 4.3 and Table 4.5). This effect was further demonstrated by the tablet surface, which was notably smoother and crack-free on reduction of lipid content. For the aforementioned reasons, sorbitol-based tablets at the ratio of 1:15 were manufactured in a greater quantity, owing to its superior powder properties. Automated tableting was possible and conducted at the rate of 20 $\pm$ 4 tablets per minute with a compression force of 3.00 - 4.50 kN.

4.3.6. Weight variation/Uniformity of proliposome tablets

Tablets manufactured from sorbitol and D-mannitol-based proliposome powders at lipid to carrier ratios of 1:10 and 1:15 w/w (with or without BDP), were further analysed using official weight variation testing, as previously described in section 2.2.16.

Sorbitol-based proliposome tablets were prepared ranging in weight from 82 - 91.97 mg for both lipid to carrier ratios (with and without BDP) (Table 4.6). The tablet weight deviation range was set at $\pm$7.5% (for the 80 - 250 mg weight range of tablets), with not more than two tablets permitted to deviate from the average weight. In sorbitol-based proliposome formulations, only two tablets exhibited deviation from the average weight by $\pm$7.5%. Of the two tablets found, each belonged to each respective ratio i.e. 1:10 and 1:15 w/w, falling in the acceptable range according to BP standards (i.e. no more than two tablets deviating in each batch from the average tablet weight) (BP., 2010e). Thus, both batches were deemed to have passed weight variation testing.

Table 4.6: Weight variation tests for sorbitol and D-mannitol-based proliposome tablets in 1:10 and 1:15 w/w lipid to carrier ratios (with and without BDP). Data are mean ± SD, n=3; p<0.05

Carrier-based proliposome tablets	BDP-incorporated formulations (mg)		Empty formulations (mg)	
	1:10 (w/w)	1:15 (w/w)	1:10 (w/w)	1:15 (w/w)
Sorbitol	91.12 ± 2.66	90.17 ± 4.30	91.97 ± 2.81	82 ± 3.10
D-mannitol	64.91 ± 3.58	65.65 ± 2.98	63.55 ± 4.02	62.66 ± 3.06

D-mannitol-based proliposome tablets for both the 1:10 and 1:15 w/w lipid to carrier ratios weighed less than 80 mg (Table 4.6). According to the BP, the deviation range for such tablets is ±10% from the average weight (BP., 2010e). It was observed that four tablets demonstrated a deviation of ±10% in weight for the 1:10 w/w D-mannitol formulations (four tablets in each of BDP incorporated and BDP free tablets). Contrastingly, only one tablet showed deviation in excess of ±10%, for the 1:15 w/w D-mannitol formulation. D-mannitol proliposome tablets with a 1:10 w/w lipid to carrier ratio showed visibly higher roughness via SEM than the 1:15 w/w formulation ratio (Figure 4.5 a, b; and Figure 4.7 a, b). The same trend was observable for sorbitol-based proliposome tablets (Figure 4.1 a, b; and Figure 4.3 a, b). A possible reason for the noted difference may be attributed to the amount of lipid present.

Tablet weight variation within the accepted range (i.e. sorbitol-based proliposome tablets in a 1:10 and 1:15 w/w lipid to carrier ratio), indicates uniform distribution of lipid during proliposome preparation using the "Slurry" method; and good flow properties of the proliposome powder, as demonstrated by AOR and Carr's compressibility index. AOR values suggested an *excellent* flow rate for both the 1:10 and 1:15 w/w sorbitol-based proliposome powder formulations, whereas a *fair* flowability for D-mannitol-based proliposomes at the 1:10 and 1:15 w/w formulations was observed (Table 4.4 and Table 4.5). In addition, Carr's compressibility index and Hausner ratio in tapped density indicated *fair to good* flowability for sorbitol formulations, and *fair* flowability for D-mannitol formulations (Table 4.1,Table 4.2 and Table 4.3); indicating that these powders would flow well to fill the tablet die allowing for easy tableting.

4.3.7. Disintegration test of proliposome tablets

Disintegration testing was conducted as detailed in section 2.2.17. Sorbitol tablets formed from proliposome powders in a 1:10 w/w lipid to carrier ratio (with BDP), showed an average disintegration time of 8.67 ± 0.88 minutes (Figure 4.9). By contrast, the same formulation without BDP exhibited a disintegration time of 10.04 ± 0.73 minutes. Upon statistical analysis this difference was deemed to be insignificant ($p>0.05$). Similarly, sorbitol-based tablets with 1:15 w/w lipid to carrier ratio showed no significant difference ($p>0.05$) between formulation containing BDP (i.e. disintegration time was 4.95 ± 0.39 minutes), and those made without BDP (i.e. disintegration time was 5.58 ± 0.39 minutes) (Figure 4.9). This indicated that, when sorbitol was used as carrier, the presence of BDP did not significantly affect the disintegration time of the tablets for each lipid to carrier ratio investigated. Additionally, presence of 2mole% of BDP to the lipid phase in the 1:10 and 1:15 w/w lipid to carrier proliposome formulations, were found to be amorphous via XRD (Section 3.3.9; Figure 3.16 and Figure 3.17).

As lipid content is the sole varying factor between the 1:10 and 1:15 ratios, it may be suggested that lipid content has influenced the disintegration time. Higher lipid content may produce tablets with higher surface hydrophobicity, which resist water penetration and subsequently may lengthen the disintegration time of the tablets. Furthermore, proliposome tablets prepared with higher lipid content i.e. 1:10 w/w lipid to carrier ratio exhibited greater adherence to the punch and die surfaces during tableting; showing prolonged disintegration times. Thus, on comparison of sorbitol-based proliposome tablets made from the 1:10 and 1:15 w/w lipid to carrier ratios, a highly significant difference ($p<0.05$) of shorter disintegration time was observed for 1:15 w/w tablets. This difference was irrespective of the presence or absence of BDP in the tablets, made from the 1:10 (i.e. 8.67 ± 0.88 or 10.04 ± 0.73 minutes) and the 1:15 w/w formulation ratios (4.95 ± 0.39 or 5.58 ± 0.39 minutes) (Figure 4.9).

Lin et al., (2001) and Lowenthal (1972) have demonstrated that in normal tablets (not proliposome tablets), particle size distribution, compression force and choice of excipients are key parameters influencing the disintegration time (Lowenthal, 1972, Lin et al., 2001). Upon examination of SEM images (Section 4.3.5), the surface roughness for tablets prepared from a 1:15 w/w ratio proliposome powder was smoother than those prepared from the 1:10 w/w lipid to carrier ratio. The raised lipid content may be the reason behind the difference in surface characteristics of the tablets (Stewart, 1980). Surface characteristics may correlate with tablet dissolution characteristics, as the surface morphology heavily influences the behaviour of tablets in a given dissolution medium. The higher lipid content in the 1:10 w/w ratio offers an explanation

behind the extended disintegration time of the tablet, compared to those manufactured using the 1:15 w/w lipid to carrier.

The added difficulty of having to manually prepare sorbitol-based proliposome tablets in a 1:10 w/w ratio, may compromise their potential for large scale production. Whereas it was found that automated manufacture of sorbitol-based proliposomes was possible when low lipid content was used (i.e. for 1:15 lipid to carrier ratio).

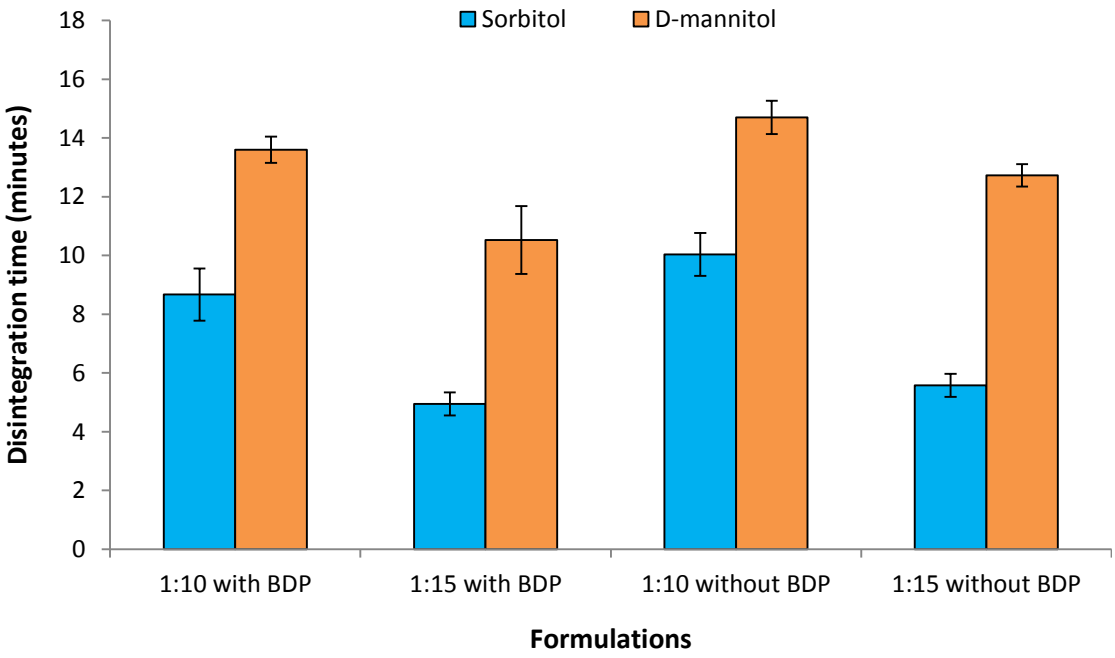


Figure 4.9: Disintegration times for sorbitol and D-mannitol-based proliposome tablets at 1:10 and 1:15 w/w lipid to carrier ratios; with or without the incorporation of BDP. Data are mean ± SD, n=3; p<0.05

In normal tablets, higher compression forces reduce porosity; and hence are known to prolong the disintegration time (Riippi et al., 1998). Whilst the increase in disintegration time is undesirable, high compression forces do produce a harder tablet (which is less liable to break). A commonly utilised technique to achieve desired tablet hardness without compromising the need for rapid disintegration is the addition of small quantities of disintegrants or super disintegrants (Colombo et al., 1981, Ibrahim, 1985, Riippi et al., 1998).

Differences in disintegration time were also observed for D-mannitol-based tablets, however these were not found to mimic the trends exhibited by sorbitol-based tablets. No significant difference ($p>0.05$) in the disintegration time was observed in proliposome tablets i.e. with or

without BDP incorporation in the 1:10 w/w lipid to carrier ratio. Similarly a trend of insignificance (*p>0.05*) in terms of disintegration was seen for the 1:15 w/w lipid to carrier ratio. However, a significant difference (*p<0.05*) was observed in the disintegration time between the 1:10 and 1:15 w/w lipid to carrier ratio formulations (i.e. 13.60 $\pm$ 0.45 minutes and 14.70 $\pm$ 0.57 minutes with and without BDP respectively for the 1:10 w/w D-mannitol-based proliposome tablets; and 10.53 $\pm$ 1.16 minutes and 12.73 $\pm$ 0.38 minutes with and without BDP for 1:15 w/w D-mannitol-based proliposome tablets correspondingly) (Figure 4.9). This confirmed that the high lipid content was the overriding reason in the extended disintegration time of proliposome tablets.

Upon direct comparison between both carriers, a highly significant difference was observed in terms disintegration time (*p<0.05*), suggesting that carrier selection plays a pivotal role in the disintegration time of tablets. The poor hardness demonstrated by D-mannitol tablets, may be attributed to the crystalline structure of D-mannitol, which was maintained even after tableting (Section 4.3.5); whereas sorbitol particles are known to deform completely (Section 4.3.5) forming harder tablets.

Sastry et al., (2000) also reported that sorbitol normal/coarse powder is highly mouldable in comparison to D-mannitol (Sastry et al., 2000). As a consequence of the poor hardness of D-mannitol-based proliposome tablets, they were unable to withstand the force applied by the hardness tester; therefore the values were below the detection limit. Additionally, D-mannitol-based proliposome tablets exhibited a longer disintegration time than sorbitol-based proliposome tablets (Figure 4.9), identifying them as unsuitable for tableting in terms of hardness and disintegration time.

The smaller weight of the D-mannitol proliposome tablets (i.e. less than 80 mg) (Table 4.6) potentially could have also improved the disintegration time; however the strong structure of the D-mannitol may have overrode this effect. Whilst disintegration times were higher for D-mannitol proliposome tablets, these were still found to disintegrate within the range specified by the BP.

To summarise, sorbitol was found to be the superior carrier for proliposome tableting, particularly at a ratio of 1:15 w/w lipid to carrier, as this was the only formulation which was able to withstand automated tableting by the Minipress tablet machine. Drug release from tablets is propagated by the process of disintegration. This facilitates the rapid fragmentation of tablets, allowing for quick release of drug and subsequent bioavailability (Zhao and Augsburger, 2005); as a consequence disintegration time is of paramount importance. In terms of disintegration, sorbitol was additionally found to be superior, showing a substantially shorter disintegration time; preferential for drug delivery.

4.3.8. Hardness testing/Crushing strength of proliposome tablets

Tablet hardness or crushing strength is known to play a key role in assessing tablets following manufacture. Crushing strength is almost directly related to the compression force employed during the tableting process, and is reported to drastically affect the mechanical properties of tablets produced (Riippi et al., 1998, El-Bagory et al., 2012).

Sorbitol-based proliposome tablets formulated at a 1:10 w/w lipid to carrier ratio were found to elicit hardness values of 76.97 ± 6.46 N for tablets with BDP and 92.47 ± 4.66 N for tablets without BDP (Table 4.7). Upon statistical analysis this difference was noted to be significant (*p<0.05*). However, no significant difference was observed between 1:15 w/w tablets formulation with and without BDP (120.67 ± 12.04N and 81.37 ± 9.26N).

Table 4.7: Crushing strength for sorbitol and D-mannitol-based proliposome tablets with and without the incorporation of BDP was measured in newtons (N) for 1:10 and 1:15 w/w lipid to carrier ratios. Data are mean ± SD, n=3; p<0.05

Crushing strength	1:10 with BDP	1:15 with BDP	1:10 without BDP	1:15 without BDP
Sorbitol	76.97 ± 6.46	120.67 ± 12.04	92.47 ± 4.66	81.37 ± 9.26
D-mannitol	0	0	0	0

Notably sorbitol-based proliposome tablets at a ratio of 1:15 w/w, showed the highest crushing values (i.e. 120.67 ± 12.04 N) out of all the formulations investigated for tableting. Whilst higher compression force does increase tablet strength; as discussed previously, it is also known to increase disintegration time (Figure 4.9). This direct relationship between the compression force and disintegration time was also suggested in normal tablets (Riippi et al., 1998). However, whilst sorbitol-based proliposome tablets made from the 1:15 w/w lipid to carrier ratio powders (with and without BDP), showed a higher crushing strength than proliposome tablets made from the 1:10 w/w ratio formulations. Disintegration time was still found to be well within the acceptable range (i.e. 4.95 - 5.58 minutes) specified by BP for standard tablets (i.e. not more than 15 minutes) (BP., 2010d) (Figure 4.9). The porous sorbitol particles were able to pack well by

compression in normal tablets, due to their high moulding properties (Sastry et al., 2000, Mizumoto et al., 2005). This is in agreement with the findings generated using sorbitol-based proliposomes tablets.

D-mannitol-based proliposome tablets at both ratios (1:10 and 1:15 w/w lipid to carrier), were unable to withstand the crushing strength of the hardness tester, showing poor hardness. This may be associated with the fact that a normal/coarse mannitol powder has low compressibility (Mizumoto et al., 2005). Moreover, whilst D-mannitol-based proliposome tablets were notably softer, they had much longer disintegration time (Figure 4.9). This was longer than disintegration times noted for sorbitol-based proliposome tablets (which are substantially harder tablets). These findings disagreed with previous studies when normal/coarse mannitol (without proliposome formulation) was reported to have low moulding upon compression, with subsequent short disintegration time (Sastry et al., 2000, Mizumoto et al., 2005).

Overall, sorbitol proliposome tablets were found to be more porous, and the reduced lipid content in the 1:15 w/w lipid to carrier ratio was found to be beneficial, with respect to the majority of the tablet testing parameters investigated; including hardness.

4.3.9. Friability of proliposome tablets

The surface roughness of tablets is considered as a crucial factor that can influence tablet friability (Riippi et al., 1998). Upon analysis, sorbitol-based proliposome tablets (BDP incorporated) demonstrated a significantly ($p<0.05$) lower friability (0.17 - 0.19%), when compared to D-mannitol-based proliposome tablets (0.24 - 0.42%) (i.e. irrespective of the lipid to carrier ratios) (Figure 4.10).

Additionally, tablets produced from sorbitol-based proliposome powders were found to be harder than D-mannitol-based tablets (Table 4.7). This may be attributed to the ability of sorbitol-based powders to deform more readily, due to their porous nature; than D-mannitol-based proliposome powders. Tablets possessing high levels of hardness are known to exhibit lower friability, as shown in Figure 4.10.

In general, the crushing strength of tablets is directly proportional to the force of compaction used. However excessive force of compaction may not further increase the tablet hardness, and in fact, it may even result in promoting tablet friability.

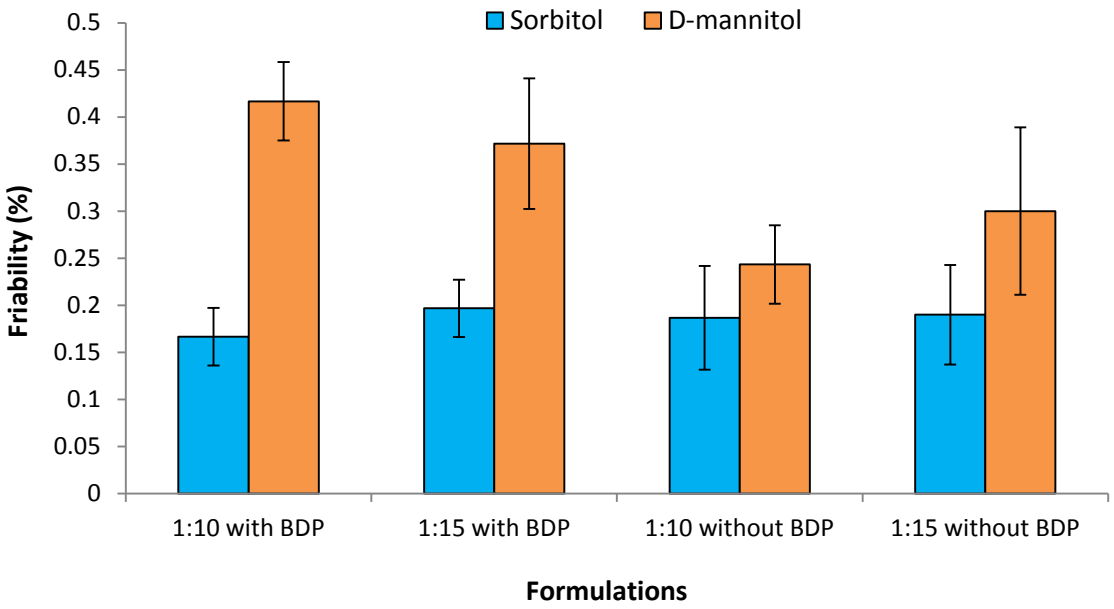


Figure 4.10: Percentage friability loss in sorbitol and D-mannitol-based proliposome tablets recorded for lipid to carrier ratios of 1:10 and 1:15 w/w, utilising tablets with and without BDP. Data are mean ± SD, n=3; p<0.05

Differences between sorbitol-based proliposome tablets at lipid to carrier ratios of 1:10 and 1:15 w/w, were found to be statistically insignificant (*p>0.05*), showing friability values of 0.17 ± 0.03% and 0.19 ± 0.06% for the 1:10 w/w ratio formulation (with and without BDP respectively); and values of 0.20 ± 0.03% and 0.19 ± 0.05% in 1:15 lipid to carrier tablets (with and without BDP respectively).

D-mannitol Proliposome tablets as shown in section 4.3.8 (Table 4.7), were found not to be able to withstand the force applied by the hardness tester, yet still showed a higher disintegration time and friability than sorbitol-based proliposome tablets (Figure 4.9). These findings contradict findings reported by Kumar et al., (2009), who demonstrated that normal tablets, manufactured using normal/coarse mannitol as diluent, exhibited lower friability with lower compression force (Kumar et al., 2009).

A significant difference (*p<0.05*) was found in friability for D-mannitol-based proliposome tablets at a 1:10 w/w lipid to carrier ratio, when BDP was incorporated and omitted (i.e. 0.42 ± 0.04% and 0.24 ± 0.04%), showing a higher level of friability for tablets with BDP. The same high friability tendency was observed for the lipid to carrier ratio of 1:15 w/w D-mannitol-based proliposome tablets (0.37 ± 0.07% with BDP and 0.3 ± 0.09% without BDP); however no significant difference was observed (*p>0.05*).

The higher friability in D-mannitol-based proliposome tablets in both the 1:10 and 1:15 w/w lipid to carrier ratios, may be attributed to the tendency of D-mannitol to retain its crystalline shape. Additionally D-mannitol crystals were also found on the tablet surface, as noted in section 4.3.5. This may cause the resultant tablets to become more fragile and exhibit higher friability.

In conclusion, on investigation of tablet friability, tablets prepared from sorbitol-based proliposomes showed the lowest loss during friability testing, particularly when using the 1:15 w/w lipid to carrier ratio. This correlates well with the findings reported by Shang et al., (2013), who stated that normal tablets (i.e. not proliposome tablets) should be strong enough to demonstrate minimal friability and retain shape and weight during production, packaging and storage (Shang et al., 2013).

4.3.10. Entrapment efficiency of BDP in liposomes generated from proliposome tablets

For entrapment efficiency studies, both sorbitol and D-mannitol-based proliposome tablets were triturated in a polythene bag followed by hydration as outlined in section 2.2.3. Following annealing and separation, BDP present in the formulations was identified and quantified using HPLC (Sections 2.2.10 and 2.2.11).

Liposomes generated from sorbitol-based proliposome tablets with 1:10 and 1:15 w/w lipid to carrier ratios, exhibited entrapment efficiencies of 53.82 $\pm$ 6.42% and 53.05 $\pm$ 7.24%. Hence, sorbitol tablets of 1:10 and 1:15 w/w ratios were deemed to have shown no significant difference ($p>0.05$) (Figure 4.11). The entrapment efficiencies in liposomes generated from D-mannitol-based proliposome tablets were found to contain 39.90 $\pm$ 4.30% and 35.22 $\pm$ 6.50% BDP, showing no significant difference ($p>0.05$). Furthermore, no significant difference ($p>0.05$) was also found with respect to BDP concentration for the drug crystal collected in spot (during separation via bench centrifuge explained in section 2.2.5 and 2.2.6); which contained free BDP crystals from the hydrated sorbitol tablets, prepared from 1:10 and 1:15 w/w lipid to carrier ratios (i.e., 43.01 $\pm$ 7.29% and 43.55 $\pm$ 6.89%). A similar trend was observed for the D-mannitol-based proliposome tablets at the 1:10 and 1:15 w/w lipid to carrier ratios, in terms of BDP spot concentration (58.19 $\pm$ 4.46 % and 61.85 $\pm$ 7.35%) (Figure 4.11).

Upon comparison of sorbitol and D-mannitol proliposome tablets; entrapment efficiency was found to be greater for sorbitol-based tablet formulations, showing a statistically significant difference ($p<0.05$) for both 1:10 and 1:15 w/w lipid to carrier tablets. This higher entrapment

efficiency may be explained by the porous nature of sorbitol, which uptake the lipid phase in the large surface area presented by the void spaces of sorbitol particles (Section 2.2.4). This consolidates the merit of sorbitol as a highly suitable carbohydrate carrier for manufacturing proliposome based tablets.

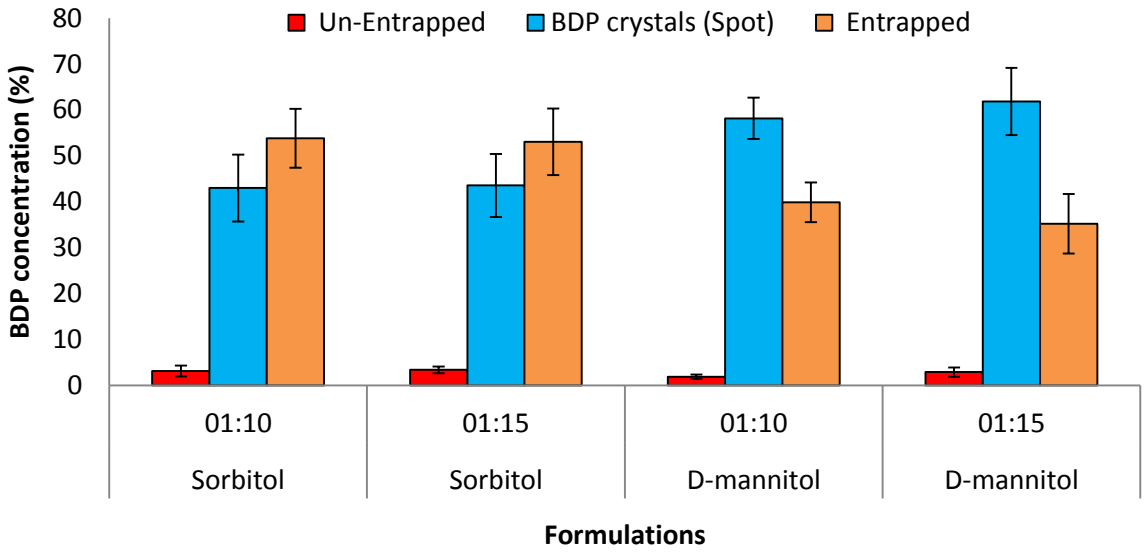


Figure 4.11: BDP concentration analysed for the entrapped, unentrapped and drug spot containing free BDP crystals; via HPLC for both sorbitol and D-mannitol-based proliposome tablets in lipid to carrier ratios of 1:10 and 1:15 w/w. Data are mean ± SD, n=3; p<0.05

4.4. Conclusions

Proliposome powder formulations utilising LMH, sucrose, sorbitol or D-mannitol were prepared in 1:5, 1:10 and 1:15 w/w lipid to carrier ratios in large quantities, via the "Slurry" method; and subsequently analysed prior to tablet manufacture. All proliposome powders (i.e. LMH, sucrose, sorbitol or D-mannitol) formulated in 1:5 w/w lipid to carrier ratios were densely coated with lipid and showed agglomeration, attributed to the sticky nature of lipids, as examined by SEM images and further substantiated by Stewart assay. Proliposome powders formulated from the same 1:5 w/w lipid to carrier ratio also showed poor compressibility, possibly due to the low density of the particles; and the soft lipid surface morphology. Additionally, entrapment efficiencies of BDP for 1:5 w/w proliposome powders were significantly lower than proliposome powders made using the 1:10 and 1:15 w/w lipid to carrier ratios ($p<0.05$) (Chapter 3). Irrespective of carrier type, the properties shown by lipid to carrier ratio in the 1:5 w/w formulations were deemed to be inappropriate for tablet manufacture and their subsequent liposomal drug delivery.

The presence of lipid causes greater surface adhesion in comparison to coarse carbohydrate carriers. This was confirmed via tapped density and angle of repose for the 1:10 and 1:15 w/w lipid to carrier ratio powders. Carr's compressibility index and Hausner ratios via tapped density identified the best proliposome formulation in terms of powder flow in the following order;

1:10 w/w lipid to carrier ratio with BDP *LMH > Sorbitol > D-mannitol > Sucrose*

1:15 w/w lipid to carrier ratio with BDP *Sorbitol > D-mannitol > Sucrose > LMH*

However, interestingly, angle of repose identified the better proliposome powder formulation in the following order;

1:10 w/w lipid to carrier ratio with BDP *Sorbitol > LMH >D-mannitol > Sucrose*

1:15 w/w lipid to carrier ratio with BDP *Sorbitol > LMH > Sucrose > D-mannitol*

Sorbitol was identified as the best proliposome powder formulation when using both the 1:10 and 1:15 w/w lipid to carrier ratios, which indicated excellent powder flowability. Sucrose-based proliposome powders with 1:10 and 1:15 w/w lipids to carrier ratios were the most poorly performing formulation in terms of proliposome powder flowability and compressibility. In addition, the compression force required to compress sucrose-based proliposome powders, resulted in picking and capping of the manufactured tablets; in all three lipid to carrier ratios (i.e. 1:5, 1:10 or 1:15 w/w). LMH-based proliposome powders, though possessing smaller powder particles were found to exhibit the same issues as sucrose-based formulations (i.e. poor flowability and compressibility). LMH-proliposome powders also exhibited comparatively less entrapment efficiency for BDP (Chapter 3) unlike sucrose; for all three lipid to carrier formulation ratios. D-mannitol-based proliposome powders showed passable flowability characteristics with satisfactory compressibility indicating unsuitability for tableting.

Sorbitol-based proliposome tablets showed a higher crushing force (hardness) than D-mannitol-based proliposome tablets; whilst D-mannitol proliposome tablets were found not to withstand the crushing force of the hardness tester. However, though harder, sorbitol-based proliposome tablets still demonstrated a shorter disintegration time than D-mannitol-based proliposome tablets. In addition, sorbitol-based proliposome tablets showed less friability than D-mannitol-based proliposome tablets. D-mannitol-based proliposome tablet's high friability was expected due to lower compression force used for tableting. The surface roughness of the tablets was substantiated by SEM images, which allowed the observation of roughness in the form of thin cracks at a high magnification, which may be influential on the surface properties of the tablets i.e. water penetration and disintegration.

Finally, the key parameter, entrapment efficiency of BDP, highlighted an inversely proportional relationship between lipid content and entrapment efficiency of BDP. High lipid concentration in the 1:10 w/w ratio formulation exhibited lower entrapment than the 1:15 w/w ratio. Interestingly, a high entrapment was found in sorbitol-based proliposome tablets with the 1:15 w/w lipid to carrier ratio. The same sorbitol-based proliposome tablets exhibited a superior surface morphology (being able to accommodate the lipid phase more readily), higher flowability, lower friability, shorter disintegration time and higher drug entrapment efficiency. This was contrary to expectation, since higher lipid content would generally promote drug entrapment, whereas the opposite has been illustrated here. This is indicative that the carbohydrate carrier has a prominent role in drug entrapment, as when carrier concentration is increased, entrapment efficiency was enhanced (this trend would require further investigation).

In general, it was concluded that the sorbitol 1:15 w/w lipid to carrier ratio proliposome tablets, complied with all official quality control tests for tablets. Additionally, these "proliposome tablets" were the only formulation that was prepared using the automated function of the Minipress machine. This provides scope for large scale industrial manufacture of proliposome tablets, which can be hydrated; and then subsequently delivered to patients via medical nebulizers.

CHAPTER 5: AIR-JET NEBULIZATION OF PROLIPOSOME POWDERS AND TABLETS

"To know what you know and to know what you don't know, that is real wisdom"

- Confucius -

5.1. Introduction

Swallowing difficulties are well known to cause poor compliance with administration of solid dosage forms, such as tablets and capsules. Moreover, alternative delivery approaches, such as; the use of parenteral administration, are not preferable due to the associated pain. The high rates of non-compliance resulting from the aforementioned reasons and many more, often result in ineffective therapy outcomes. However, recent advances in drug delivery systems have endeavored to enhance the efficacy of API's, as well as offering alternative routes of administration, which ultimately achieve enhanced patient compliance (Kumar et al., 2009).

Aerosol formulations have been commonly implemented to achieve drug delivery of therapeutic agents directly to the pulmonary system, in the treatment of immune mediated and other respiratory diseases (Waldrep, 1998). The respiratory system offers a large surface area which facilitates rapid absorption into the circulation system (Farr et al., 1987, Adjei and Gupta, 1994). Additionally, the majority of inhaled macromolecules offer low oral bioavailability, due to enzymatic degradation and low permeability (Jorgensen and Nielson, 2010). Moreover, evolving technologies have been developed for drug delivery, including: DPIs and pMDIs; where the formulations are required to meet stringent requirements. The usage of nebulizer to aid in pulmonary drug delivery eliminates the aspect of patient competence in the use of administration devices, as the aerosol generated via nebulizers is achieved using an air compressor.

Whilst devices are known to play a pivotal role in drug delivery, drugs may be formulated in various manners in order to aid and modify drug delivery. Liposomes are known for their ability to encapsulate APIs, modifying their release in the pulmonary system, for the treatment of respiratory conditions (Taylor and Fan, 1993). As pulmonary delivery carriers, liposomes are associated with a number of benefits, including: their ability to encapsulate/entrap hydrophilic and hydrophobic drugs, resulting in a reduction in local irritation of the tissue in contact with the formulation, uniform drug deposition in the lung; as well as reduced systemic and local toxicities (such as candidiasis and dyspnoea associated with glucocorticoids) (Darwis and Kellaway, 2001) and sustained drug release (Parthasarathy et al., 1999). As mentioned previously, devices such as inhalers are associated with a number of steps in formulation and administration. However, nebulizers have been demonstrated to successfully deliver liposomes with enhanced pulmonary deposition; enabling their sustained release and slow clearance of the therapeutic agent (Niven and Schreier, 1990, Saari et al., 1999).

The characteristics of aerosol formation from liposome formulations are notably dependent on a myriad of factors, such as; nebulizer design, nebulization and sputtering times, aerosol output and the size of the aerosol droplets produced throughout nebulization (Niven et al., 1991). Aside from influence from aerosol formulation, the composition of liposome formulation is equally important. The selection and nature of the chosen phospholipid, as well as their concentration greatly dictates the resultant aerosolised droplets, which impacts heavily upon the degree of lung deposition and subsequent success of therapy (Niven and Schreier, 1990). This is supported by the atomisation theories which predict that aerosol size and output characteristics are dependent not only upon operating principle and design of the nebulizer, but also upon the physicochemical properties of the nebulized fluid (Mercer, 1981).

Whilst highly noted for their ability to modify drug release and aid in drug delivery, liposomes are associated with stability issues. This may entail drug leakage, liposome fusion and aggregation upon prolonged storage. Leakage is identified as a critical issue during formulation of liposomes for nebulization, which may partially or completely eliminate the therapeutic advantages of using liposomes via inhalation (Niven and Schreier, 1990). Combative actions against stability problems have included freeze drying (lyophilisation) of liposomes prior to nebulization (Darwis and Kellaway, 2001). However, lyophilisation has also been reported to cause drug leakage; resulting in poor feasibility as an industrial process (Desai et al., 2002). A facile approach to overcome these problems was achieved by the introduction of proliposomes (Payne et al., 1986b). Proliposome formulations involve the incorporation of carbohydrate carriers which are coated with lipid, to aid in prolonging the stability of the formulation; essentially resulting in a dry formulation, which may be hydrated to produce stable liposomes.

The growing popularity of liposomes and the need for novel pulmonary formulations form the foundation of research for this chapter. This chapter outlines research, which was conducted to evaluate the effect of different proliposome preparations e.g. proliposome powder and tablets prepared from sorbitol and/or D-mannitol powders in 1:10 and 1:15 w/w lipid to carrier ratios; on nebulization.

Performance of both carriers for subsequent nebulization of the hydrated formulations was assessed using a Pari LC Sprint air-jet nebulizer. Nebulizer performance was further analysed by evaluating the sputtering time, output rate and mass output. The aerosol generated from the Pari LC Sprint air-jet nebulizer was also examined for particle size (also referred to as VMD), size distribution (SPAN) and fine particle fraction (FPF); utilising a Malvern Spraytec (laser diffraction technology). Liposomes size analysis was conducted prior and post-nebulization to allow for

comparison. A TSI was also utilised to measure the size of liposome and BDP concentration in the nebulizer reservoir, and upper and lower compartments of the TSI.

5.2. Methodology

5.2.1. Proliposome formulation

Proliposomes were prepared utilising sorbitol and D-mannitol carbohydrate carriers in 1:10 or 1:15 w/w lipid to carrier ratios. Weights of excipients per ratio were as follows: For the 1:10 ratio, the lipid content was 250 mg and carrier content was 2,500 mg. Whereas for the 1:15 ratio; the lipid content was maintained at 250 mg and the carbohydrate carrier content was elevated to 3,750 mg. A drug concentration of 2 mole% of BDP to lipid phase (i.e., 4.48 mg of BDP) was maintained for both formulations (Table 2.1). SPC and cholesterol made up the lipid phase in a 1:1 mole ratio. These formulations were prepared according to section 2.2.2, in a RBF via the novel "Slurry" method, introduced in this project. Post-manufacture, proliposome formulations were stored at -18°C to ensure stability.

5.2.2. Proliposome tablet manufacture

Following preparation of sorbitol and D-mannitol-based proliposome powders, the respective formulations were then manufactured into tablets using a Riva Minipress tablet machine (Section 2.2.15). As stated sorbitol-based tablets in the 1:15 w/w ratio were prepared through the automated function, whereas all other formulations were tabletted manually.

5.2.3. Proliposome hydration for aerosolisation via a Pari air-jet nebulizer

For the purpose of nebulization, 150 mg of a given proliposome formulation (i.e. proliposome powder or tablets) was transferred in to a reservoir chamber of a Pari LC Sprint air-jet nebulizer (i.e. containing Pari Turbo Boy Master Compressor). This was followed by the addition of 5 ml DW producing a liposome suspension with concentration of 30 mg/ml; identical volume as utilised by

Steckel and Eskandar (2003) and Elhissi et al., (2007). The produced liposome suspension was subsequently employed for assessment of nebulization performance (Steckel and Eskandar, 2003, Elhissi et al., 2007). Nebulization studies were conducted on all proliposome formulations (i.e. powder and tablets) for both carriers at lipid to carrier ratios of 1:10 and 1:15 w/w. Following nebulization, the reservoir chamber was examined for the presence of solid residues, after which findings were recorded. All formulations were nebulized in triplicate.

5.2.4. Determination of nebulizer performance

Nebulization time, was defined as the total time required for the entire liposome suspension to be nebulized. This time frame also included sputtering time (intermittent nebulization behaviour of the machine in the final few minutes, prior to complete formulation nebulization), which was also recorded (Section 2.2.22).

5.2.5. Aerosol mass output and output rate

Aerosol mass output was determined gravimetrically by calculating the difference between the weight of nebulizer containing liposomal suspension, prior and post complete nebulization (referred to as time to "dryness") (Section 2.2.22). The percentage mass output was then established according to the following equation 5.1:

$$Mass\ output(\%) = \left(\frac{Weight\ of\ nebulized\ formulation}{Weight\ of\ formulation\ present\ in\ the\ nebulizer\ prior\ to\ nebulization}\right) X100 (Equ\ 5.1)$$

Aerosol output rate was determined in accordance to section 2.2.22, where the amount of nebulized formulation was analysed and measured until complete nebulization (i.e. "dryness") was achieved. The efficiency of air-jet nebulizer was than calculated in mg/minute for all formulations via the following equation 5.2:

$$Aerosol\ output\ rate = \left(\frac{\text{Weight of nebulized formulation}}{\text{Complete nebulization time}}\right) \quad \dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots\dots . (Equation\ 5.2)$$

5.2.6. Size analysis of aerosol droplets via laser diffraction

Size or VMD (50% undersize), and size distribution (SPAN), were both determined for the aerosols via a Malvern Spraytec instrument; which employs laser diffraction technology to conduct size analysis, as illustrated in section 2.2.7. Particles with a 5.4 µm size are denoted as "respirable" fraction or fine particle fraction (FPF), and were measured in percentage. All measurements were taken for the complete duration of nebulization, at intervals of 1, 10, 20 and 25 minutes. Moreover, the nebulizer was kept at a fixed position to ensure uniform aerosol droplet generation and measurement, while the generated aerosol cloud passed through the centre of the Spraytec's laser beam.

5.2.7. Pulmonary drug deposition assessment via Two-stage Impinger

A TSI was used (an artificial lung model, representing the upper and lower stages as the upper and lower respiratory tract), in order to collect the nebulized formulation as it would deposit in the upper and lower stages of the TSI. The respective compartments were filled with 7 and 30 ml of the DW prior to nebulization (Section 2.2.20). Liposome suspensions (5 ml) were then transferred to the air-jet nebulizer for aerosol production. The nebulizer was then placed next to the mouthpiece adapter of the TSI, to ensure that the entire liposome formulation was nebulized into the impinger. Aerosol samples were then collected from both the upper and lower compartments (i.e. stages), and analysed for drug content using HPLC (Section 2.2.10 and 2.2.11).

5.2.8. Liposome characterisation prior and post nebulization

Liposome size and SPAN for all proliposome formulations (as powders and tablets) were investigated prior and post nebulization, including for samples collected from the nebulizer reservoir and upper and lower compartments of the TSI; via laser diffraction using a Malvern Mastersizer 2000. The samples were collected for size analysis following the achievement of "dryness". Liposomes size in the two stages of TSI, were compared in terms of size to the liposomes generated prior to nebulization.

5.2.9. Output efficiency determination

BDP was employed as model API in the proliposome powder and tablet formulations. A liposome suspension of 5 ml was transferred to the nebulizer (i.e. 150 mg powder or tablets weight dissolved in 5 ml of aqueous medium, maintaining a 30 mg/ml concentration standard in this research). The output efficiency of BDP utilising the Pari air-jet nebulizer, was then determined by calculating the amount of drug delivered via analysis of the drug quantity in the upper and lower stages of the TSI, as well as the nebulizer reservoir. BDP concentrations determined in each compartment using HPLC method (Section 2.2.10 and 2.2.11) were compared with the original liposome suspension prior to nebulization.

5.3. Results and discussion

5.3.1. Nebulization time for proliposome powder and tablets

The time required from the beginning of aerosol production using the Pari LC Sprint air-jet nebulizer to complete cessation of mist (droplet) formation, including "sputtering time", was considered as the "nebulization time". Thus, nebulization was commenced and continued to "dryness".

Direct comparison of the formulation nebulization times, Figure 5.1 illustrated no significant difference ($p>0.05$) between the proliposome powder formulations based on D-mannitol or sorbitol carriers. Contrastingly, upon examination of the nebulization times for proliposome tablets, a higher nebulization time was observed for D-mannitol-based proliposome tablets than sorbitol-based proliposome tablets for both the 1:10 and 1:15 w/w lipid to carrier ratios (Figure 5.1). Additionally, sorbitol-based proliposome tablets at the 1:15 w/w ratio (24.18 $\pm$ 0.16 minutes), exhibited a significant difference ($p<0.05$) in terms of a lower nebulization time than both sorbitol and D-mannitol-based proliposome powders with the 1:10 (i.e. 25.95 $\pm$ 0.82 and 26.77 $\pm$ 1.06 minutes) and 1:15 (26.27 $\pm$ 0.93 and 26.98 $\pm$ 0.77 minutes) w/w lipid to carrier ratio formulations.

Shorter nebulization times (24.18 $\pm$ 0.16 minutes) were exhibited by sorbitol (1:15 w/w) tablets, indicating a significant difference ($p<0.05$) when compared to D-mannitol-based proliposome tablets at both 1:10 and 1:15 w/w ratios (31.39 $\pm$ 2.66 and 31.36 $\pm$ 1.46 minutes respectively)

(Figure 5.1). This difference may be attributed to lower viscosity exhibited by the hydrated sorbitol formulations prior to nebulization. Lower viscosity may allow for easier nebulization of the hydrated proliposome formulation. Differences in carbohydrate carrier structure may also influence the resultant viscosity of the hydrate proliposome suspension. Sorbitol's porous structure possesses the ability to uptake the lipid component with ease, whereas, D-mannitol's smooth surface exhibits the adherence of lipid to the carrier surface as confirmed by SEM analysis (Section 3.3.1). Higher contents of lipid in suspensions have been theorised to increase viscosity. The elevated presence of lipid on the D-mannitol carrier's surface (as particle size of D-mannitol is smaller than sorbitol mentioned in section 2.2.1), results in a greater quantity of lipid in the liposome suspension as confirmed by Stewart assay (Table 3.1), potentially resulting in an increase in viscosity. Moreover, it has been observed, that the decrease in temperature in the air-jet nebulizer may also contribute to increasing the viscosity of the nebulization solution (Steckel and Eskandar, 2003). The negative effect of higher viscosity upon nebulization was discussed by Finlay et al., (2000), who explained that a higher viscosity would result in a greater adherence of aerosol droplets to the inner walls of the nebulizer, as well as reduce deflection of these droplets back into the nebulizer reservoir, greatly affecting nebulization efficiency (prolonging nebulization time) (Finlay et al., 2000).

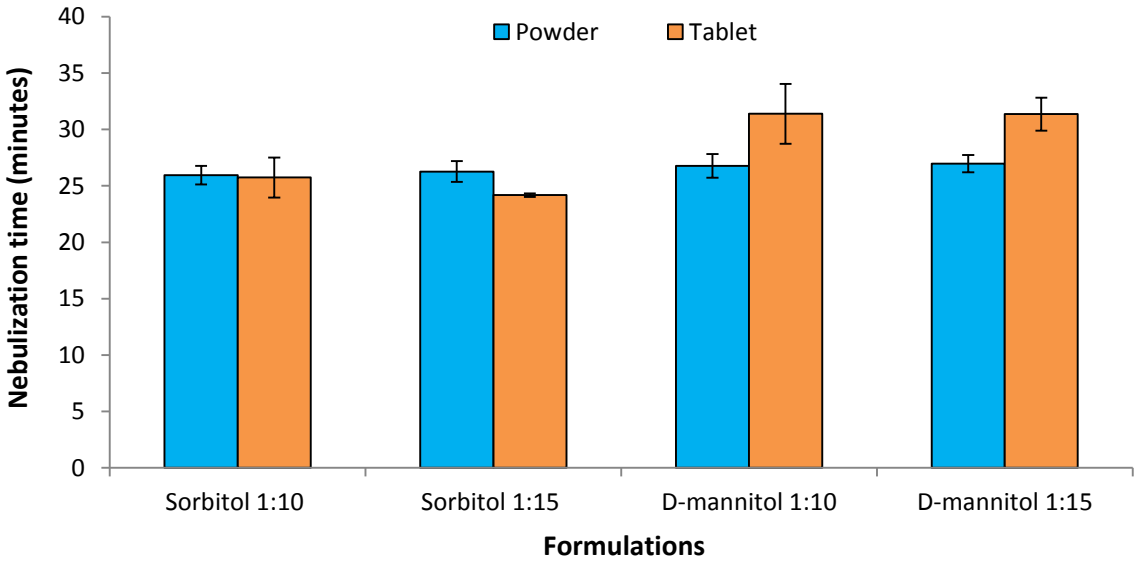


Figure 5.1: Nebulization time of BDP inclusive sorbitol and D-mannitol-based proliposome powders and tablets, utilising a Pari LC Sprint air-jet nebulizer. Data are mean ± SD, n=3; $p<0.05$

The difference in nebulization time (i.e. liposome generated from sorbitol and D-mannitol-based proliposome) may therefore also be attributed to the carbohydrate carrier morphology (Section

3.3.1). This would further suggest that D-mannitol-based carriers produce a more viscous suspension upon hydration, as the nebulization time was longer for both ratios (i.e. 1:10 and 1:15 w/w ratio) of tablets and powders, when this carrier was used; compared to sorbitol. Elevated nebulization times have been previously attributed to higher fluid viscosity (McCallion et al., 1995). Moreover, liposomes analysed in terms of size from the nebulizer reservoir were larger than those successfully nebulized (observed for D-mannitol-based tablet formulations at 1:10 and 1:15 w/w lipid to carrier ratios) (Section 5.3.6 and Figure 5.9), which offers an explanation of the prolonged nebulization time. Larger liposomes are more likely to deflect into the nebulizer reservoir, during nebulization and end up as a component of the nebulizer's residual or dead volume.

Higher levels of lipid phase in liposome suspensions, may reduce the surface tension of the suspension (Elhissi et al., 2013a, Elhissi et al., 2013b), and hence shorten the nebulization time (Ghazanfari et al., 2007). This proposed reduction in nebulization time was not observed for any of the proliposome powders or tablets investigated.

Overall, on examination of the proliposome powder formulations, sorbitol-based proliposome powders in a 1:15 w/w lipid to carrier ratio, exhibited the shortest nebulization time, making it the most efficient proliposome formulation for nebulization.

5.3.2. Nebulization sputtering time determination

Sputtering time is the time period where nebulizers exhibit an intermittent aerosolisation of the liposome suspension in the nebulizer, usually towards the end of complete nebulization time. This may also produce a specific sound, which is another indication of sputtering time (Section 2.2.22).

Similar trends to nebulization times were observed on examination of sputtering time. No significant difference ($p>0.05$) was noted when using the Pari air-Jet nebulizer amongst sorbitol and D-mannitol-based proliposome powder formulations; and similar results were observed for sorbitol and D-mannitol-based proliposome tablets. However differences in sputtering time were noted when comparing proliposome tablet and powder formulations. Sputtering time was higher for all proliposome powders as opposed to proliposome tablets. On further analysis, the sorbitol-based proliposome powders formulated at the 1:15 w/w lipid to carrier ratio were found to nebulize significantly ($p<0.05$) slower than the same formulation in tablet form (i.e. 4.27 $\pm$ 0.47

minutes and 2.42 ± 0.54 minutes respectively). Marked differences in sputtering time for tablet formulations were found in the following order:

Sorbitol 1:15 < D-mannitol 1:10 < D-mannitol 1:15 < Sorbitol 1:10

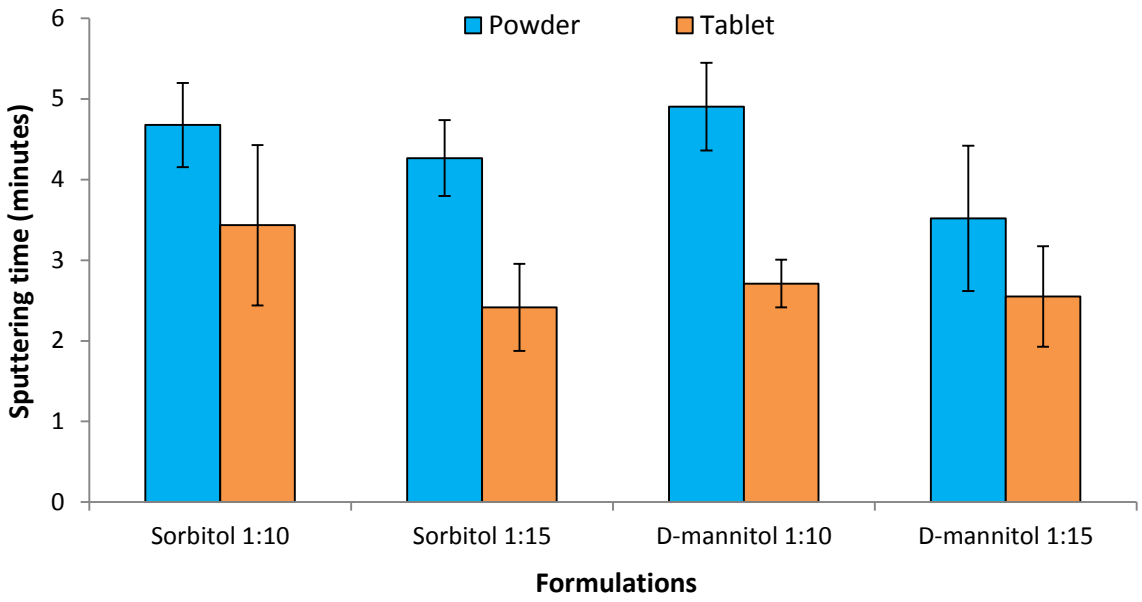


Figure 5.2: Sputtering time for sorbitol and D-mannitol-based proliposome powders and tablets made from 1:10 and 1:15 w/w lipid to carrier ratio formulations via Pari LC Sprint air-jet nebulizer. Data are mean ± SD, n=3; p<0.05

Sputtering behaviour was also found not to exert a notable effect on the VMD of the aerosol droplets produced by the Pari air-Jet nebulizer (Section 5.3.5), which contradicts previous research; which demonstrated a notable increase in both VMD and SPAN during sputtering of the air-jet nebulizer (Elhissi et al., 2012). According to Elhissi et al., (2012) droplet size of liposomes measured by laser diffraction, were similar during nebulization. However, the size and size distribution of the aerosol were found to increase during sputtering (Elhissi et al., 2012); conflicting with the current research findings for droplet size analysis (Section 5.3.6).

On comparison of all formulations investigated, the sputtering time exhibited by sorbitol-based proliposome tablets (1:15 w/w) was noted to be the shortest (i.e. 2.42 ± 0.54 minutes), i.e. the remaining low volume of suspension was nebulized quickly in the latter stages of nebulization. Whilst this formulation apparently had the shortest sputtering time, statistical analysis revealed with respect to the other formulations in the study, that the difference represented only a trend (i.e. was not statistically significant).

5.3.3. Aerosol Mass Output Investigation

In order to establish the mass output of the formulated aerosol, the Liposome suspension in the nebulizer is weighted prior and post nebulization; followed by calculating the percentage difference in weight, this provides the mass of the solution nebulized (Section 2.2.22).

For this research, in addition to nebulization time and sputtering time; mass output percentage (aerosol mass output) (Section 2.2.22) was established for sorbitol and D-mannitol-based proliposome powders and tablets (at both 1:10 and 1:15 w/w lipid to carrier ratios). Nebulization was performed to "dryness", however, complete atomization of the liposome suspension was not achieved, and therefore 100% aerosol mass output was not attained, due to the dead volume (i.e. the residual amount of liposome suspension which remained in the nebulizer reservoir post nebulization) (Clay et al., 1983, Elhissi et al., 2006). Large size liposomes have a tendency to remain in the reservoir as part of the residual nebulizer content contributing to this effect (Elhissi et al., 2012).

For proliposome powder formulations, a significant difference ($p<0.05$) was found between formulation ratios of 1:10 and 1:15 w/w for sorbitol (i.e. 87.20 $\pm$ 1.17% and 84.30 $\pm$ 0.58% respectively). Moreover, a statistically significant difference ($p<0.05$) was found between sorbitol 1:15 w/w (i.e. 84.30 $\pm$ 0.58%) and D-mannitol 1:10 proliposome powder (89.01 $\pm$ 0.57%); exhibiting a lower aerosol mass output rate for sorbitol-based proliposome powder in a 1:15 w/w ratio. These findings are in concordance with research conducted by Elhissi and Taylor (2005 and 2006), who reported an output higher than 80% using a Pari nebulizer (Elhissi and Taylor, 2005, Elhissi et al., 2006).

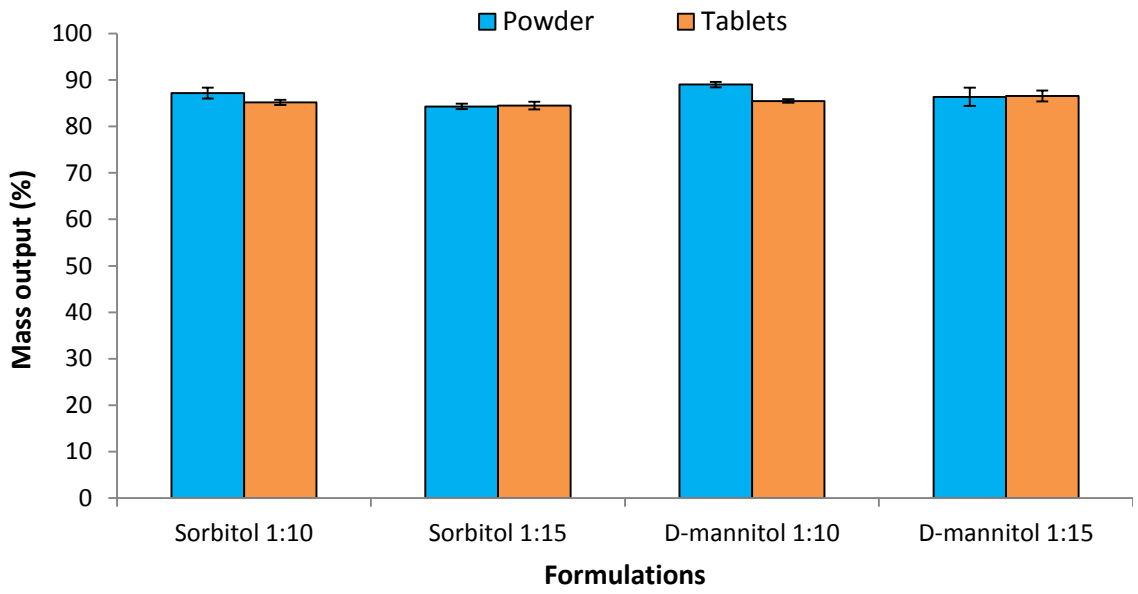


Figure 5.3: Percentage aerosol mass output for Pari LC Sprint air-jet nebulizer, for sorbitol and D-mannitol-based proliposome powder and tablets; prepared from 1:10 and 1:15 w/w ratio formulations. Data are mean ± SD, n=3; p<0.05

On comparison of tablets, formulated at both ratios using both carriers, differences in aerosol mass output were found to be insignificant (*p>0.05*) (Figure 5.3). Both sorbitol-based powder and tablets exhibited similar mass output for the 1:15 w/w ratio (i.e. 84.30 ± 0.58 and 84.48 ± 0.82%). This trend was common for the D-mannitol-based proliposome powders and tablets at the 1:15 w/w ratio formulations (86.37 ± 1.97 and 86.57 ± 1.18%). When tablet and powder proliposome formulations were compared in terms of mass output, a higher value was found for D-mannitol-based proliposome powder in a 1:10 w/w formulation (Figure 5.3).

Overall, the aerosol mass output was observed to be in excess of 80% for sorbitol and D-mannitol-based proliposome powder and tablets in 1:10 and 1:15 w/w ratio formulations. This was indicative of successful nebulization of the formulations, and deposition in the respiratory tract.

5.3.4. Aerosol Output rate

Aerosol output rate is the determination of the rate at which the total aerosol is nebulised. This assesses the efficiency of the nebulizer, and is calculated in terms of mg per minute, in accordance to section 2.2.22.

Maximum aerosol output rate was shown by the sorbitol-based (1:15 w/w) proliposome tablets i.e. 180.20 ± 0.31 mg/minute. In contrast, D-mannitol-based proliposome tablets in a 1:10 and 1:15 w/w proliposome tablets had the lowest aerosol output rate (i.e. 140.92 ± 11.88 and 142.16 ± 8.04 mg/minute) via Pari Sprint air-jet nebulizer. No significant difference (*p>0.05*) was found amongst the proliposome powder formulations. Similar results were demonstrated by tablet preparations, however a highly significant difference (*p<0.05*) was observed between sorbitol and D-mannitol 1:15 w/w tablets; sorbitol showing the higher output of the two at the 1:15 w/w ratio (Figure 5.4).

A significant difference (*p<0.05*) was also noted for the aerosol output rates, between the sorbitol powder and tablet formulations with 1:15 w/w ratio. In this instance output rate was higher for the tablet formulation as oppose to the proliposome powder tested (Figure 5.4). D-mannitol powder and tablet formulations at 1:10 and 1:15 lipid to carrier ratios also showed a statistically significant difference in terms of output rate. Interestingly, here, output rate was higher for the powder formulations, and lower for the tablet, at each respective ratio (Figure 5.4).

Mass output rate data published by Elhissi et al., (2006 and 2007) using air-jet nebulizer with liposomes generated from sucrose proliposome powders, exhibited similar results to those illustrated in Figure 5.4, where liposomes were generated from sorbitol and D-mannitol-based proliposome powder and tablets (Elhissi et al., 2006, Elhissi et al., 2007).

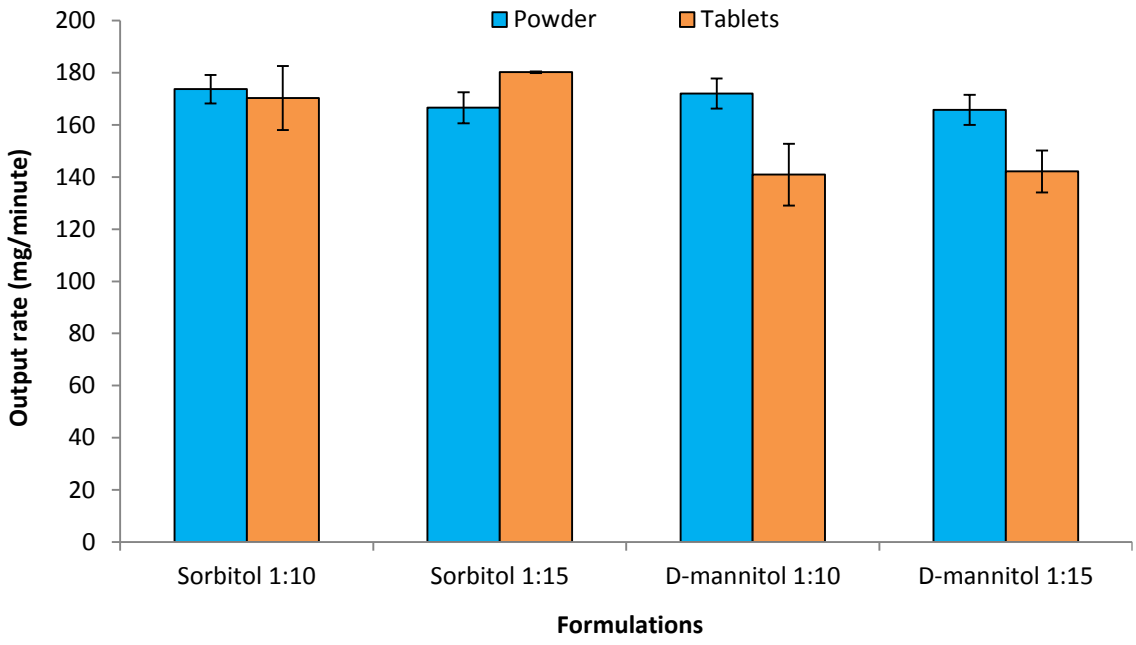


Figure 5.4: Aerosol output rate for sorbitol and D-mannitol-based proliposome powder and tablets at 1:10 and 1:15 w/w ratios. Data are mean ± SD, n=3; p<0.05

The shorter nebulization time exhibited by sorbitol-based tablet formulations (Section 5.3.1) may account for the higher aerosol output rate. By contrast, D-mannitol-based formulations had longer nebulization times and hence lower aerosol output rates. Decreased D-mannitol output rates in tablets may also be due to the possible increase in the viscosity of the formulation (as the small particle size of the D-mannitol carriers results in a greater concentration of exposed lipid on the surface, giving rise to greater viscosity upon hydration) (Section 5.3.1), as well as its ability to form crystals in water (Sastry et al., 2000, Yoshinari et al., 2003), which may result in drug retention within the nebulizer reservoir. This may cause a reduction in the amount of the formulation released from the nebulizer.

In terms of the best performing formulation, sorbitol-based proliposome tablets (1:15 w/w) were found to be superior with respect of aerosol output. As constant parameters (i.e. nebulizer type and suspension volume) were used, it can be stated that the enhanced performance was attributed only to the formulation and was independent of additional varying factors. Higher output rates typically results in better compliance from patients due to the shorter time required to nebulize a given volume of the liquid dispersion, hence this is a desirable property.

5.3.5. Determination of droplet Volume Median Diameter, SPAN and Fine Particle Fraction using laser diffraction

Different proliposome powders and tablets formulations were compared for VMD, FPF and SPAN of generated aerosol droplets, at varied time intervals e.g. 1, 10, 20 and 25 minutes. For each time interval, formulations elicited similar droplet size (Figure 5.5), indicating that possible differences in physicochemical properties between the formulations had no effect on droplet size emitted from the nebulizer.

The VMD recorded at minute 1, was smaller for all proliposome powder formulations (sorbitol or D-mannitol with 1:10 and 1:15 w/w lipid to carrier ratios), ranging from 4.16 - 4.71 µm, indicating no significant difference ($p>0.05$) (Figure 5.5). However, shortly after the 1st minute, the VMD of aerosol droplets for sorbitol-based proliposome powders (1:10 w/w lipid to carrier ratio) increased suddenly to 14.69 $\pm$ 1.17, 14.92 $\pm$ 0.99 and 15.93 $\pm$ 1.37 µm; at time intervals of 10, 20 and 25 minute. The same trend of increased VMD values was found for the sorbitol-based proliposome powder at a 1:15 w/w lipid to carrier ratio. Additionally, no significant difference ($p>0.05$) was found for the VMD between sorbitol-based proliposome powders at 1:10 and 1:15 w/w lipid to carrier ratios, at time intervals of 10, 20 and 25 minutes (Figure 5.5).

On nebulization using an air-jet nebulizer, Finlay et al., (2000) indicated the droplet size initially increased, after which it either decreased (Steckel and Eskandar, 2003) or plateaued (Mc Callion and Patel, 1996). This trend was observed across various nebulizers throughout nebulization, with little alteration in droplet size, even during sputtering (Mc Callion and Patel, 1996). The initial increase, followed by decrease in droplet size, may be attributed to the sudden change in temperature following initial nebulization (Niven, 1996). The latent heat of evaporation may account for the temperature decrease during nebulization (Steckel and Eskandar, 2003). Approximately a 7°C decrease in the initial two minutes of nebulization was observed in the nebulizer (Steckel and Eskandar, 2003). The drop in the temperature of the nebulizer fluid may also potentially result in an increase in the viscosity, due to evaporation of the continuous phase solvent.

SPAN measurements were also recorded for the complete duration of nebulization, at time intervals of 1, 10, 20 and 25 minutes. With respect to SPAN of the droplets, on comparison between all proliposome powders and tablet formulations (both sorbitol and D-mannitol), no significant difference ($p>0.05$) was observed at time intervals of 1, 10 and or 25 minutes (independent of lipid to carrier ratio). The only exception in SPAN was when the aerosol of D-mannitol-based proliposome powder formulations (at a ratio of 1:15 w/w), elicited higher SPAN values of 17.63 $\pm$ 0.29 for 20 minutes (Table 5.1). This was mimicked by a higher SPAN value at 20 minutes, which was also found for D-mannitol-based proliposome tablets, at a 1:10 w/w lipid to carrier ratio (16.81 $\pm$ 0.84), this difference with respect to the alternative formulations tested was found to be statistically significant ($p<0.05$). At time intervals of 25 or 31 minutes (representing sputtering time) a decreasing trend in droplet SPAN was observed. However contrastingly, increasing SPAN was found at 25 minutes for sorbitol-based tablets at the 1:10 w/w ratio (16.80 $\pm$ 1.48).

Furthermore at 31 minutes, a SPAN value of 14.76 $\pm$ 0.99 was noted for D-mannitol-based proliposome tablets at the 1:15 w/w ratio, which was higher than the other formulations tested. However, these changes in SPAN were not statistically significant ($p>0.05$) (Table 5.1). As mentioned in section 5.3.1, D-mannitol 1:10 and 1:15 w/w (lipid to carrier ratios) proliposome tablets, offer a longer nebulization time of approximately 31 minutes, indicating slow aerosol delivery (Figure 5.6), undesirable for patient administration.

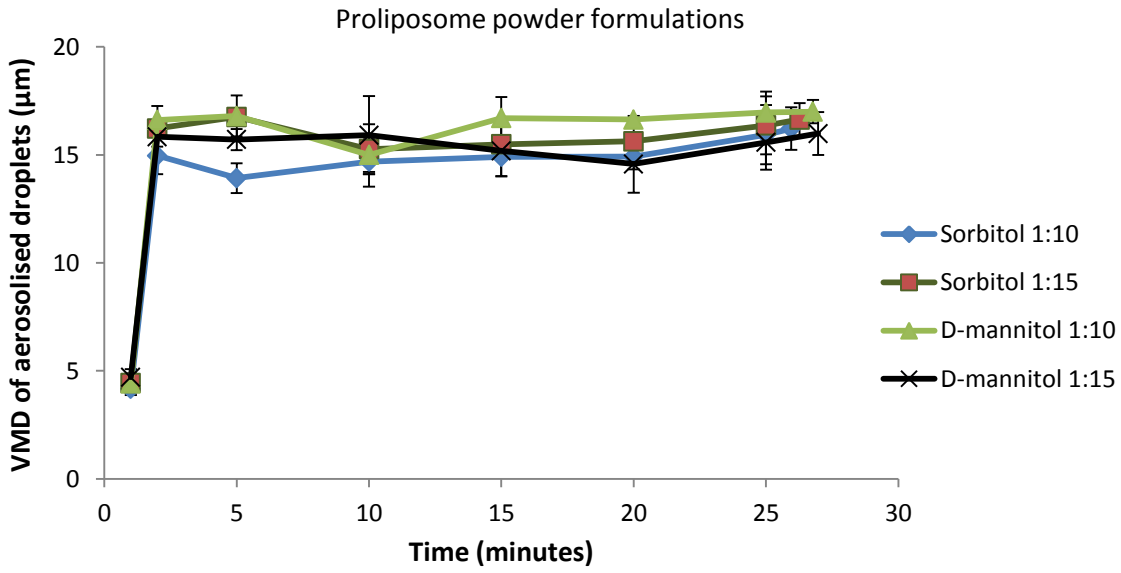


Figure 5.5: Size of aerosol droplets generated during nebulization from sorbitol and D-mannitol-based proliposome powder formulations, at 1:10 and 1:15 w/w lipid to carrier ratios. Data are mean ± SD, n=3; p<0.05

Compressed proliposome tablets manufactured from sorbitol and D-mannitol demonstrated no significance difference (*p>0.05*) in terms of VMD, at time intervals of 1, 10, 20 and 25 minutes (Figure 5.6). Sorbitol and D-mannitol-based proliposome tablets at 1:10 and 1:15 w/w (lipid to carrier ratios) exhibited no significance difference (*p>0.05*) in droplet size ranging from 3.04 - 3.75 µm at the 1st minute (Figure 5.6). The VMD increased rapidly after the 1st minute. Statistically, no significant difference (*p>0.05*) in VMD was found for sorbitol-based proliposome tablets at the 1:10 w/w (lipid to carrier ratio), at time intervals of 10, 20 and 25 minutes (these are 14.69 ± 0.98, 15.23 ± 1.40 and 15.03 ± 2.52 µm).

Figure 5.6 also illustrated no significance (*p>0.05*) in VMD between time intervals of 10 and 25 minutes, for sorbitol-based proliposome tablets at the 1:15 w/w ratio (16.63 ± 0.83 and 17.61 ± 0.62 µm). Nebulization time was shorter than 25 minutes, for sorbitol-based proliposome tablets at the 1:15 w/w ratio (Section 5.3.1). Formulation at 1:10 and 1:15 w/w lipid to carrier ratios for sorbitol-based proliposome tablets, exhibited no significant difference in VMD, for the 10 and 25 minute time intervals.

Conflicting results were found for the droplet size as a result of differences in viscosity and surface tension of the liposome suspensions. According to McCallion et al., (1995), viscosity is inversely proportional to droplet size (i.e. increasing viscosity results in decreasing droplet size), produced by an air-jet nebulizer. Moreover, excessively viscous solutions can resist droplet formation, and may cause nebulization to cease (McCallion et al., 1995, Ghazanfari et al., 2007).

These findings were re-enforced by the earlier observation of Davis (1978), who stated that when using an air-jet nebulizer, the high viscosity solutions produced smaller aerosol droplets (Davis, 1978, Steckel and Eskandar, 2003). These findings were further in concordance to Finlay (2001), who stated that reduction in size of nebulized droplets could be achieved by reducing the surface tension of formulation, via the addition of surfactants (Finlay, 2001).

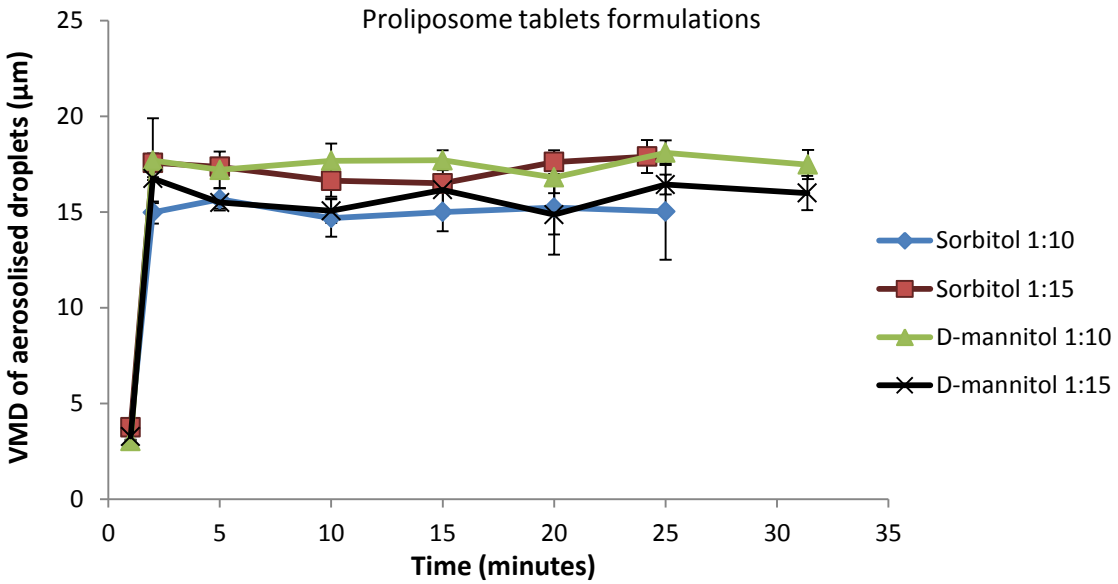


Figure 5.6: Size of aerosol droplets generated during nebulization from sorbitol and D-mannitol-based proliposome tablets formulations, at 1:10 and 1:15 w/w lipid to carrier ratios. Data are mean ± SD, n=3; $p<0.05$

Table 5.1: SPAN of aerosol droplets analysed during nebulization, via Pari LC Sprint air-jet nebulizer for sorbitol and D-mannitol-based proliposome formulations, using 1:10 and 1:15 w/w lipid to carrier ratios formulated powders and tablets. Data are mean ± SD, n=3; p<0.05

Formulations	Proliposome powders (Lipid to carrier ratios) (w/w)				Proliposome tablets (Lipid to carrier ratios) (w/w)			
Time (minutes)	Sorbitol 1:10	Sorbitol 1:15	D-mannitol 1:10	D-mannitol 1:15	Sorbitol 1:10	Sorbitol 1:15	D-mannitol 1:10	D-mannitol 1:15
1	1.96 ± 0.47	2.17 ± 0.57	1.78 ± 0.61	2.15 ± 0.57	1.80 ± 0.59	1.89 ± 0.82	1.72 ± 0.44	1.64 ± 0.43
10	15.37 ± 0.53	14.37 ± 0.89	15.90 ± 0.55	15.93 ± 0.69	14.66 ± 0.64	14.48 ± 0.60	14.43 ± 0.34	15.05 ± 0.62
20	14.72 ± 0.63	15.50 ± 0.74	14.49 ± 0.87	17.63 ± 0.29	14.10 ± 1.00	13.42 ± 0.84	16.81 ± 0.84	14.10 ± 1.14
24	-	-	-	-	-	14.66 ± 0.90	-	-
25	14.18 ± 1.98	14.18 ± 2.93	15.15 ± 1.07	17.07 ± 0.91	16.80 ± 1.48	-	15.84 ± 0.55	14.16 ± 1.88
31	-	-	-	-	-	-	15.79 ± 0.76	14.76 ± 0.99

Aerosol droplets were analysed for their FPF during nebulization of the proliposome powder formulations, at time intervals of 1, 10, 20 and 25 minutes (Figure 5.7). A significant difference *(p<0.05)* in FPF was observed at 1 minute, with FPF values in the range of 57.27 - 64.08%, when compared with remaining time intervals tested (i.e. 10, 20 and 25 minutes). Moreover, the FPF did not show any significant difference *(p>0.05)* at time intervals of 10 and 20 minutes, with the exception of sorbitol powder 1:15 w/w at 25 minutes. Sorbitol-based proliposome powders (1:15 ratio) exhibited a significant decrease (*p<0.05*) in FPF values (i.e. 14.87 ± 0.71 um or %), when compared to the 1:10 w/w ratio (18.25 ± 1.38 µm or %) (Figure 5.7).

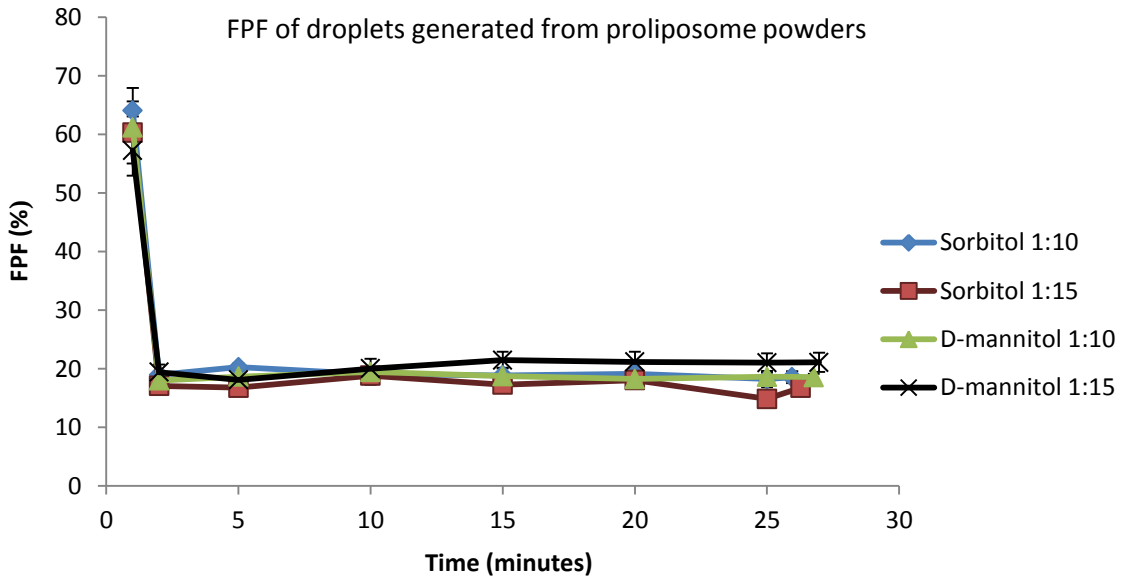


Figure 5.7: FPF of aerosol droplets analysed through Spraytec using Pari LC Sprint air-jet nebulizer, for sorbitol and D-mannitol-based proliposome powder formulations at lipid to carrier ratios of 1:10 and 1:15 w/w. Data are mean ± SD, n=3; p<0.05

Similar to proliposome powder formulations, aerosol droplets were examined at 1, 10, 20 and 25 minutes, in order to measure FPF for proliposome tablet formulations. D-mannitol-based proliposome tablets at 1:10 and 1:15 w/w lipid to carrier ratios (80.99 - 82.08%), showed higher values and were found statistically different (*p<0.05*) when compared to sorbitol-based proliposome tablets at the same ratios (i.e. 3.69 - 3.75%) at the 1st minute (Figure 5.8). However, shortly after the 1st minute, the FPF of aerosol droplets for D-mannitol-based proliposome tablets (1:10 lipid to carrier ratio), dropped dramatically (22.79 ± 1.34, 22.37 ± 0.94, 20.25 ± 1.01 and 21.25 ± 1.35% for the time intervals of 10, 20, 25 and 31 minutes). The same trend of sudden decrease in FPF, was found for D-mannitol-based proliposome tablets at the 1:15 w/w lipid to carrier ratio. Additionally, no significant difference (*p>0.05*) was found in the FPF between D-

mannitol tablets at 1:10 and 1:15 w/w lipid to carrier ratios, at time intervals of 10, 20, 25 and 31 minutes (Figure 5.8).

Sorbitol-based proliposome tablets (at the 1st minute) exhibited a low FPF for both the 1:10 and 1:15 w/w lipid to carrier ratios (i.e. 3.69 ± 0.40 and 3.75 ± 0.15 µm or %). Statistically, no significant difference (*p>0.05*) in FPF was found for the 1:10 w/w lipid to carrier ratio, at time intervals of 10, 20 and 25 minutes (these are 14.69 ± 0.98, 15.23 ± 1.40 and 15.03 ± 2.52%). Figure 5.8 also illustrated no significant difference (*p>0.05*) in FPF for the sorbitol 1:15 w/w ratio tablets, at time intervals of 10 and 20 minutes (16.63 ± 0.83 and 17.61 ± 1.02% respectively). Thus, the 1:10 and 1:15 w/w lipid to carrier ratios for sorbitol-based proliposome tablets exhibited no significant difference in FPF for 10 and 20 minutes of time intervals. Sorbitol-based proliposome tablets at a 1:15 w/w lipid to carrier ratio exhibited a nebulization time less than 25 minutes (Section 5.3.1).

The sudden drop in FPF may be attributed to plume formation (coalescence of droplets resulting in slow propulsion of the aerosol droplets), which is caused by the collision and fusion of smaller droplets into larger droplets (Hui et al., 2009). High FPF (i.e. a large fraction of droplets below the size of 5.4 µm) indicated that aerosol droplets were smaller, and hence deposition in the lower respiratory airways was more likely (i.e. alveolar region). The deposition of liposome vesicles in the pulmonary system is dependent upon the aerosol droplet size rather than liposome size. Droplets size below 5-6 µm may effectively penetrate the bronchioles and alveoli (Stahlhofen, 1980).

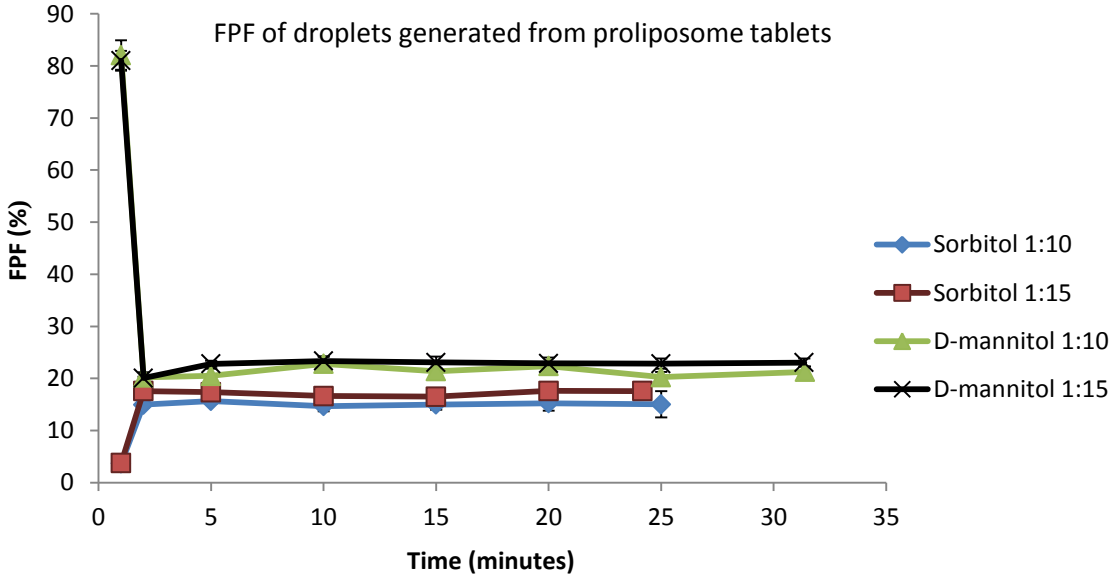


Figure 5.8: FPF of aerosol droplets analysed through Spraytec using Pari LC Sprint air-jet nebulizer, for sorbitol and D-mannitol-based proliposome tablet formulations at lipid to carrier ratios of 1:10 and 1:15 w/w. Data are mean ± SD, n=3; p<0.05

Overall, it was found that at each time interval of 1, 10, 20 and 25 minutes, no difference (*p>0.05*) in VMD was observed between the proliposome powder formulations, and similar trend was observed between proliposome tablet formulations; regardless of carrier type and lipid to carrier ratio (Figure 5.5 and Figure 5.6). The smaller the aerosol VMD, the better the deposition and accumulation in the lower respiratory tract.

A similar trend of statistical insignificance (*p>0.05*) for powder and tablet formulations (at both the 1:10 and 1:15 w/w lipid to carrier ratios), was also noted for SPAN values at each time interval (i.e. at 1, 10 and 25 minutes). However at 20 minutes, a significant difference (*p>0.05*) was noted between D-mannitol-based proliposome powders (at a 1:15 w/w lipid to carrier ratio) and the remaining powder formulations; showing a higher SPAN value (17.63 $\pm$ 0.29). Similarly, at 20 minutes, D-mannitol tablets (at a 1:10 w/w ratio) also demonstrated higher SPAN values (16.81 $\pm$ 0.84), which were comparatively higher than the remaining tablet formulations tested (*p<0.05*) (Table 5.1).

Post-nebulization, examination of the proliposome powder formulations showed a high FPF at the 1st minute, followed by a sudden decrease; and then a plateau, till complete nebulization time (Figure 5.7). Contrastingly in sorbitol-based proliposome tablets at both ratios (1:10 and 1:15 w/w ratio formulations), initial FPF values were very low at minute 1. Following the 1st minute FPF increased, followed by a plateau in values for the 10, 20 and 25 minute time intervals (Figure 5.8). Whilst, D-mannitol-based proliposome tablets showed a similar trend to the D-mannitol proliposome powder, with a high FPF at minute 1, followed by decrease and subsequent plateau till 25 minutes. Sorbitol-based proliposome tablets at a 1:15 w/w lipid to carrier ratio, was the only formulation exhibiting a nebulization time of less than 25 minutes.

5.3.6. Effect of nebulization upon liposome size and size distribution delivered to a Two-stage Impinger

The effect of nebulization on VMD (i.e. size of liposome), and SPAN (size distribution) were examined in this study, using sorbitol and D-mannitol-based proliposome powders and tablets; at lipid to carrier ratios of 1:10 and 1:15 w/w utilising a TSI.

Measurements of VMD and SPAN were taken prior and post completed nebulization of the liposome suspensions. The contents of the upper and lower compartments of the TSI, as well as

the residual contents of the nebulizer reservoir, were examined for liposome size following nebulization (Table 5.2 and Table 5.3).

As may be seen in Figure 5.9, D-mannitol-based proliposome tablet formulations elicited a highly significant difference ($p<0.05$), with respect to the liposome size (which was considerably larger in the residual volume than comparable sorbitol tablet formulations). Additionally, in tablets manufactured using sorbitol and D-mannitol-based proliposome powders, no significant difference ($p>0.05$) in VMD was observed between both ratios of each carrier, whereas a significant difference ($p<0.05$) was observed between both sets of carrier-based tablets at each respective ratio.

As may be seen in Figure 5.9 and Table 5.2, regardless of carrier type and lipid to carrier ratio, larger liposome size was found in the nebulizer reservoir, followed by smaller sized liposomes in the upper compartment of the TSI; and the smallest size of liposomes in the lower compartment of the TSI. The trend of decreasing liposome size deposited in the compartment post nebulization can be seen as:

Nebulizer reservoir > Upper compartment > Lower compartment

Examination of the nebulized D-mannitol-based proliposome tablets, revealed larger liposomes in the nebulizer reservoir when compared with sorbitol-based proliposome tablets; the VMD of liposomes analysed were observed to be unaffected by the lipid to carrier ratio. Moreover, by comparing sorbitol tablets (1;10 and 1:15 w/w ratios) with D-mannitol tablets (1:10 and 1:15 w/w ratios), a significant difference ($p<0.05$) was found in the nebulizer reservoir, showing larger sized liposomes for the D-mannitol based formulation, as compared to sorbitol (Figure 5.9).

In proliposome powder formulations, statistically no significant difference ($p>0.05$) was noted in the size of liposomes, in the nebulizer reservoir, between sorbitol 1:10 (9.67 $\pm$ 0.35 μm), sorbitol 1:15 (9.06 $\pm$ 1.81 μm) and D-mannitol 1:10 (8.33 $\pm$ 0.53 μm) lipid to carrier ratio formulations.

As discussed previously, D-mannitol possesses smaller particle size, and their surface characteristics may allow coating of higher amounts of lipid, which may lead to an increase the viscosity of the resultant liposome suspension. Increases in viscosity, may result in an increase in the nebulization time, as well as reduce the nebulizer output. Additionally, as a result of reduced nebulizer output, liposomes may aggregate or fuse, resulting in the formation of larger sized particles, within the nebulizer reservoir.

The reduced level of lipid present on sorbitol (a comparatively larger carbohydrate carrier than D-mannitol) carrier surfaces was confirmed by Stewart assay (Section 3.3.2), potentially resulting in a less viscous suspension, which is easier to nebulize; resulting smaller liposomes in the residual volume.

Furthermore, prior to nebulization of both sorbitol and D-mannitol-based proliposome powders and tablets, liposomes identified were smaller than the liposomes present in the residual volume (remaining volume in the nebulizer reservoir), post centrifugation (Table 5.2). This may be attributed to aggregation and fusion, during continuous deflection of a proportion liposomes, and final settlement in the nebulizer reservoir in the form of vesicle aggregates. On examination of the TSI, the upper compartment exhibited comparatively larger liposomes when proliposome tablets were nebulized, as oppose to proliposome powders (regardless of lipid to carrier ratios). However, the lower compartment of TSI showed smaller liposomes for both proliposome powder and tablet formulations (irrespective lipid to carrier ratio) (Figure 5.9). The smallest liposome size (2.14 + 0.91 μm) in the lower compartment of TSI was found for sorbitol-based proliposome tablets at a 1:15 w/w ratio.

Moreover, between the two ratios of the carrier-based formulations investigated, no significant difference (*p>0.05*) in liposome size was found in the upper stage of impinger for proliposome tablet formulations (sorbitol 1:10 and 1:15 w/w ratios 5.78 $\pm$ 0.82, 6.28 $\pm$ 0.90 respectively; and D-mannitol 1:10 and 1:15 w/w ratios (5.44 $\pm$ 0.29 and 6.29 $\pm$ 0.32 μm) (Figure 5.9). This trend was observed for all sorbitol and D-mannitol-based proliposome powder formulations in the upper stage of impinger. The exception in liposome size to this trend was noted on comparison of sorbitol 1:10 w/w (5.66 $\pm$ 0.82 μm) and D-mannitol 1:15 w/w ratio (4.59 $\pm$ 0.14 μm) proliposome powder formulations, following deposition in the upper impinger (Figure 5.9). Smaller liposomes were found in the lower stage of impinger, exhibiting no significant difference (*p>0.05*) in size of liposome between proliposome powders and tablets (regardless of carrier type and lipid to carrier ratio in Figure 5.9).

The presence of smaller liposomes may be attributed to a shearing effect, generated by the compressed air through the inlet of the Pari air-jet nebulizer; which converts the liposomal suspensions into droplets, which are then propelled through the nebulizer. Many droplets impact upon the nebulizer's baffle, resulting in vesicle fragmentation (i.e. size reduction) (Taylor et al., 1990, Saari et al., 1999).

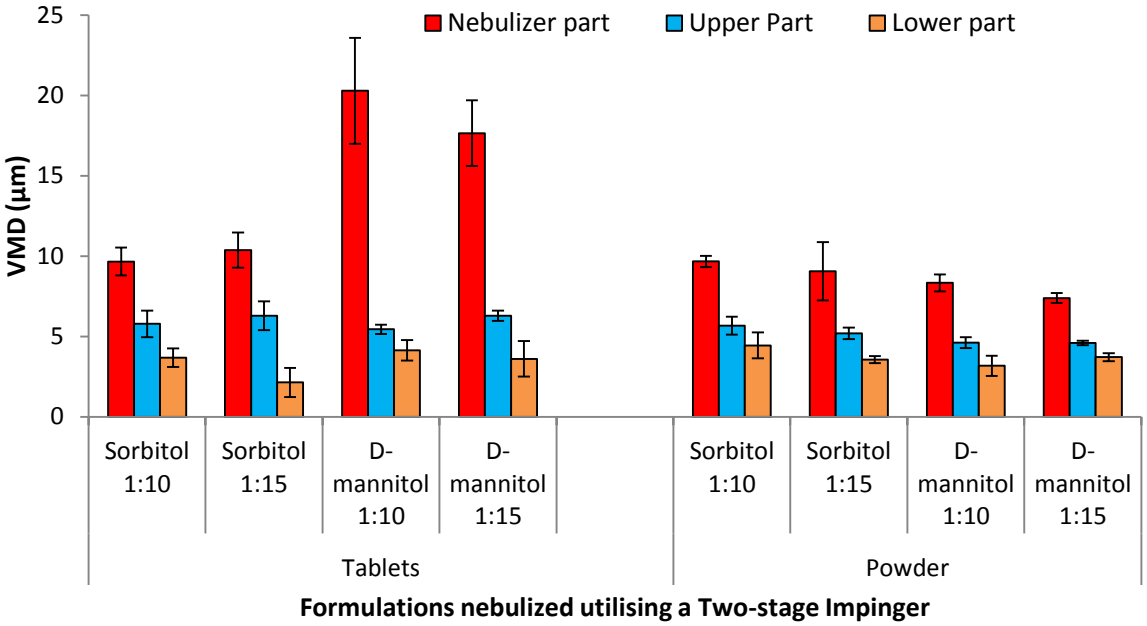


Figure 5.9: Liposome size for sorbitol and D-mannitol-based proliposome powder and tablet formulations, at 1:10 and 1:15 w/w ratios, after nebulization via a Pari LC Sprint air-jet nebulizer at 60 litres/minute. Data are mean ± SD, n=3; p<0.05

Previous research findings have indicated that air-jet nebulization of conventional liposomes can result in liposome in a size reduction of 1 µm, as a consequence of high shearing force within the nebulizer (Saari et al., 1999). These smaller sized aerosol droplets are deemed more likely to reach the alveolar region and hence are considered therapeutically useful.

Table 5.2: Size of liposome prior and post nebulization, using a Pari LC Sprint air-jet nebulizer, for sorbitol and D-mannitol-based proliposome powder and tablet formulations at 1:10 and 1:15 w/w ratios. Data are mean ± SD, n=3; p<0.05

Formulations	Before nebulization	After nebulization		
	Liposome suspension	Nebulizer reservoir	Upper compartment	Lower compartment
Proliposome Powders				
Sorbitol 1:10	5.49 ± 1.78	9.67 ± 0.35	5.66 ± 0.56	4.44 ± 0.81
Sorbitol 1:15	5.68 ± 1.55	9.06 ± 1.81	5.19 ± 0.35	3.56 ± 0.21
D-mannitol 1:10	5.43 ± 0.74	8.33 ± 0.53	4.62 ± 0.34	3.71 ± 0.25
D-mannitol 1:15	5.18 ± 0.93	7.39 ± 0.31	4.59 ± 0.14	3.17 ± 0.62
Proliposome Tablets				
Sorbitol 1:10	5.21 ± 0.74	9.66 ± 0.36	5.78 ± 0.82	3.68 ± 0.58
Sorbitol 1:15	5.85 ± 0.86	10.38 ± 1.11	6.28 ± 0.91	2.14 ± 0.91
D-mannitol 1:10	7.25 ± 0.70	20.29 ± 3.29	5.44 ± 0.29	4.13 ± 0.63
D-mannitol 1:15	7.31 ± 0.54	17.65 ± 2.04	6.29 ± 0.32	3.6 ± 1.10

Sorbitol and D-mannitol-based proliposome powder and tablets presented no significant difference (*p>0.05*) when compared directly in terms of SPAN, regardless of carrier type and lipid to carrier ratio (Table 5.3). In proliposome tablets, a significant difference was found in SPAN between the 1:10 w/w lipid to carrier ratios of sorbitol (3.63 ± 1.21) and D-mannitol (1.72 ± 0.44) formulations; sorbitol showing larger SPAN values. On examination of the lower compartment of the impinger, regardless of carbohydrate type, the 1:15 w/w powder formulations elicited lower SPAN values (*p<0.05*) when compared to the 1:10 w/w proliposome powders (Table 5.3).

In the lower stage of the TSI, no significant difference (*p>0.05*) in the SPAN values was noted between proliposome powder and tablet formulations. However, a significant difference was seen between the sorbitol 1:10 w/w powder (3.70 ± 0.60) and sorbitol 1:10 w/w tablet (1.72 ± 0.64) formulations.

Table 5.3: SPAN of liposomes prior and post nebulization in the impinger compartments utilising Pari LC Sprint air-jet nebulizer. Data are mean ± SD, n=3; p<0.05

Formulations	Before nebulization	After nebulization		
	Liposome suspension	Nebulizer reservoir	Upper compartment	Lower compartment
Proliposome Powders				
Sorbitol 1:10	2.42± 0.81	2.58 ± 0.27	3.07 ± 1.26	3.70 ± 0.60
Sorbitol 1:15	2.62 ± 0.75	2.39 ± 0.32	2.33 ± 0.36	2.02 ± 0.39
D-mannitol 1:10	3.80 ± 0.79	2.60 ± 0.29	2.40 ± 0.33	3.50 ± 0.80
D-mannitol 1:15	3.46 ± 0.97	2.46 ± 0.15	2.16 ± 0.50	2.24 ± 0.21
Proliposome Tablets				
Sorbitol 1:10	2.49 ± 0.57	3.78 ± 1.39	3.63 ± 0.24	1.72 ± 0.64
Sorbitol 1:15	3.62± 0.52	2.46 ± 0.53	2.51 ± 1.21	1.18 ± 0.96
D-mannitol 1:10	2.41 ± 0.55	2.54 ± 1.14	1.72 ± 0.44	2.48 ± 1.07
D-mannitol 1:15	2.34 ± 0.67	3.02 ± 1.78	3.41 ± 0.69	2.88 ± 0.94

Generally, prior to nebulization, liposome size for D-mannitol-based proliposome tablets was significantly larger for the 1:10 and 1:15 w/w ratios (7.25 ± 0.70 µm and 7.31 ± 0.54 µm), when compared to sorbitol-based tablets (5.21 + 0.74 µm and 5.85 + 0.86 µm), irrespective of carrier type and lipid to carrier ratio. An identical trend was noted for the powder formulations of both carriers. Additionally post nebulization, liposome size also increased in the nebulizer reservoir for D-mannitol-based proliposome tablets (including both the 1:10 and 1:15 w/w lipid to carrier ratios). Moreover, the VMD generated for both sorbitol and D-mannitol tablets and powder formulations were notably smaller in the upper and lower compartments of the TSI. Smaller liposome sizes in the lower stage of TSI indicated higher deposition in the lower part of pulmonary system (i.e. alveolar region).

5.3.7. Nebulizer output efficiency of BDP via Two-stage Impinger

Nebulization performed via the Pari air-jet nebulizer and TSI was used to determine BDP concentrations in the impinger stages and nebulizer reservoir.

Upon observation of Figure 5.10, it is notable, that amongst the proliposome powder formulations, no significant difference ($p>0.05$) was observed for BDP concentrations in the nebulizer reservoir; when comparison was conducted between sorbitol and D-mannitol 1:10 w/w ratio formulations. Contrastingly, a significant difference ($p<0.05$) was found between the sorbitol and D-mannitol 1:15 w/w proliposome powder formulations; showing a higher concentration in the nebulizer reservoir, for the sorbitol 1:15 w/w formulations. With respect to the tablet formulations, a significantly higher concentration of BDP (66.38 $\pm$ 1.15%) in the nebulizer reservoir was observed for the sorbitol 1:10 w/w tablet formulation; in comparison to the alternative tablet formulations tested. Overall, however, a higher BDP concentration compared to other compartments (i.e. in the upper or lower compartment of TSI) was found in the nebulizer reservoir, for all proliposome powder and tablet formulations, irrespective of the carrier type or formulations ratio (Figure 5.10). This may be explained by the greater tendency of larger liposomes to be retained in the nebulizer reservoir (Bridges and Taylor, 2000). The presences of larger liposomes in the nebulizer reservoir were also reported in the current research (Table 5.2).

For proliposome powders, in the upper compartment of the TSI, a significant difference ($p<0.05$) in BDP concentration was detected between sorbitol and D-mannitol-based powder formulations, at a ratio of 1:15 w/w; namely, a higher concentration of BDP was noted for the sorbitol formulation (22.19 $\pm$ 1.81%) in comparison to D-mannitol powder (18.19 $\pm$ 1.33%). However, for the tablet formulations, a significantly lower ($p<0.05$) concentration of BDP (16.17 + 1.01%) in the upper compartment was observed for the sorbitol 1:10 w/w tablet formulation; with reference to the alternative tablet formulations tested.

Examination of the lower compartment of the TSI showed no significant difference ($p>0.05$) between the carrier-based powder formulations investigated at both ratios (Figure 5.10). However, for the tablet formulations, a significantly lower concentration of BDP (17.45 + 1.99%) was observed for the sorbitol 1:10 w/w tablet formulation; with respect to the alternative tablet formulations tested.

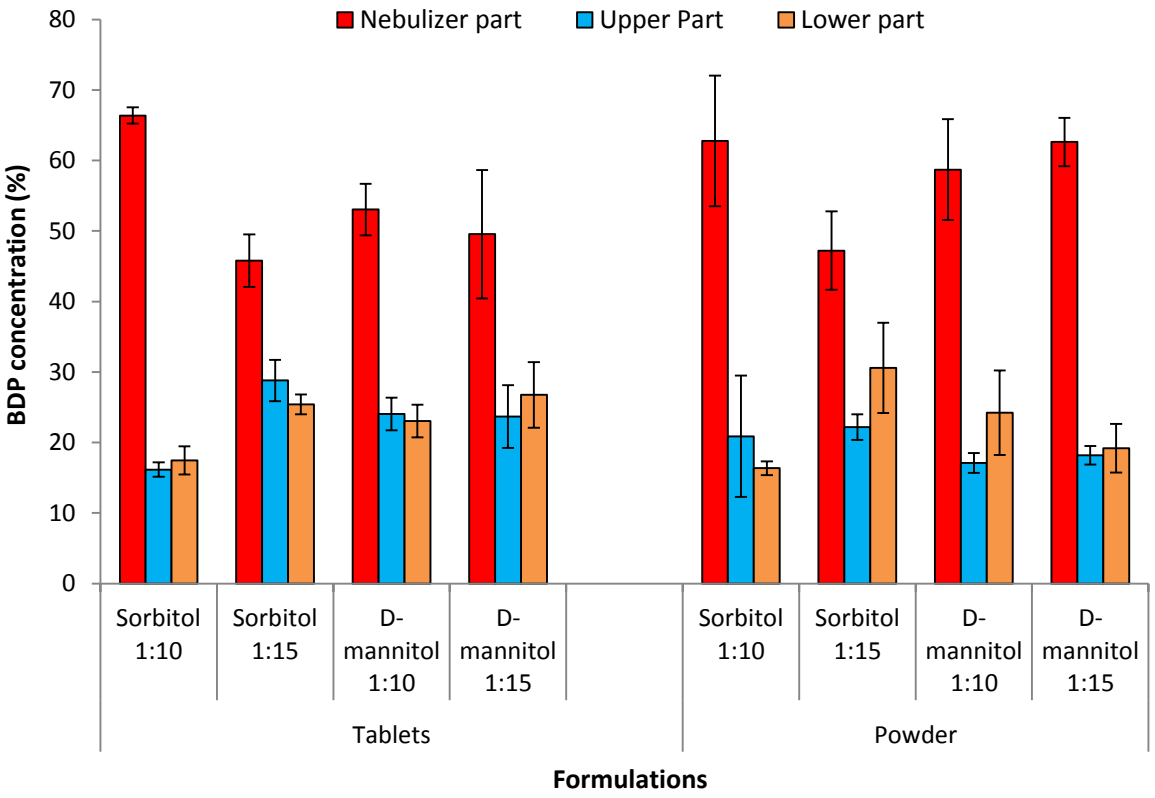


Figure 5.10: Concentration of BDP post-nebulization via a Pari LC Sprint air-jet nebulizer; utilising a Two-stage Impinger for sorbitol and D-mannitol-based proliposome powder and tablet formulations (in 1:10 and 1:15 w/w lipid to carrier ratios). Data are mean ± SD, n=3; $p<0.05$

As shown in Figure 5.11, the highest BDP concentration in the nebulizer reservoir was found for the sorbitol 1:10 w/w tablet formulation (66.38 ± 1.15%). This may be due to shear forces generated by the air-jet nebulizer, which may cause damage to the concentric bilayers of the liposomes; resulting in drug leakage (Taylor et al., 1990, Elhissi et al., 2007). The lowest concentration of BDP found was 45.78 ± 3.72%, for the same carrier (i.e. sorbitol) in the 1:15 w/w tablet formulations (i.e. in the nebuliser reservoir).

Moreover, the undelivered BDP concentration in the nebulizer reservoir was compared with the combined delivered BDP concentration (in the upper and lower compartments) (Figure 5.11). Direct comparison of sorbitol with D-mannitol-based proliposome powders and tablets (comparing identical lipid to carrier ratios), demonstrated no significant difference ($p>0.05$), with regards to the BDP concentration in the nebulizer reservoir (Figure 5.11). Results generally indicated that BDP found in the nebulizer reservoir was of a higher concentration, than that of the combined upper and lower compartments of the TSI.

It is well known that during nebulization the concentration of drug solution increases gradually (O'Callaghan and Barry, 1997). According to a study by Steckel and Eskandar published in 2003;

the concentration rises in the air-jet nebulizer reservoir. This observation could be attributed to the temperature change in the nebulizer solution during nebulization (Steckel and Eskandar, 2003). A reduction in temperature of the nebulizer solution may be the reason behind the increase of concentration in the residual volume, when using an air-jet nebulizer (Cockcroft et al., 1989, Dennis et al., 1990). A larger volume of residual liposome suspension, generally indicated the presence of larger liposomes; and hence an elevated concentration of BDP was observed (Figure 5.11).

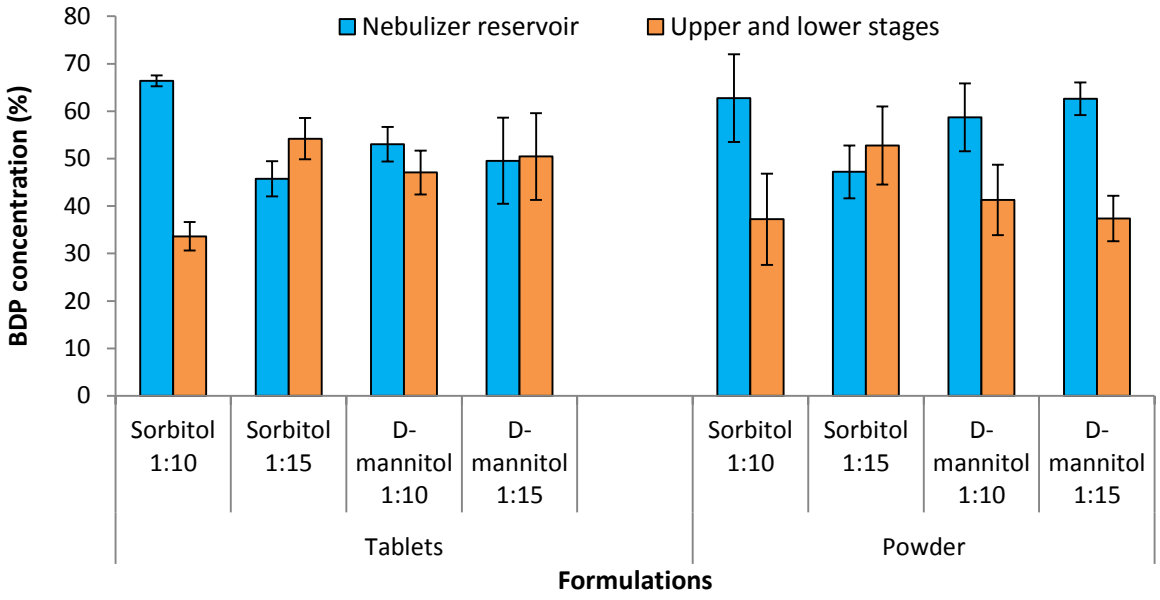


Figure 5.11: BDP concentration observed in the nebulizer reservoir (i.e., undelivered) and combined upper and lower stages (i.e., delivered) of Two-stage Impinger; from proliposome powder and tablet formulations at various ratios, nebulized via a Pari LC Sprint air-jet nebulizer. Data are mean ± SD, n=3; $p<0.05$

According to Figure 5.11, the sorbitol 1:15 w/w ratio proved to be the best formulation in both proliposome powder and tablet form, in terms of drug delivery; the formulation demonstrated a net delivery of 52.78 ± 8.23% and 54.22 ± 4.34% (BDP concentration in powder and tablet formulations), respectively. If the VMD of droplets is larger, whilst maintaining a low SPAN, this indicates a higher deposition of drug in the impinger compartments using liposomes (Elhissi et al., 2013b). This trend was only seen in the sorbitol 1:15 w/w proliposome powder and tablet formulations (Section 5.3.5; Figure 5.5 and Figure 5.6). On general comparison of proliposome tablet formulations, enhanced BDP delivery into the TSI compartments was seen as compared to the proliposome powder formulations.

The order of BDP delivered concentration for powder formulations in Figure 5.11 was denoted as:

Sorbitol 1:15 > D-mannitol 1:10 > D-mannitol 1:15 > Sorbitol 1:10

The trend for BDP delivery in tablets (Figure 5.11) was found to differ slightly, showing:

Sorbitol 1:15 > D-mannitol 1:15 > D-mannitol 1:10 > Sorbitol 1:10

BDP delivered concentration was highest for sorbitol-based proliposome formulations (both powder and tablets) at a 1:15 w/w lipid to carrier ratio, using a TSI. Thus, sorbitol was identified as a superior carrier for formulating proliposomes for pulmonary drug delivery.

5.4. Conclusions

The aim of this piece of research was to evaluate the effect of various proliposomes powder and tablet formulation type (i.e. using sorbitol and D-mannitol as carbohydrate carriers with 1:10 and 1:15 w/w lipid to carrier ratio) upon the nebulization of BDP.

Various parameters were investigated in order to assess this, including: nebulization time, sputtering time, mass output, aerosol output rate and liposome size etc. Upon examination of these variables, sorbitol-based proliposomes (both 1:10 and 1:15 w/w lipid to carrier ratio) were found to perform better than other proliposome formulations used in this study. This was independent of whether they were formulated as a powder or tablet. Additionally, when sorbitol was formulated in a 1:15 w/w ratio, superior performance was noted compared to all investigated formulations.

Nebulization and sputtering times were notably shorter for sorbitol-based proliposome tablet formulations, particularly at the 1:15 w/w ratio, indicating a high efficiency in terms of nebulization capacity. Moreover, BDP delivery was also observed to be highest for the 1:15 w/w lipid to carrier ratio (sorbitol-based proliposome powder and tablet formulations), when using a TSI. These properties are highly important, as they indicate the maximum amount of formulation delivered in the shortest nebulization time, paramount for maintaining efficient drug delivery and patient compliance.

Tableting of the proliposome formulations yielded mixed results. Whilst no difference was observed in terms of aerosol droplet size at varied time intervals (i.e. 1, 10, 20 and 25 minutes), SPAN was found to differ for both ratios of the D-mannitol-based proliposome powders; which

showed higher values than the alternate formulations investigated (i.e. sorbitol-based formulations).

Differences in aerosol FPF values during the first minute are challenging to explain, as the only difference in testing is the formulation carrier and formulation form; all additional parameters were kept constant. Both sorbitol and D-mannitol powder formulations, and D-mannitol tablet; regardless of lipid to carrier ratios, exhibited a high FPF value at the 1st minute. Whereas, sorbitol-based proliposome tablets (at both 1:10 and 1:15 w/w lipid to carrier ratios) showed low FPF values. These FPF values may have been influenced by the viscosity of the solution. Conflicting research indicates that higher viscosity may be beneficial or detrimental to droplet formation, influencing nebulization efficiency. In this instance, it is possible that the presence of D-mannitol, aids in droplet formation, whereas in the sorbitol-based formulation the carrier negates this. As a consequence, higher FPF values are noted for D-mannitol-based formulations, within the first minute of nebulization; as oppose to sorbitol. Alternatively, there is possibility that prior to change in nebulizer, temperature may have affected the FPF values (as the temperature is known to vary during air-jet nebulization).

Interestingly, liposome size was noted to differ prior and post nebulization, increasing following nebulization (in the reservoir). This was theorised to be due to larger liposomes being poorly nebulized; falling back into the reservoir due to lack of momentum, as well as liposome fusion.

Conclusively, sorbitol was identified as an ideal carrier for proliposome powder and tablet formulation; and subsequently for nebulization. Carrier type was found to notably influence the nebulization properties of the formulations; moreover ratio of lipid to carrier was also found to elicit a change in nebulization properties.

CHAPTER 6: STABILITY STUDIES OF SORBITOL-BASED PROLIPOSOME TABLETS

"Its not that I am so smart, its just that I stay with problems longer"

- Albert Einstein -

6.1. Introduction

Whilst there are thousands of formulations present in the market which are clinically effective, stability of these products or shelf-life, remains a paramount factor in clinician prescribing or production selection (Labvantage Solutions, 2011). Many manufacturers strive to ensure lengthy stability of their formulated product. There are a number of explanations for this; firstly, a longer shelf-life is ultimately more beneficial to the patient, they are able to keep the formulation longer, larger numbers of doses may be included in the formulation. This is more cost-effective for prescribers, as medicines which are wasted (often due to expiration) is a prevalent problem, costing in the region of millions to the NHS in the UK (Trueman et al., 2010).

In terms of formulations, solid dosage forms, particularly tablets are perhaps the most widely available formulation in the pharmaceutical industry. This is not purely by serendipity, there are a number of key advantages associated with tablet formulations which make them wholly desirable as a formulation to manufacture and sell. High patient compliance, generally ensures high sales, moreover these solid dosage forms are packaged and transported with ease, unlike many liquid formulations. However, a key advantage associated with these is enhanced stability; many tablets are inherently stable due to the lack of ingredients, or presence of certain thermo or moisture stable excipients. Despite this, should a tablet formulation be sensitive to any of these factors (temperature, moisture and light), blister packing often remedies a number of these problems, and the addition of desiccants into these blisters also aids in the protection of moisture sensitive drugs or excipients.

The exposure of a pharmaceutical product to any of the aforementioned factors (i.e. high temperature and moisture conditions) may result in degradation of the API, which may result in ineffective treatment, or cause harm to the patient. The same may be noted for excipients, as many of the excipients used, may be attractive to microorganisms, once again compromising stability. For these reasons, it is crucial to establish product stability, calculate the duration for which the product is suitable and develop ways in which this may be enhanced.

Though liposome formulations are known for their instability, proliposome formulation of liposomes has been demonstrated to be a viable method to ensure formulation stability (Gupta et al., 2008, Ekins and Xu, 2009, Jaiswal, 2013). Whilst, stability has been ensured to an extent, current proliposome formulations are typically provided in a powder form. This makes ensuring packaging, transport, patient use, and stability challenging. Firstly, powders are bulky in terms of handling making dosing difficult and also elevating packaging and transport costs. From a stability

perspective, the addition of a desiccant is problematic, and there is the additional factor of the large surface area associated with powders, which provides a greater opportunity of exposure to external factors; which may influence product stability (such as moisture, heat and light).

As tablet formulations are widely manufactured and known for their stability over alternative formulations available on the market, the suggestion is made that tableting of proliposome formulations may offer a solution to the previously discussed issues. Compacted dosage forms may be more stable due to their reduced surface area (the converse of which is a pitfall for proliposome powders).

Hence, in this project proliposomes have been formulated as tablets. This chapter sets out to investigate the stability of the developed formulations in this research (in tablet form), using a variety of methods and conditions, in order to establish the viability of a proliposome tablet formulation in today's current market as a novel and competitive product.

For the purpose of this chapter, sorbitol-based tablets (1:15 w/w lipid to carrier ratio) have been assessed as these were deemed to be the most successful proliposome tablet formulation, based on the test conducted in chapters 4 and 5, using a range of proliposome formulations. Samples were subjected to three temperatures sets (Fridge, room or high temperature; 6, 22 or 40 $\pm$ 1°C, respectively) over a period of six months. Throughout this duration samples were extracted and tested for a number of parameters such as, liposome size and size distribution, liposome surface charge, weight variation of formulated tablets, disintegration time of tablets, tablet hardness, tablet friability and importantly, entrapment efficiency of BDP in liposomes.

6.2. Methodology

6.2.1. Proliposome tablet manufacturing

Proliposome powders containing BDP were prepared via the slurry method (Section 2.2.2). The prepared powders (Sorbitol-based proliposomes at a 1:15 w/w lipid to carrier ratio), were then compacted into proliposome tablets in accordance to section 2.2.14 and 2.2.15. Following tableting, the proliposome tablets were hydrated in D_2O and annealed as described in section 2.2.3.

6.2.2. Stability studies

The freshly prepared sorbitol-based proliposome tablets, in a 1:15 w/w lipid to carrier ratio were used as a control, being tested in accordance to BP and USP guidelines. The remaining freshly prepared proliposome tablets were placed in triplicate, at three varied temperatures to allow for comparison over a six month period for stability.

Room Temperature (RT), i.e. 22°C

Fridge Temperatures (FT), i.e. 6°C

High temperature (HT), i.e. 40°C

6.2.2. Tablet morphology

Sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio) were physically examined and photographed following manufacturing (i.e. control tablets). Proliposome tablets stored at the various temperature conditions, were physically inspected at intervals during the stability testing period for colour change; as well as any changes in physical surface and tablet integrity.

6.2.3. Liposome Characterisation

Liposomes generated from the proliposome tablets were initially examined for VMD (also referred to as size of liposome vesicle), and SPAN (known as size distribution) using the Malvern Mastersizer 2000 (Section 2.2.7). Zeta potential of the liposome vesicles was also analysed using the Malvern Zetasizer Nanoseries (Section 2.2.8).

6.2.4. Tablet specification and testing

Sorbitol-based proliposomes tablets at a 1:15 w/w (lipid to carrier ratio) were processed and tested in accordance to guidelines documented in the BP and USP for standard uncoated tablets. These included; weight variation, disintegration, hardness or crushing strength and friability tests, as outlined in chapter 2.

6.2.5. Liposome entrapment efficiency

The entrapped concentration of BDP into liposome vesicles, unentrapped BDP and free BDP crystals were analysed by HPLC. However initially, proliposome tablets were hydrated and annealed in D_2O (Section 2.2.3). This was followed by centrifugation (explained in chapter 3), after which the BDP in each separate part was quantified through HPLC, explained in section 2.2.10 and 2.2.11.

6.3. Results and discussion

Upon fresh batch preparation of the proliposome tablets, several tablets were randomly selected and characterised using the aforementioned techniques. These tested tablets were utilised as a control sample (i.e. were characterised and their generated readings noted), the remaining tablets from the manufactured batch were then divided between the three outlined temperature conditions (in triplicate) randomly, and stored for a six month period. All results generated from subsequent monthly interval analysis of tablets, at varied conditions were directly compared to the results generated for the control proliposome tablets characterised. Testing was conducted on a monthly basis, with comparison made each time to the control sample, as well as results generated from preceding months.

6.3.1. Proliposome morphology

Physical examination of the tablets was further achieved by the aid of a Digital camera (Canon EOS 100D). This process was conducted for the controlled tablets (i.e. freshly prepared tablets), photographs showed the tablets to be white in colour for FT, RT and HT stored samples. These tablets were then subsequently photographed after keeping them in the varied temperature conditions for 3 month. All photographs showed the tablets were white in colour. The colour was maintained by the tablets for the three month at varied temperature conditions. However, after six months of stability testing; the colour was maintained in the proliposome tablets stored at FT and RT. However, for tablets stored at HT, a change in colour was noted after six months, tablets were found off-white to yellow in appearance in comparison to the control tablets (Figure 6.1). This trend was mirrored upon examination of the cross-sections of the stored tablets. Both sets of

tablets stored at FT and RT were observed to be homogenous in colour and were distinctively white in colour. Comparatively, the cross-section of the HT stored tablets after six months, exhibited a notably off-white to yellowish appearance. Yellowing of the tablets surface or cross-section was not noted at the three month interval, but only the six month. As sorbitol powder is known not to discolour at elevated temperatures (Newman et al., 1999, Nabors, 2001), it is suggested that discolouration may as a consequence of lipid component degradation due to the HT storage conditions.

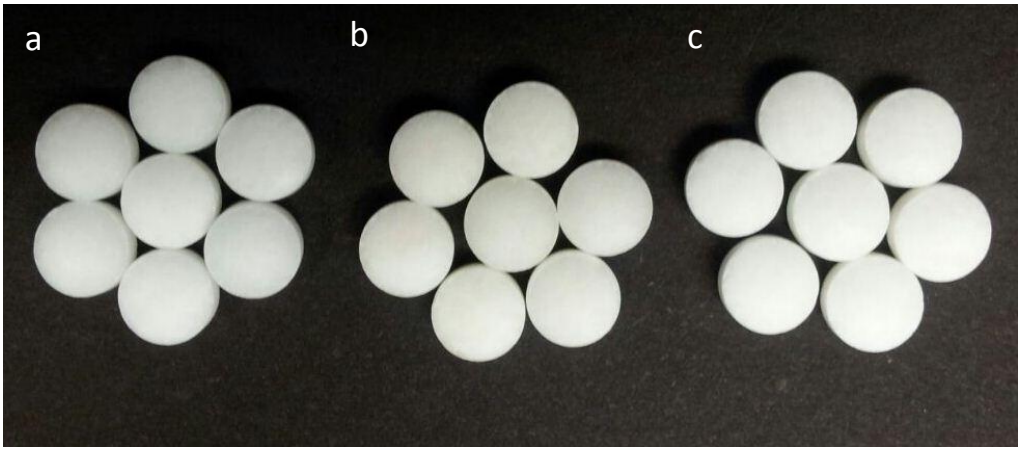


Figure 6.1: Photographs showing the colour of proliposome tablets upon preparation, and storage under various conditions for the six month stability testing: (a) FT of 6°C, (b) HT of 40°C and, (c) RT of 22°C. These photographs are typical of three such different experiments

6.3.2. Liposome size analysis

6.3.2.1. Control and individual temperature condition comparison

The VMD generated (in D_2O after hydration and annealing) of control tablets were compared with the VMD of liposomes generated from all six months samples (where samples were stored at variant temperature conditions i.e. FT, RT or HT).

On statistical analysis, in terms of liposome size, for the proliposome tablets stored at RT, no significant difference ($p>0.05$) was noted between the VMD of the liposomes generated from control, compared to all the RT stored samples at each of the six months (Figure 6.3). Furthermore no statistical difference ($p>0.05$) was noted upon direct comparison of the RT stored samples with each other for the various tested months. An identical trend was noted, in terms of

VMD, for samples stored at FT, when compared with each other and when compared to the control sample (Figure 6.3).

Differences in the aforementioned trends were noted upon comparison of the HT stored samples with one another and with the control sample. In terms of VMD, a statistically significant difference (*p<0.05*) was noted between the control sample (5.9 ± 0.7 µm) and HT samples stored for 4, 5 and 6 months (9.48 ± 0.73, 10.37 ± 0.56 and 9.19 ± 0.81 µm, respectively) i.e. VMD was found to increase as storage time at HT increased (Figure 6.2). Upon direct comparison of the HT samples at monthly intervals with one another, a statistically difference (*p<0.05*) was observed in terms of liposome VMD. This was observed between months 1 and 2 when compared with months 4, 5 and 6.

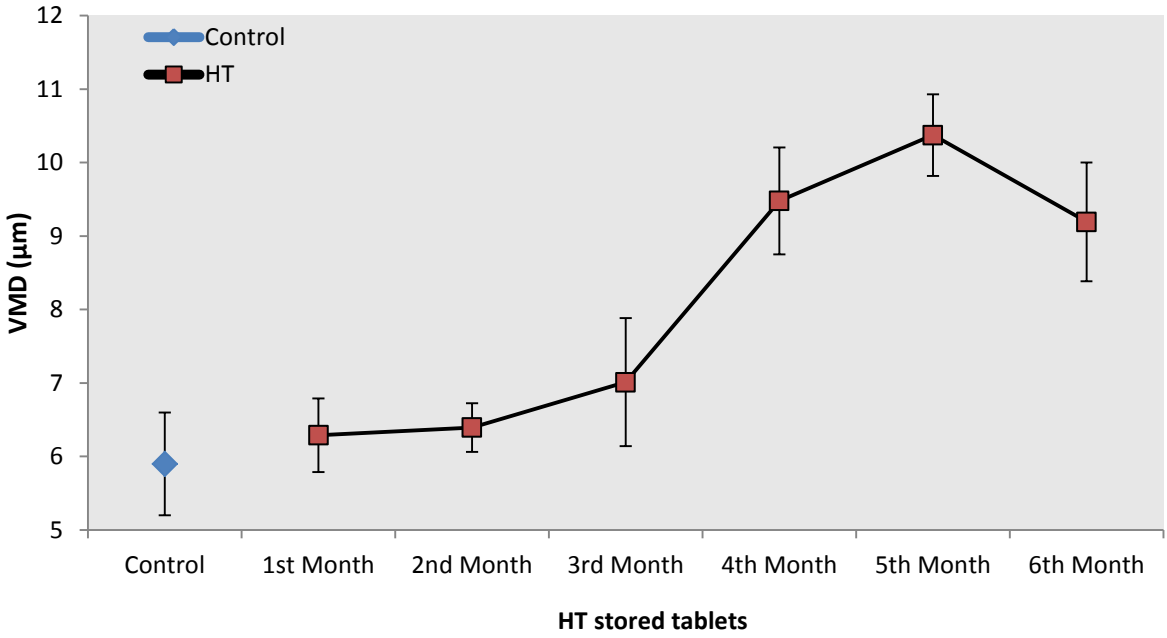


Figure 6.2: Volume median diameter (VMD) of liposomes generated from control sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio) compared to the liposomes generated from sorbitol-based proliposome tablets stored at HT. Data are mean ± SD, n=3; p<0.05

6.3.2.2. Month by month temperature condition comparison

Statistical significance was assessed upon comparison of each formulation at each temperature condition with the respective formulation at the alternative conditions i.e. month 1 sample at FT, RT and HT were compared with one another to assess statistical significance, using the control sample as a control/reference once again.

Upon comparison of the VMD generated from all samples at the three temperature conditions with each other, at months 1 and 2, no statistically significant difference was observed (*p>0.05*) (Figure 6.3). However at month 3, a statistically significant difference (*p<0.05*) was noted between samples stored at HT (7.01 $\pm$ 0.87 μm) and RT (4.73 $\pm$ 0.63 μm) (Figure 6.3), exhibiting larger liposome size at the HT.

Further differences were noted upon increasing storage time, liposome size was observed to increase at HT as storage time increased. Consequently a statistically significant difference (*p<0.05*) was noted at months 4, 5 and 6 when comparing VMDs generated from HT stored samples showed larger liposome size than those stored at RT and FT (Figure 6.3). This difference in VMD is mirrored by differences in tablet colouration (Section 6.3.1), tablets were found to discolour (from white to an off-white and yellowish colour) in the latter months i.e. months 4, 5 and 6 at HT storage conditions. Storage at high temperature was suggested to be responsible for the discolouration as a result of possible degradation of the lipid component.

Previous research has indicated that high temperature as well as physical instability (of lipid component) may cause liposome fusion or aggregation, resulting in larger liposomes as well as potential degradation of the concentric bilayers in liposomes (Lasic, 1998, Jain, 2012). However, it is noteworthy that this literature does not utilise stored dry formulations of liposomes (i.e. proliposome tablets), but in fact focuses attention upon the generated liposomes in comparison to dry formulation storage conditions.

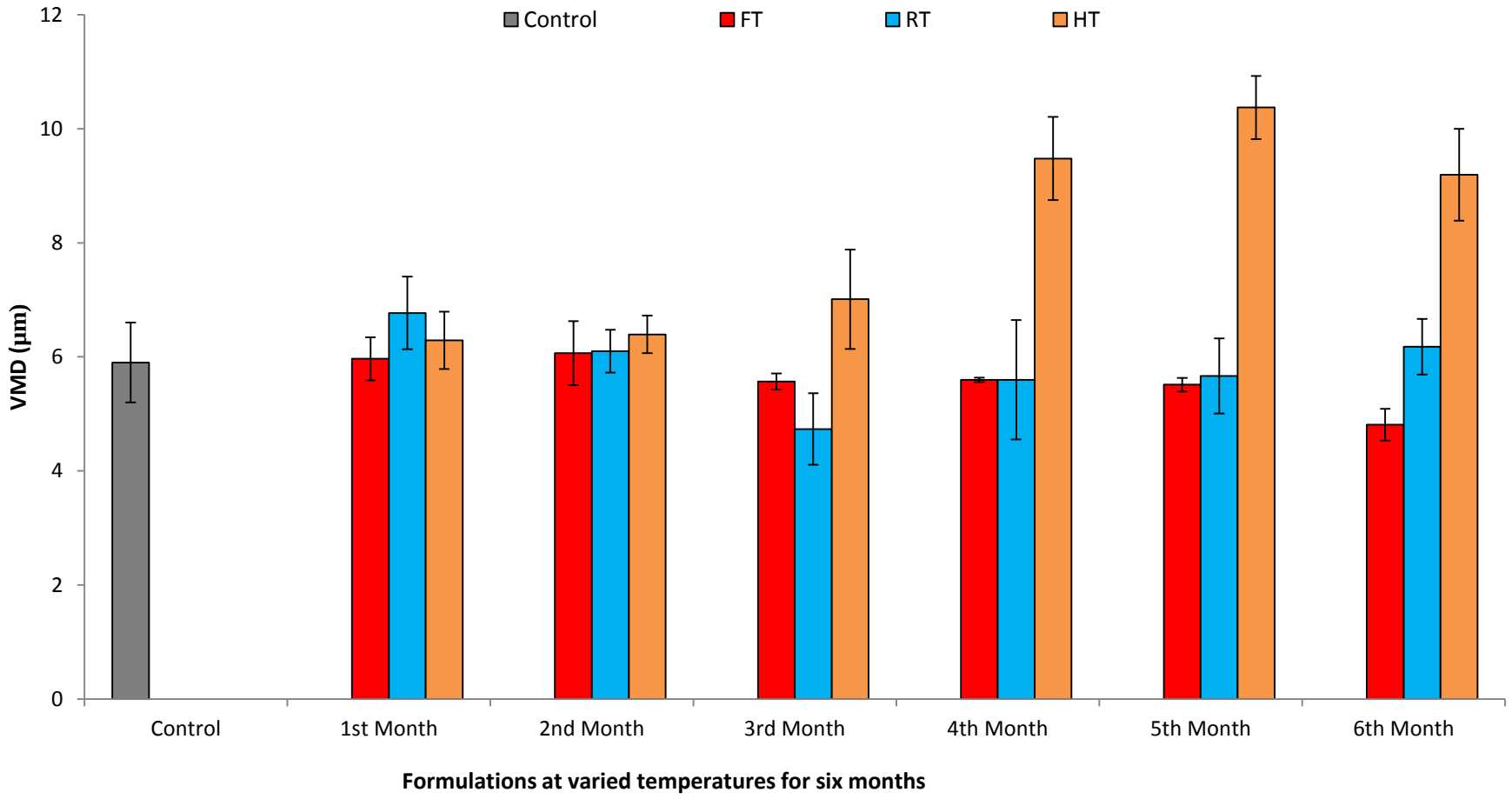


Figure 6.3: Volume median diameter (VMD) of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control tablets (freshly prepared) and tablets placed at three different temperatures condition (i.e. RT, FT and HT for six months) for comparison. Data are mean ± SD, n=3; $p<0.05$

6.3.3. Size distribution of liposomes

6.3.3.1. Control and individual temperature condition comparison

The SPAN of liposomes generated from the control tablets (using D_2O for hydration and followed by annealing) were compared with the SPAN values of liposomes generated from each of the monthly set of tablets for the six month stability period (where samples were stored at variant temperature conditions i.e. FT, RT and HT).

Upon analysis of the SPAN values generated from the FT, RT and HT, no significant difference (*p>0.05*) was noted for any of the stored samples when direct comparison was made with the control sample (Figure 6.4).

6.3.3.2. Month by month temperature condition comparison

Further analysis demonstrated no significant difference (*p>0.05*) in terms of liposome SPAN, when formulations from different temperature sets were compared directly with their monthly counterparts (i.e. month 1 FT with month 1 RT and month 1 HT).

Whilst in the previous sections (i.e. 6.3.1 and 6.3.2), differences were noted in terms of liposome size as well as the morphology of the tablets manufactured (and stored under variant conditions), SPAN was observed not to be different statistically. However, towards the latter two months of the stability testing period, SPAN was observed to increase in the HT conditions (Figure 6.4) though still not significant statistically (*p>0.05*). The discolouration in the tablets observed under these conditions for the latter months i.e. months 4, 5 and 6 (Section 6.3.1) may be linked to this as it was previously suggested that this may be a result of degradation of the lipid component. Differences in this component may directly influence the quality, or in this case, the consistency of the liposomes generated, resulting in a greater variance in liposome size distribution (i.e. SPAN).

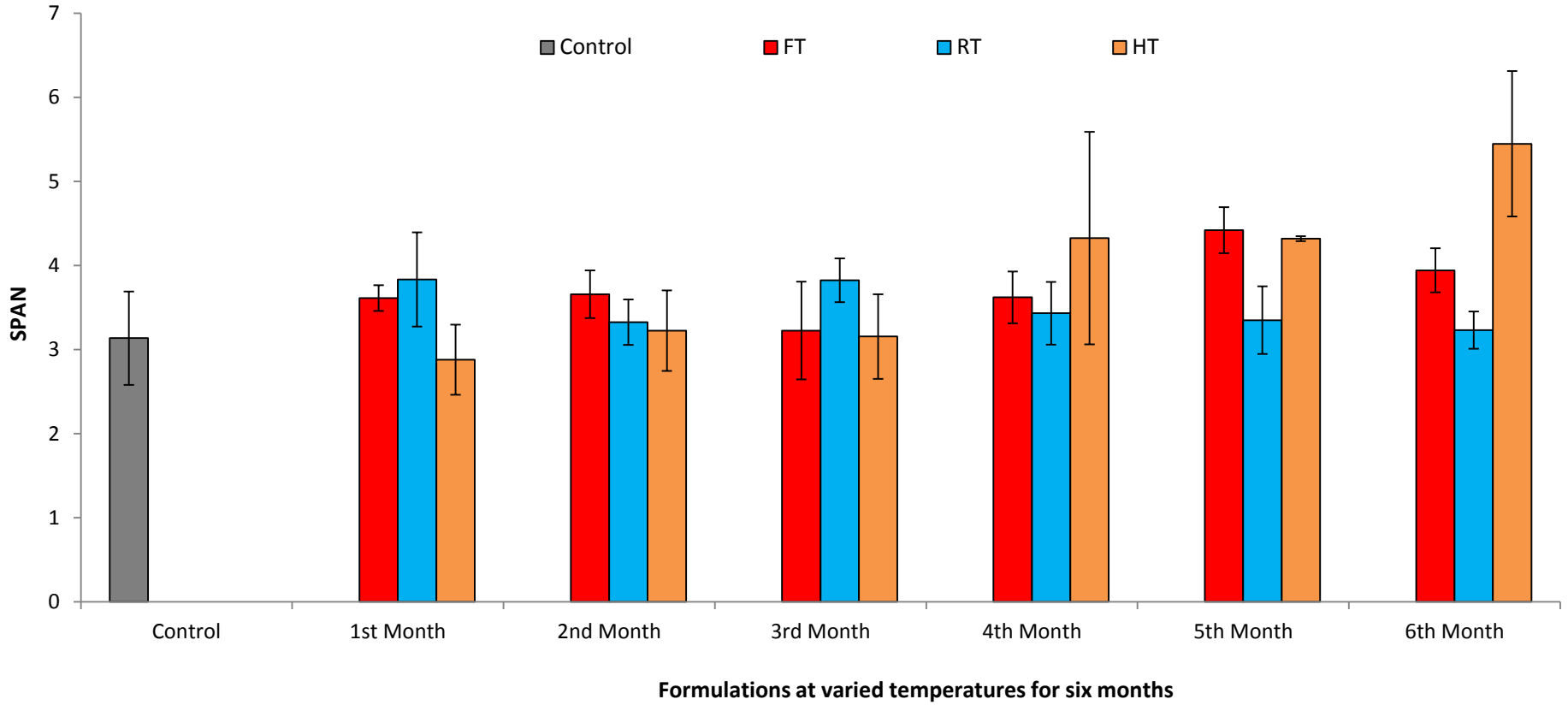


Figure 6.4: SPAN of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control tablets (freshly prepared) and tablets placed at three different temperatures condition (i.e. RT, FT and HT for six months) for comparison. Data are mean ± SD, n=3; $p<0.05$

6.3.4. Zeta potential of liposomes

6.3.4.1. Control and individual temperature condition comparison

The zeta potential of liposomes generated from the control tablets (in D_2O after hydration and annealing) were compared with the zeta potential values of liposomes generated from each of the monthly set of tablets for the six month stability period (where samples were stored at variant temperature conditions, i.e. FT, RT or HT).

For samples stored in FT conditions, a statistically significant difference ($p<0.05$) was observed when comparing samples stored at month 3 and 6 (i.e. -2.31 $\pm$ 0.07 and -3.99 $\pm$ 0.25 mV) with the control sample (-3.08 $\pm$ 0.26 mV) (Figure 6.5). Differences in zeta potential were not only noted for samples when compared with the control. A statistically significant difference ($p<0.05$) was observed upon comparison of months 5 and 6 (-3.37 $\pm$ 0.18 and -3.99 $\pm$ 0.25 mV) showing a high zeta potential for months 1, 3 and 4 (-2.34 $\pm$ 0.22, -2.31 $\pm$ 0.07 and -2.37 $\pm$ 0.03 mV respectively). Month 6 was also found to differ significantly ($p<0.05$) with month 2 in addition to the aforementioned months. The general trend was of increasing zeta potential with storage time, as this was found to be substantially higher in the latter months of stability testing. However, upon examination of Figure 6.5, it can be observed that month 3 generated zeta potential values were distinctly different from the overall trend of zeta potential values. However, taking into account the scale of zeta potential utilised, this difference may appear to be more exaggerated than its reality.

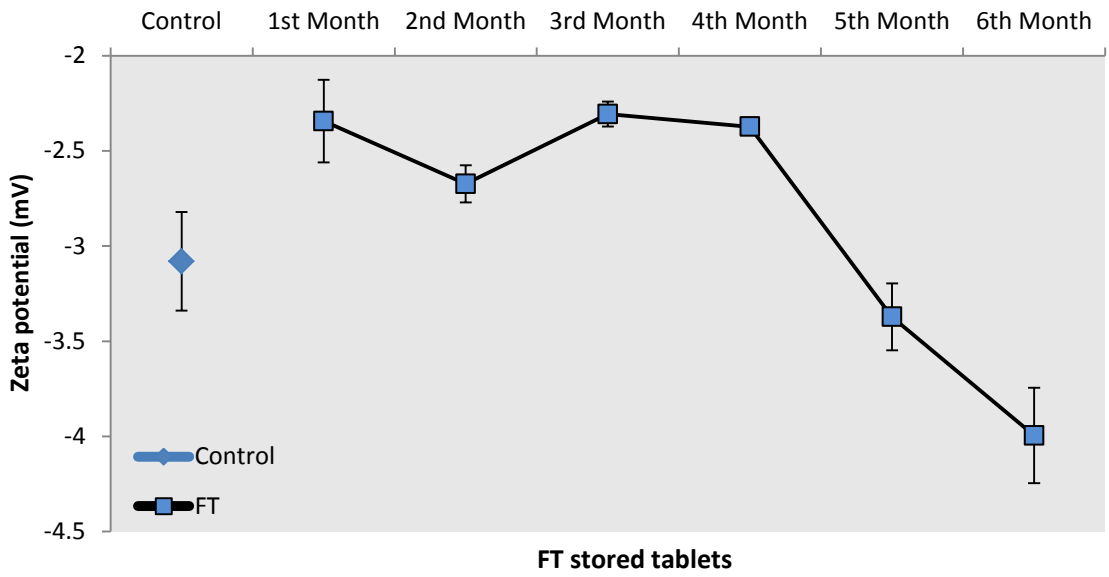


Figure 6.5: Zeta potential of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control tablets (freshly prepared) and tablets placed at FT storage conditions for comparison. Data are mean ± SD, n=3; p<0.05

With respect to samples stored in RT conditions, differences (*p<0.05*) were observed between the control sample (-3.08 ± 0.26 mV) and samples from month 5 and 6 (-4.00 ± 0.22 and -6.31 ± 0.27 mV), exhibiting higher charge values than control sample (Figure 6.6). Additionally, measurements conducted in month 5 and 6 were found to be significantly higher (*p<0.05*) to the alternate months tested (i.e. 1, 2, 3 and 4). Overall, as may observed from Figure 6.6 a general trend of increasing zeta potential was observed with increasing time, which accelerated in the latter months (i.e. month 5 and 6). This possibly indicates that under the RT storage conditions the lipid component is stable for the initial four month storage period. By contrast, in month 5 and 6 yet again, higher values in zeta potential may suggest a change or degradation of the lipid.

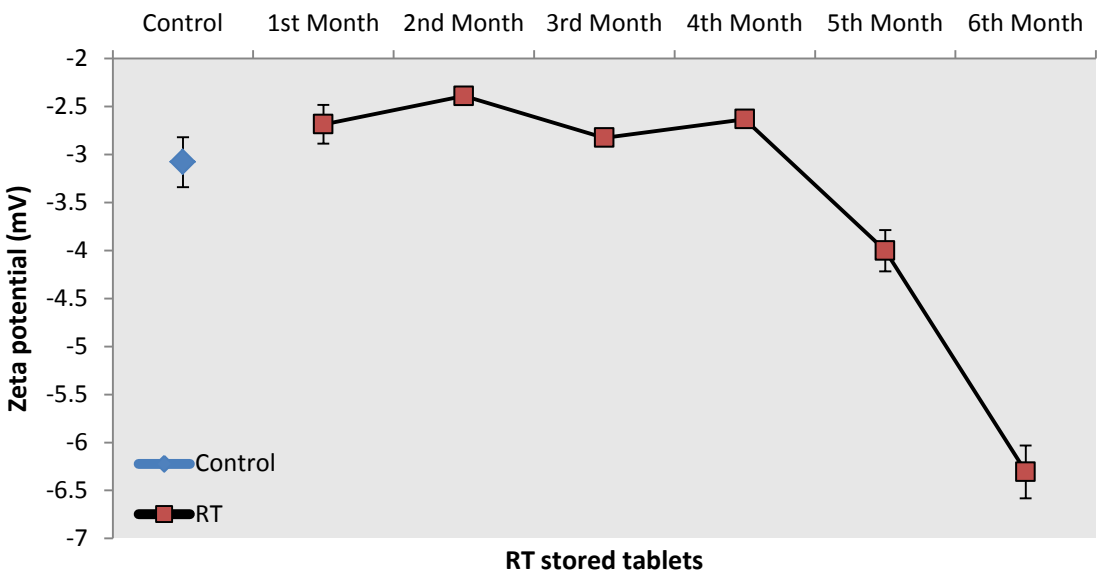


Figure 6.6: Zeta potential of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control tablets (freshly prepared) and tablets placed at RT storage conditions for comparison. Data are mean ± SD, n=3; p<0.05

Samples stored under HT conditions were found to exhibit a stronger trend in terms of increasing zeta potential in comparison to the samples stored in FT and RT. Initially, a statistically significant difference (*p<0.05*) was noted between the control sample (-3.08 ± 0.26 mV) and samples from months 3, 4, 5, and 6 (-4.52 ± 0.17, -6.39 ± 0.40, -6.84 ± 0.13 and -7.93 ± 0.25 mV respectively) showed a higher zeta potential under storage in the HT condition (Figure 6.7). Distinctively, following the third month of storage, zeta potential was found to dramatically increased reaching values of -7.93 ± 0.25 mV, as can be observed from (Figure 6.7). This contrasts with the range of zeta potential reached from samples tested using RT and FT, which even in the latter two months only exhibited zeta potential values of -6.31 ± 0.27 mV for FT and -3.99 ± 0.25 mV for RT. This rapid rise in zeta potential under HT conditions may be attributed to the effect of elevated temperature on formulation. Under the previously discussed storage conditions (i.e. RT and FT) zeta potential was found to increase further in the latter two months of the stability testing. This was particularly more so for the RT stored proliposomes tablets, therefore the effect of temperature upon zeta potential was evident. Elevated temperatures shortened the stability of the formulation in terms of the zeta potential of the liposomes generated.

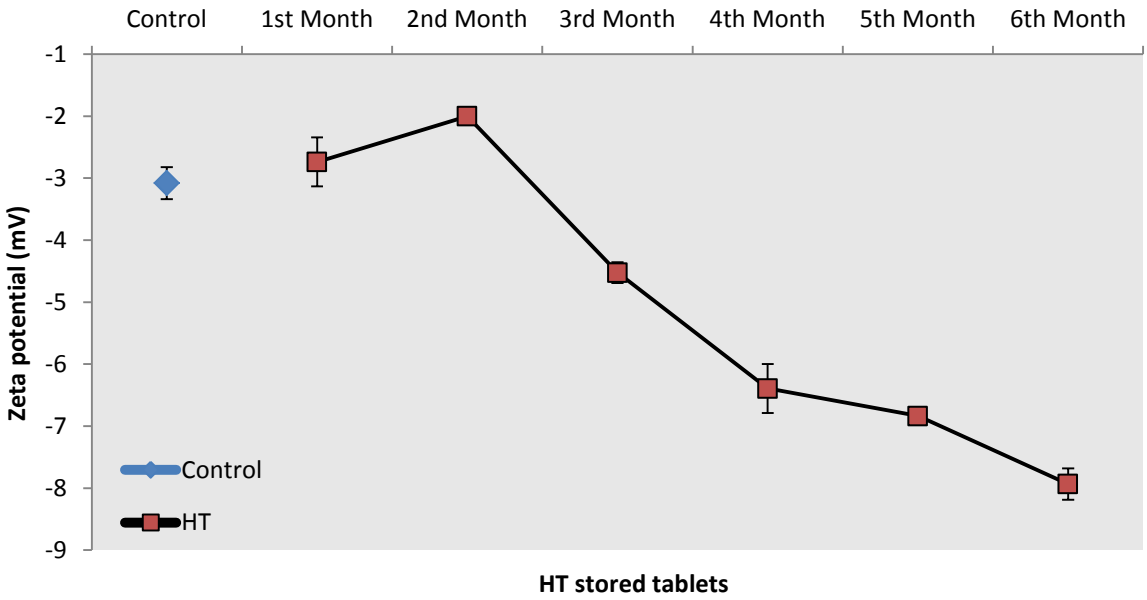


Figure 6.7: Zeta potential of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control tablets (freshly prepared) and tablets placed at HT storage conditions for comparison. Data are mean ± SD, n=3; $p<0.05$

Interestingly, phosphatidylcholine has been described as a neutral phospholipid pertaining little to no electrostatic charge (Hrdina et al., 2008). This however is dependent on the material from which the phospholipid is derived. In this case SPC is derived from soya and the control sample zeta potential indicates a neutral to mildly negative charge. Depending upon the material derived from phase transition temperature of PC may vary, which in turn may dictate their behaviour under elevated temperature. Thus, although zeta potential was found to increase over time this was observed to be more drastic following the third month of storage. Linked with the discolouration of tablets (Section 6.3.2), this further supports the suggestion that the lipid component is degrading with time, and that this process is partially temperature dependant (i.e RT and HT conditions). Further studies would attempt to identify the cause of lipid degradation; a potential cause may be oxidation of the lipid components resulting in discoloration under HT storage conditions.

6.3.4. Month by month temperature condition comparison

Comparison of the month by month formulations for zeta potential at varied conditions was also made to assess statistical significance of the difference amongst the liposomes generated from

proliposome tablets stored at various set temperatures for the six month period. Zeta potential values for month 1 showed no statistically significant difference (*p>0.05*) when compared to the counterpart formulations at different temperatures (i.e. FT, RT and HT); a similar trend was exhibited by samples stored at month 2 (Figure 6.8 and Figure 6.9). Alternatively, zeta potential values generated for samples stored (FT and RT conditions) for months 3, 4 and 5 were found to be statistically different (*p<0.05*) to HT stored samples (Figure 6.8 and Figure 6.9). Comparatively, differences in zeta potential (*p<0.05*) were observed between the month 6 FT, RT and HT (-3.99 $\pm$ 0.25, -6.31 $\pm$ 0.27 and -7.93 $\pm$ 0.25 mV) stored samples individually.

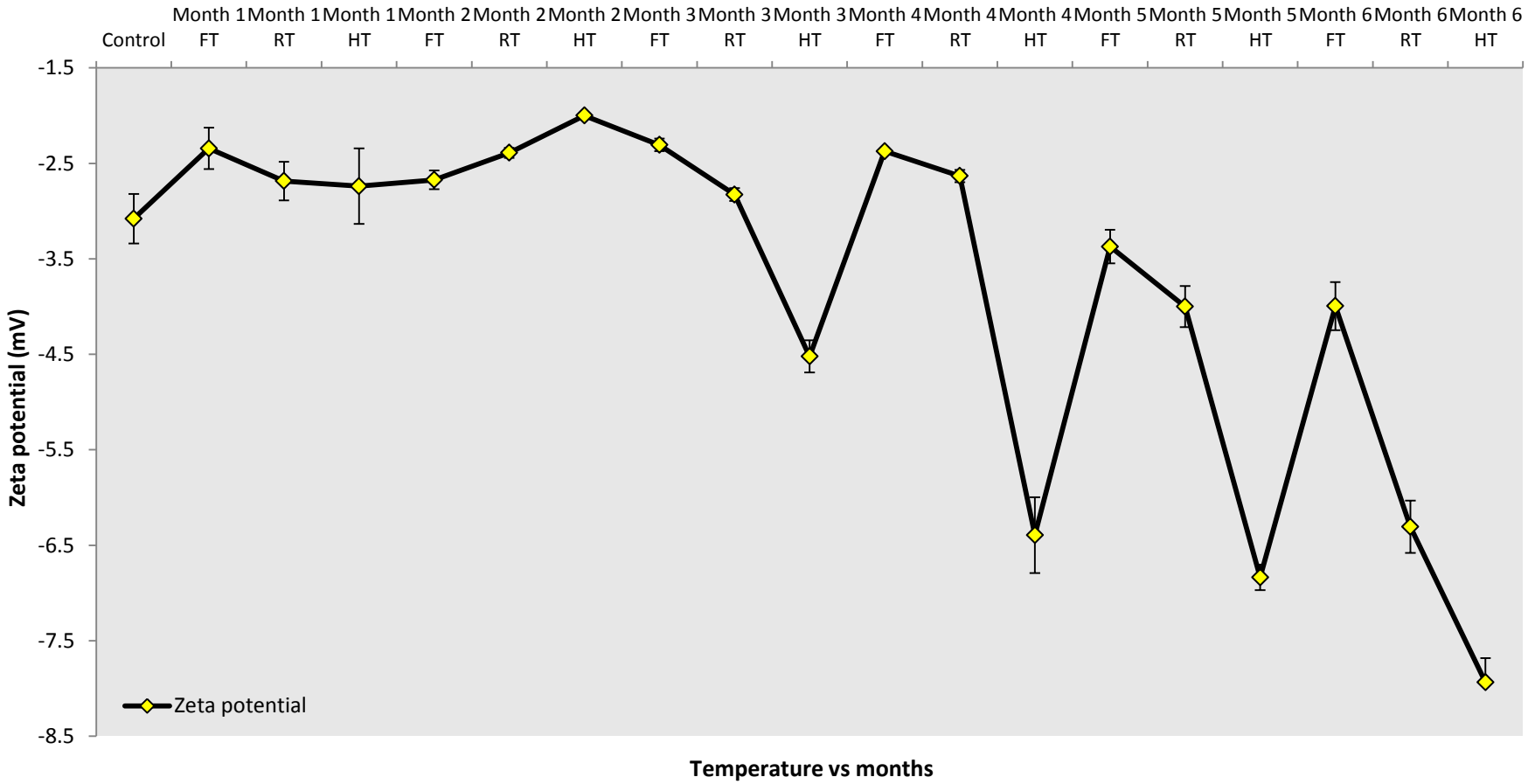


Figure 6.8: Zeta potential of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio), when proliposome tablets as a control (freshly prepared) were compared with proliposome tablets stored in CT, RT and HT conditions for month by month (i.e. six months). Data are mean ± SD, n=3; $p<0.05$

Overall irrespective of the storage condition, zeta potential was found to increase over time (Figure 6.8 and Figure 6.9), suggesting time dependant stability, although marked differences were observed under elevated temperature. Zeta potential is an established method, utilised in the prediction of stability of a given system. Should the charge be sufficiently high, (> ± 30 mV), whether negative or positive, particles in the system should display repulsion to one another, avoiding tendencies of aggregation. Contrastingly, particles with low zeta potential are more pre-disposed to flocculation due to their lack of repulsion (Hrdina et al., 2008, Laouini et al., 2012). Studies have indicated that a negative zeta potential in excess of 20 mV will suffice to prevent coalescence of vesicles (Wiacek and Chibowski, 1999, Sun et al., 2013). Consequently, a change in charge (as observed through storage under the various temperature conditions particularly HT), may cause aggregation of liposomes, resulting in larger liposome size (Section 6.3.2) with potential impact upon the actual drug delivery. Smaller liposomes are associated with lower charge, as mentioned charge in excess of ±30 mV is deemed to be sufficient to prevent aggregation of liposomes. Low charge values have also been indicated as a cause of liposome aggregation in other studies (Kurakula et al., 2012).

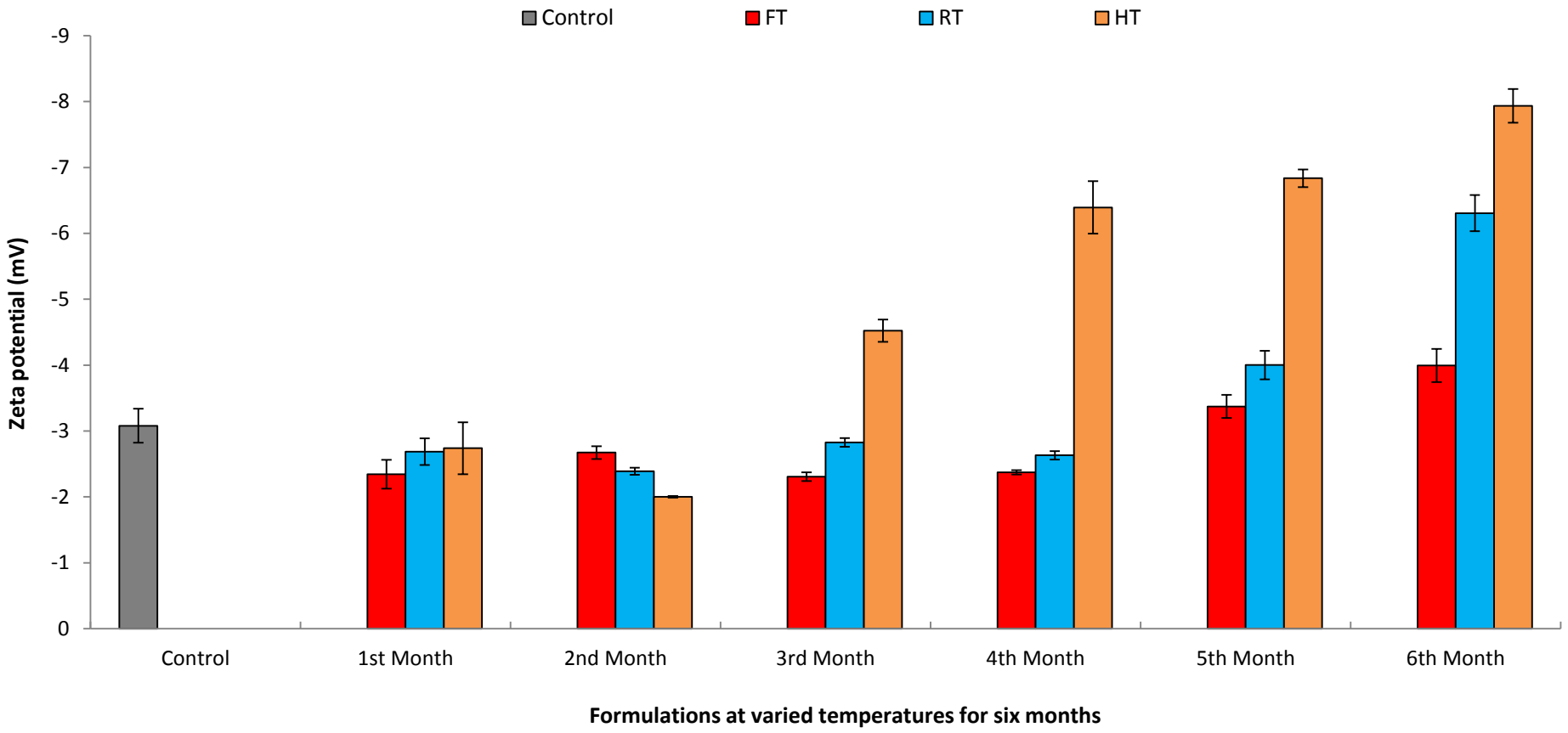


Figure 6.9: Zeta potential of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio), when proliposome tablets as a control (freshly prepared) were compared with proliposome tablets stored in CT, RT and HT conditions for six months. Data are mean ± SD, n=3; $p<0.05$

6.3.5. Weight variation of tablets

Weight variation of batches of tablets were assessed in accordance with BP testing (BP., 2010e). Comparisons in terms of weight variation were made between each monthly set of tablets (under each set of storage conditions i.e. FT, RT and HT) for the entire six month duration, with the control batch of tablets. Moreover, batches in each set of storage condition were compared directly in terms of weight variation with equivalent batches in alternative storage conditions (i.e. between month 1 FT, RT and HT).

6.3.5.1. Control and individual temperature condition comparison

Upon comparison of the control sample with each monthly sample at each condition (i.e. FT, RT and HT) no significant difference ($p>0.05$) was observed in terms of weight variation between the batches (Figure 6.10). This indicated that tablet weight remained relatively constant irrespective of the storage time or temperature condition.

6.3.5.2. Month by month temperature condition comparison

On comparison, of each month at each temperature condition (i.e. month 1 FT, RT and HT) no significant difference ($p>0.05$) was observed in terms of weight variation. Further reiterating that tablets weight remained consistent, irrespective of temperature condition or storage duration (Figure 6.10). This suggests that tablet weight remains stable under various conditions and durations.

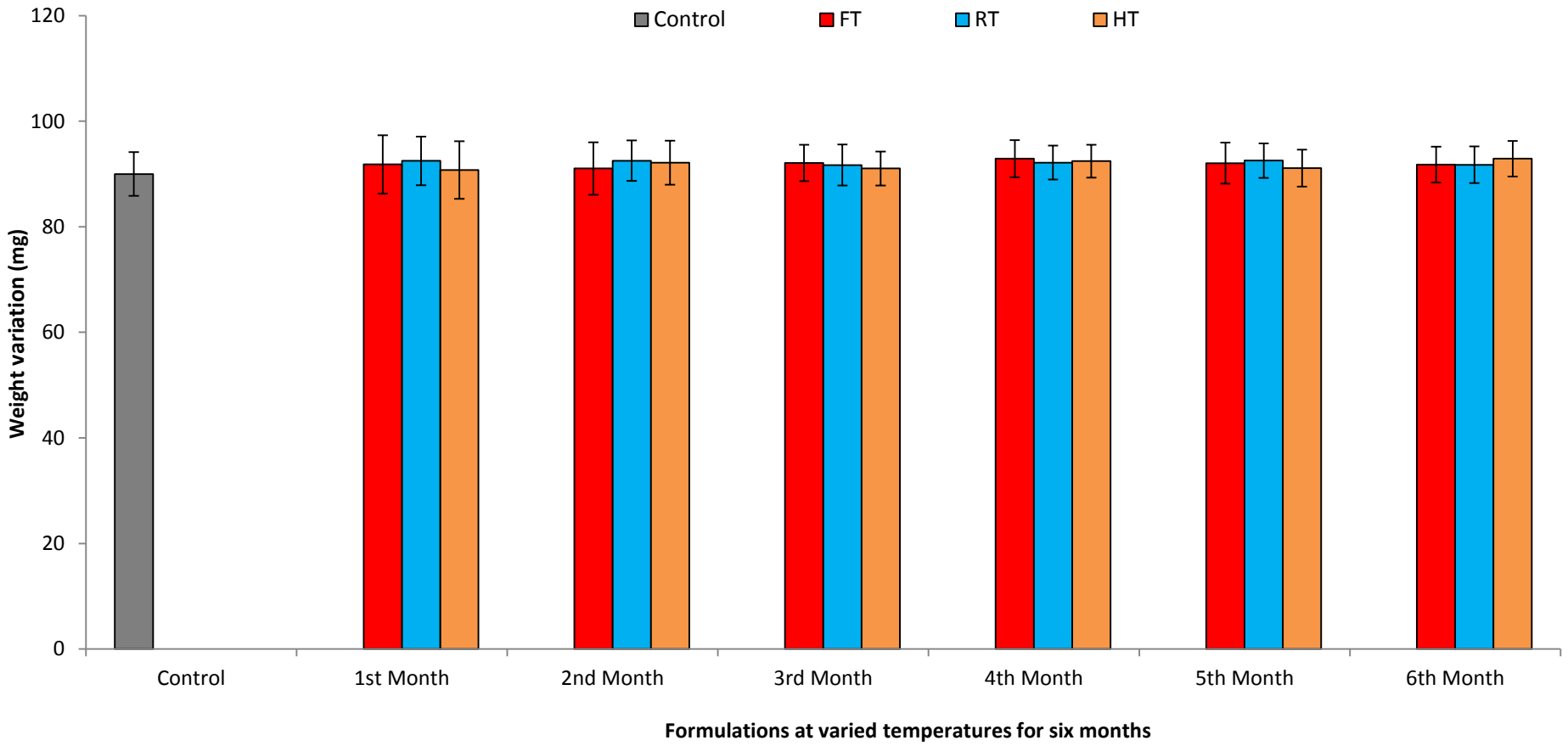


Figure 6.10: Weight variation of sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio), when proliposome tablets as a control (freshly prepared) were compared with proliposome tablets placed at three varied storage temperatures (i.e. RT, FT and HT). Data are mean ± SD, n=3; $p<0.05$

6.3.6. Disintegration of tablets

Disintegration testing conducted upon batches of tablets were assessed in accordance with BP guidelines (BP., 2010b). Comparisons in terms of disintegrations times of tablets were made between each monthly set of tablets (under each set of storage conditions i.e. FT, RT and HT) for the entire six month duration, with a control batch of tablets. Moreover, batches in each set of storage condition were compared directly in terms of disintegration time with equivalent batches in alternative storage conditions (i.e. between month 1 FT, RT and HT).

6.3.6.1. Control and individual temperature condition comparison

Comparisons in terms of disintegration time were made between each formulation at each set of conditions at monthly intervals with the control sample. Upon examination of the FT stored samples at various monthly intervals, no significant difference ($p>0.05$) was found between the control sample and any of the monthly tested sample in FT conditions in terms of disintegration time. This was suggestive that under FT conditions, disintegration time did not change.

However, upon examination of the RT stored formulations, no significant difference ($p>0.05$) was found between the control sample (5.85 $\pm$ 0.66 minutes) and the stored samples for months 1, 2, 3, 4, 5 and 6 (i.e. 6.46 $\pm$ 0.13, 7.02 $\pm$ 0.49, 6.12 $\pm$ 0.56, 6.55 $\pm$ 0.04, 4.79 $\pm$ 0.50 and 5.09 $\pm$ 0.07 minutes, respectively). Observation of Figure 6.11 demonstrates a slight decrease in disintegration for months 5 and 6 compared to the former monthly samples. Additionally, RT stored samples tested for month 5 were found to have significantly lower ($p<0.05$) disintegration times than samples tested for month 1, 2 and 4. Lastly, on comparison of month 6 with the alternative monthly samples, month 6 was found to hold a lower disintegration time than all the alternative monthly formulations (Figure 6.11). However, a significant difference ($p<0.05$) was found between month 6 (5.09 $\pm$ 0.07 minutes) and month 2 (7.02 $\pm$ 0.49 minutes) (the latter having a notably longer disintegration time than all other formulations).

The variance in disintegration time over an extended period, suggests that as a parameter disintegration time is not stable throughout storage under RT conditions, particularly for month 5 and 6. However, it is noteworthy that the degree of change in time is relative, and the disintegration conforms well within BP guidelines for uncoated standard tablets, even though it is seen to decrease over time (Figure 6.11). A lower disintegration time is also not necessarily perceived as a disadvantage; however tablet constituent quality is of the paramount of

importance. Should the reduction in disintegration time be as a consequence of degradation of the tablet constituents (i.e. lipid component), this may be detrimental to the formulation characteristics. Therefore further testing is indicated to further elaborate upon this trend.

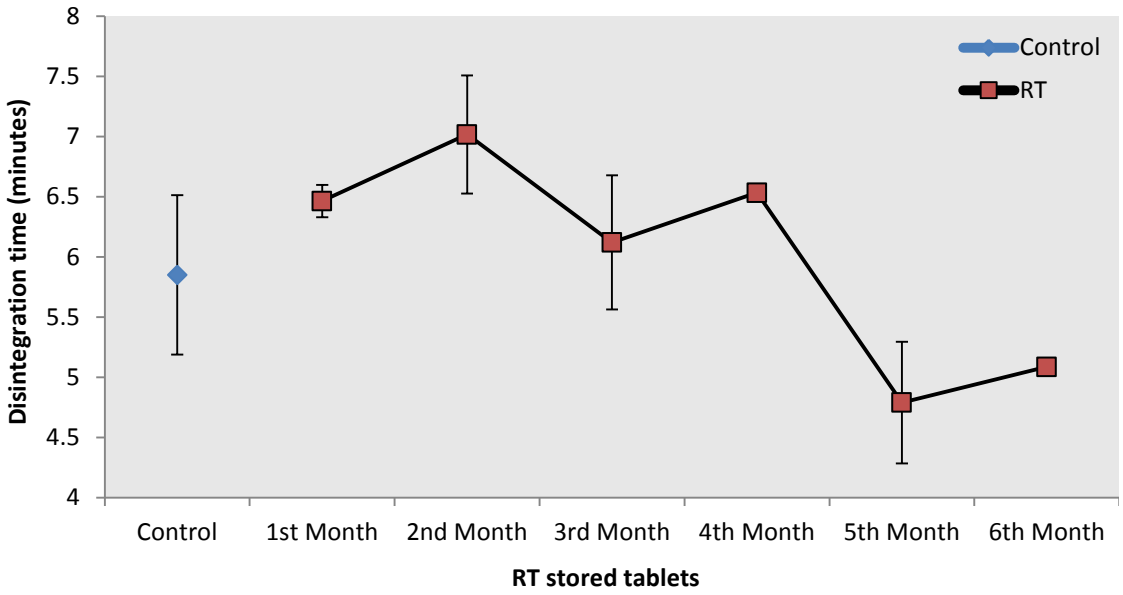


Figure 6.11: Weight variation of sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control tablets (freshly prepared) and tablets placed at RT storage conditions for comparison. Data are mean ± SD, n=3; p<0.05

Samples stored under HT conditions were assessed in a similar fashion with comparison to the control sample. Upon comparison, a statistically significant difference ($p<0.05$) was found between samples tested at month 6 (3.79 ± 0.55 minutes) exhibited lower disintegration time than the control sample (5.85 ± 0.66 minutes) (Figure 6.12). As may be observed in Figure 6.12, the disintegration time of samples tested at month 6 was substantially lower than that of the disintegration time of the control sample; moreover an overall trend of reducing disintegration time was noted on increasing storage duration. Potentially, storage at the elevated temperature may affect the lipid coating on the proliposome tablets, resulting in decreased disintegration time. This may be attributed to the degradation of the lipid component, which in turn may reduce the hydrophobicity of the tablet, hence reduce disintegration time.

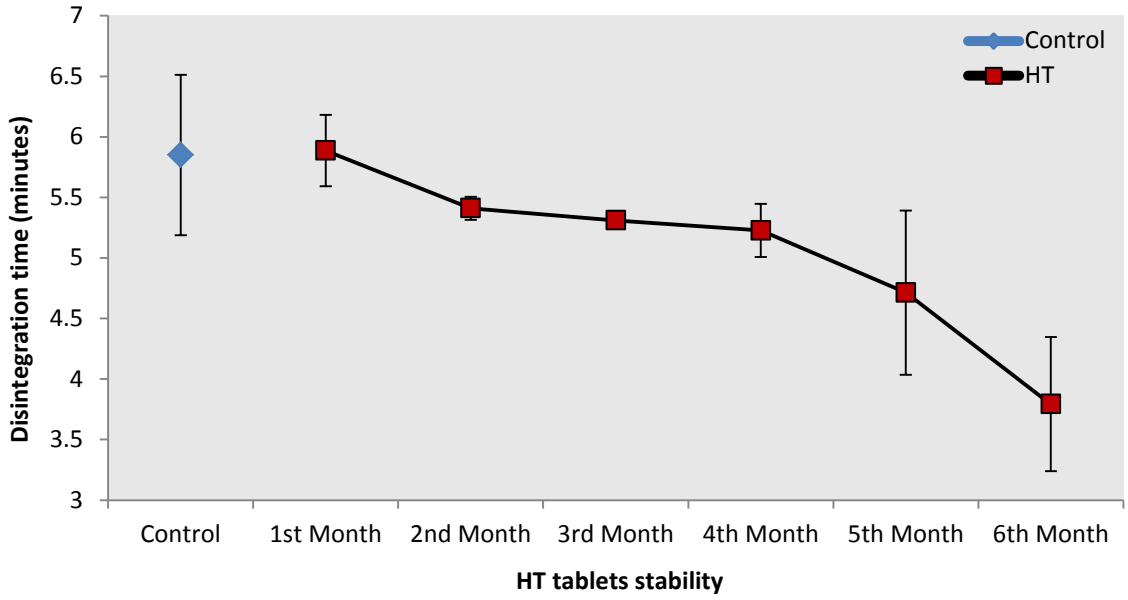


Figure 6.12: Weight variation of sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control tablets (freshly prepared) and tablets placed at HT storage conditions for comparison. Data are mean ± SD, n=3; p<0.05

Tablets stored under FT conditions were found to overall, marginally increase in disintegration time (Figure 6.13), though the difference was not significant (*p>0.05*). In contrast, formulations stored under RT and HT conditions were found to exhibit decreasing disintegration time as storage time increased. Therefore, a time dependant effect was not established in terms of disintegration time. It is clear that temperature was the overriding factor due the opposite trends observed in HT and RT samples, when compared to FT stored samples tested.

6.3.6.2. Month by month temperature condition comparison

On comparison, of each month at each temperature condition (i.e. month 1 FT, RT and HT) no differences were observed in terms of disintegration time. Between months 1, 2, 3 and 4 an insignificant difference (p>0.05) was observed between each storage condition (i.e. FT, RT and HT). However on examination of month 5 and 6, disintegration times for FT stored samples was significantly higher (*p<0.05*) than the disintegration times for samples stored under RT and HT conditions, respectively. This may be as a result of SPC has converting into free fatty acids, hence reducing surface hydrophobicity and making water penetration into the tablet easier; resulting in more rapid disintegration.

Sorbitol has been utilised for over a decade as a directly compressible sugar in the manufacture of normal/standard tablets (specifically chewable tablets). The only limitation pertaining to its use is its hygroscopic nature, this is negated upon tableting, as the smooth surface generated when sorbitol is used, is relatively impervious to moisture penetration (Basedow et al., 1986, Bolhuis et al., 2009). In this instance, the additional incorporation of a lipid component (i.e. in the formulation of proliposome tablets) further minimises moisture penetration due to its hydrophobic nature. In accordance with Bolhuis et al., (2009), sorbitol tablets are known to disintegrate in approximately 3.3 minutes (200 seconds), whereas lubricated sorbitol tablets (i.e. with 0.5% magnesium stearate) take circa 5 minutes (300 seconds). Similar findings were observed for proliposome tablets based upon using sorbitol carriers. In this instance, the lipid phase may influence disintegration (a time of 5.85 $\pm$ 0.66 minutes) of the freshly prepared proliposome control tablets (Figure 6.13).

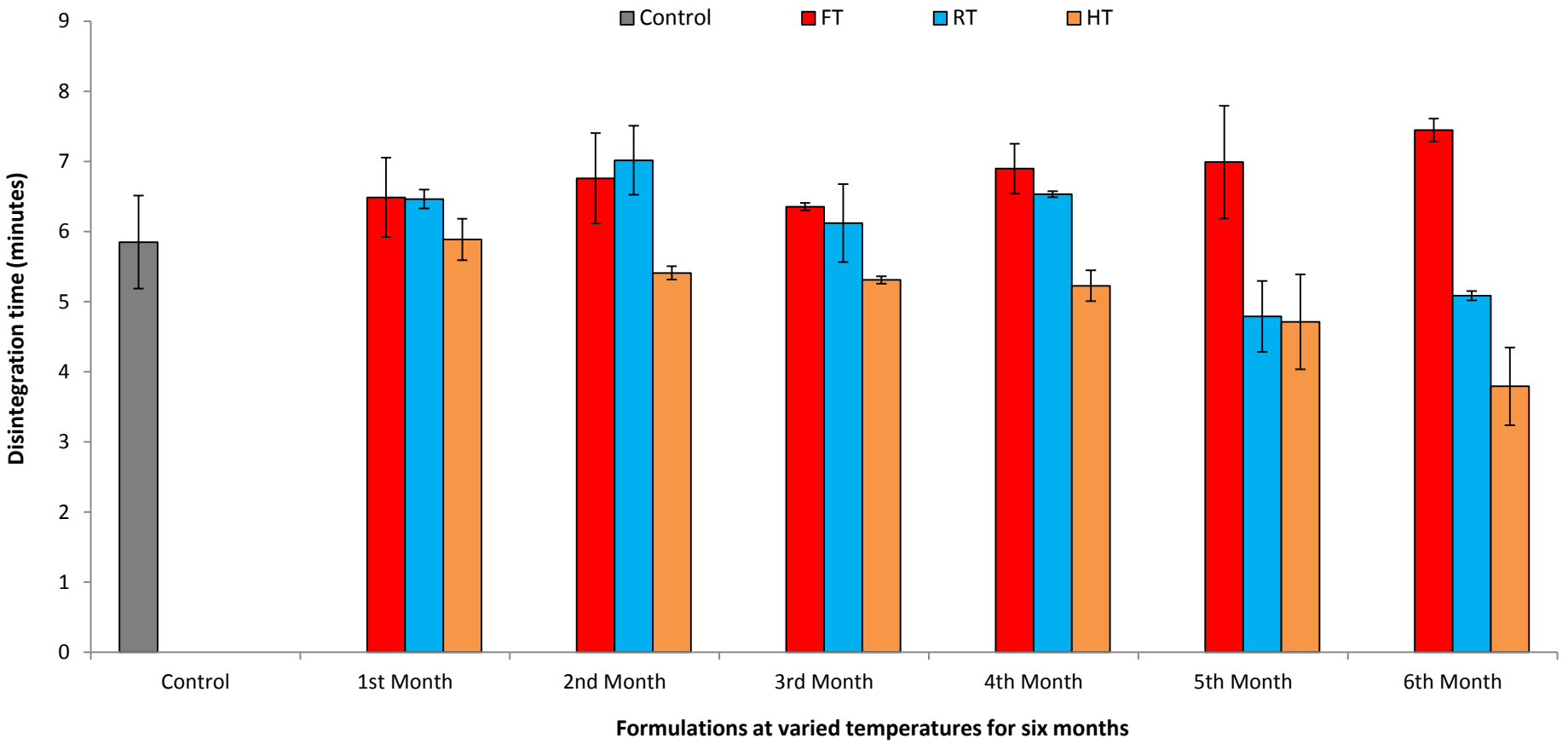


Figure 6.13: Disintegration time of sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio), when proliposome tablets as a control (freshly prepared), were compared with proliposome tablets placed at three varied storage temperatures (i.e. RT, FT and HT). Data are mean ± SD, n=3; $p<0.05$

6.3.7. Hardness or crushing strength of tablets

Hardness or crushing strength measurements for the prepared tablets were conducted in accordance to BP guidelines (BP., 2010c), using a hardness tester. Hardness values were determined for each monthly set of stored tablets (i.e. those stored in FT, RT and HT conditions) for the entire six month duration, and compared directly with hardness values generated for freshly prepared control tablets, as well as the hardness values for the each of the monthly stored samples; in the same storage conditions. In addition to comparison with the control set of tablets, batches at each set of storage conditions were compared directly for differences or similarities in terms of crushing strength i.e. equivalent batches in alternative storage conditions were compared with one another (i.e. between month 1 FT, RT and HT).

6.3.7.1. Control and individual temperature condition comparison

Upon comparison of each of the stored formulations (i.e. under FT, RT and HT conditions), at each monthly interval with the control sample, no significant difference ($p>0.05$) in the hardness of the tablets stored was observed (Figure 6.14). Moreover, comparison of each of the monthly intervals within each set of storage conditions (for example, month 1 RT with month 2, 3, 4, 5 and 6 RT) further showed no significant difference in terms of crushing strength. This would indicate that the structure formed upon compression remained intact throughout storage even in alternate temperature conditions i.e. elevated and lowered temperatures. In terms of hardness the tablets were deemed to exhibit a good degree of stability suggesting that they would retain their structural integrity.

6.3.7.2. Month by month temperature condition comparison

On comparison, of each month at each temperature condition (i.e. month 1 FT, RT and HT with each other), no differences ($p>0.05$) were observed in terms of crushing strength for any of the stored formulations investigated (Figure 6.14). In addition to previous findings showing a lack of difference between the control sample and any of the store formulations, this would further indicate that the structure of the tablets remained intact and showed stability across the six month period, irrespective of the condition stored at.

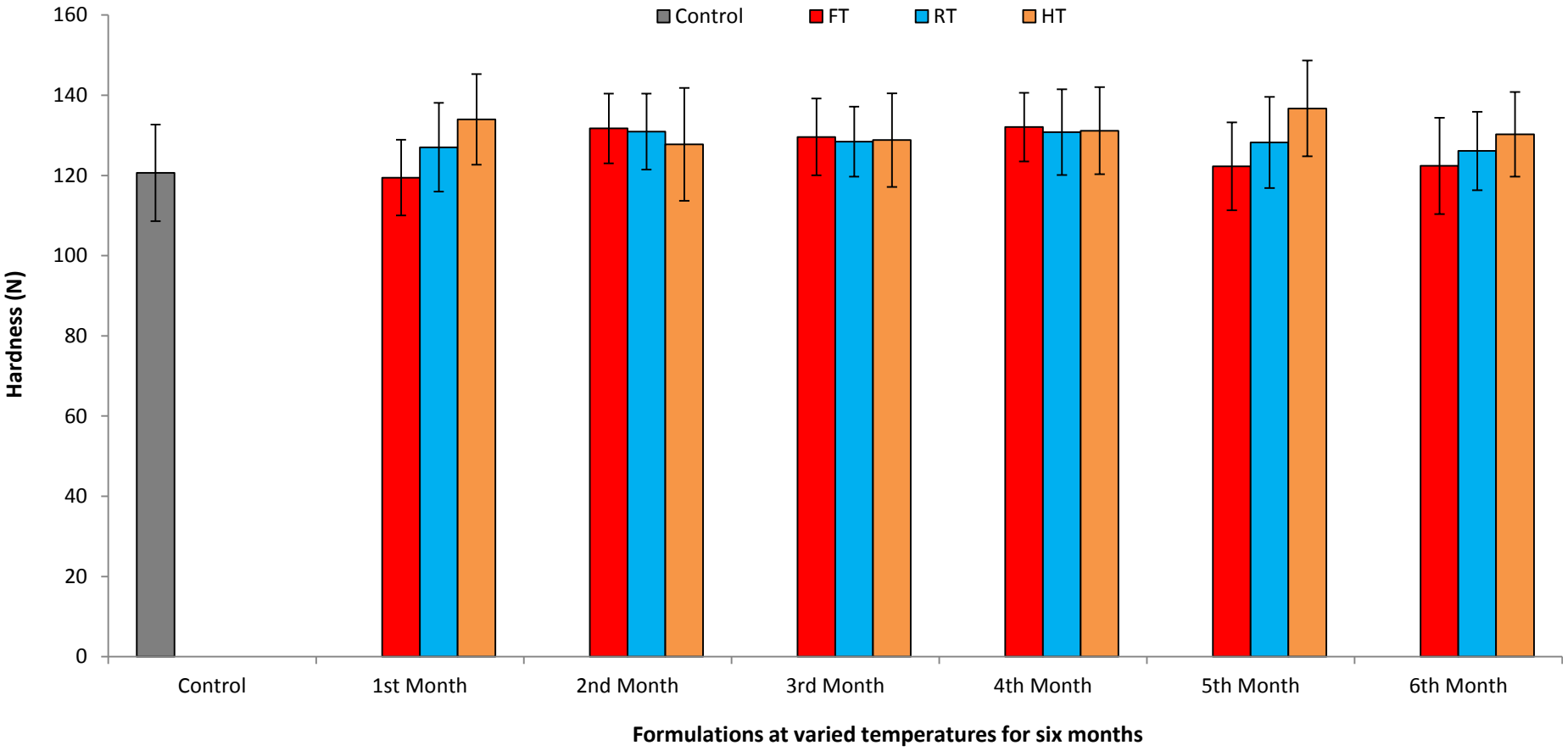


Figure 6.14: Hardness or crushing strength of sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio), when proliposome tablets as a control (freshly prepared) were compared with proliposome tablets placed at three varied storage temperatures (i.e. RT, FT and HT). Data are mean ± SD, n=3; $p<0.05$

6.3.8. Friability of tablets

Whilst friability may be described as an additional technique for indicating tablet hardness, its purpose is more to provide information as to whether the tablet may be able to withstand the handling involved during blistering and transportation. The parameters for passing this test are stringent, as the BP outlines that no more than 1% weight loss is considered acceptable after four minutes (i.e. 25 revolutions per minutes) of testing using a specified apparatus (BP., 2010c). Much like previous tests conducted (hardness etc.), friability values were derived from each monthly set of stored tablets (i.e. those stored in FT, RT and HT conditions) for the six month stability period, and compared directly with friability values generated for freshly prepared control tablets. Comparisons were further made between the friability values generated for each set of monthly stored samples in the same storage conditions (i.e. FT, RT and HT) e.g. month 1 RT compared with months 2, 3, 4, 5 and 6 RT. Yet again, in addition to comparison with the control sample, the stored samples were compared with one another in terms of equivalent batches (i.e. month 1 FT, RT and HT were compared directly in terms of friability).

6.3.8.1. Control and individual temperature condition comparison

None of the stored tablets were found to differ significantly in terms of friability with the control sample ($p>0.05$), and all were found to show less than 1% weight loss indicating a pass in terms of friability testing, as may be observed from Figure 6.15. Moreover, comparison of each of the monthly intervals within each set of storage conditions (for example, month 1 RT with month 2,3,4,5 and 6 RT) further showed no significant difference ($p>0.05$) in terms of friability. This would indicate that all the tablets would be able to withstand manual handling during blistering and storage for up to six months irrespective of the storage condition.

6.3.8.2. Month by month temperature condition comparison

Upon comparison of the stored formulations with each other i.e. month 1 FT with RT and HT, no significant difference ($p>0.05$) was observed in terms of friability. This would indicate that in terms of friability the formulations retained stability regardless of the storage condition.

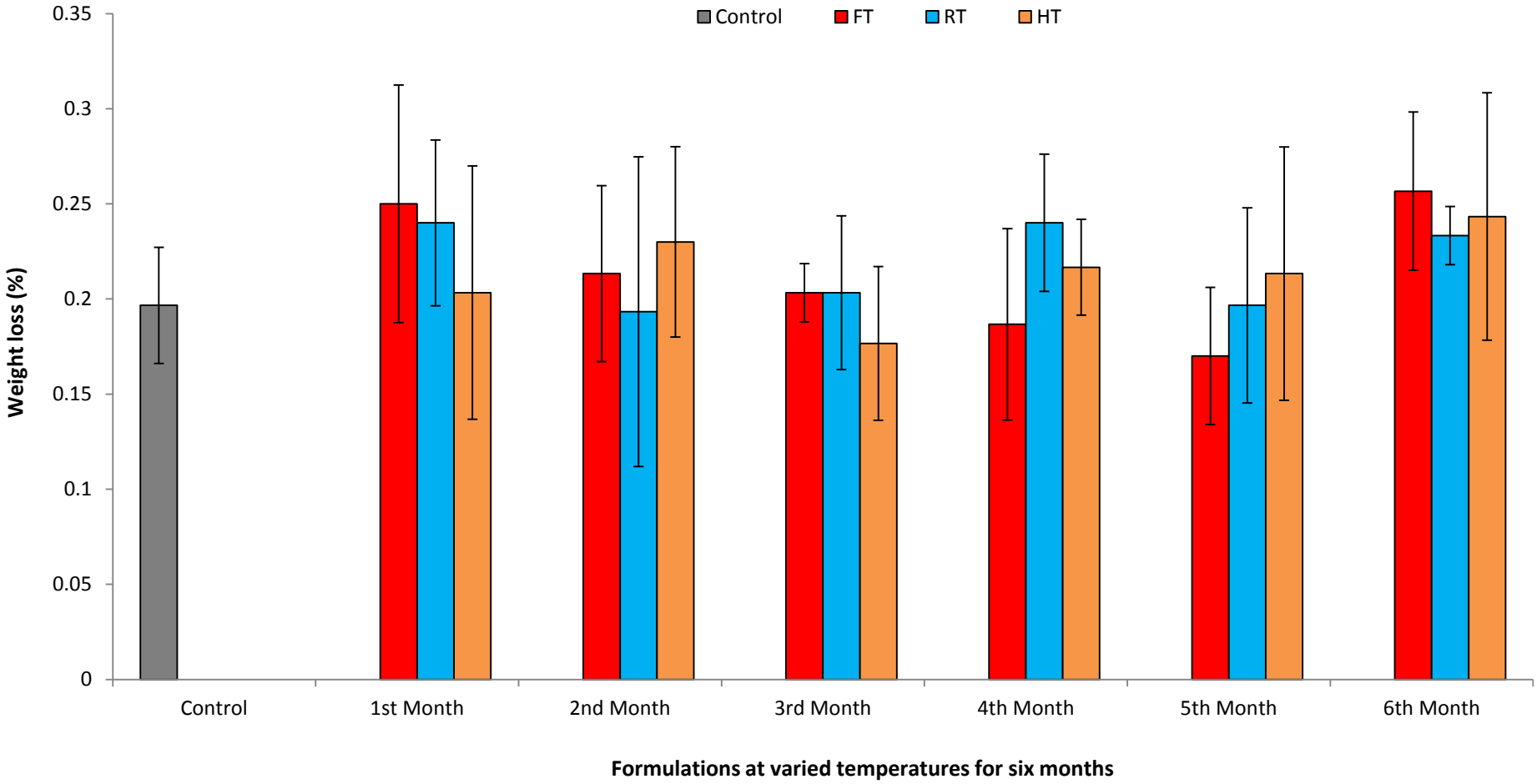


Figure 6.15: Weight loss during friability of sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio), when proliposome tablets as a control (freshly prepared), were compared with proliposome tablets placed at three varied storage temperatures (i.e. RT, FT and HT). Data are mean ± SD, n=3; $p<0.05$

6.3.9. Drug entrapment efficiency

Whilst the aforementioned stability tests are of great importance, entrapment efficiency may be described as one of the most crucial parameters measured in terms of formulation stability. This is simply because, differences in entrapment efficiency will ultimately greatly impact upon the concentration of drug which reaches the patient from the formulation. Entrapment efficiency as described in section 2.2.9 and 2.2.10, was established using HPLC. Here, stored proliposome tablets (in FT, RT and HT conditions) were hydrated in D_2O, centrifuged, and the relevant fractions were quantified using HPLC to establish drug concentration. This was additionally conducted for a freshly prepared batch of proliposome tablets, with which comparison was also made as well comparison between each of the stored formulations at equivalent time intervals (i.e. month 1 FT with RT and HT stored samples); for the total six month period.

6.3.9.1. Control and individual temperature condition comparison

Comparisons in terms of entrapment efficiency were achieved by directly comparing the entrapped component, the unentrapped part as well as the drug spot with the control samples. Initially, no significant difference ($p>0.05$) was observed upon comparison of the RT stored formulations at each month with the control sample. This indicated that at RT samples retained good stability in terms of entrapment efficiency.

For the FT stored samples, no significant difference ($p>0.05$) was noted between entrapment values generated for the control sample and the remaining formulations (all monthly intervals for the six months) tested in terms of the entrapped and unentrapped component or the drug spot. However a significant difference ($p<0.05$) was observed upon comparison of month 6 FT (6.83 $\pm$ 1.62%) and month 1 FT (2.45 $\pm$ 0.78%), the unentrapped drug concentration was found to be significantly higher for the month 6 FT stored sample than the month 1 FT stored sample (Figure 6.16). These findings indicate that a greater concentration of drug was found to be unentrapped as storage time was increased.

Samples stored under HT conditions, exhibited some similar trends to the previously tested samples, however differed in other trends. Firstly, no significant difference ($p>0.05$) in terms of entrapment efficiency (i.e. entrapped, unentrapped and spot concentration) was observed upon comparison of the control sample with the majority of the HT stored samples at each monthly interval (Figure 6.16). However month 5 and 6 (i.e. 29.30 $\pm$ 3.63 and 32.56 $\pm$ 5.47%) showed a

notably lower entrapment ($p<0.05$) than the control sample (57.43 ± 9.12%), indicating lower stability in the latter months of storage under HT conditions, in terms of entrapment efficiency. A notable drop in entrapment efficiency was observed following the third month of stability testing, and this dropped was maintained in the latter tested months. Month 5 and 6 were also found to exhibit notably lower entrapment ($p<0.05$) than months 1 and 2 (Figure 6.17).

6.3.9.2. Month by month temperature condition comparison

Comparisons in terms of entrapment efficiency were made between each month in corresponding storage conditions e.g. Month 1 FT with month 1 RT and HT.

Upon comparison of month 1 samples stored at the varied conditions (e.g. FT, RT and HT) no significant difference ($p>0.05$) was observed in entrapment efficiency (i.e. using entrapped, unentrapped and drug spot concentration values). This trend was observed for months 2, 3 and 4; indicating stability in terms of entrapment efficiency to this point.

At month 5 HT, entrapment efficiency (29.30 ± 3.63%) was significantly different ($p<0.05$) to the comparable monthly stored formulations in FT and RT (56.19 ± 1.94 and 55.86 ± 2.76%) conditions. At month 5 the entrapment efficacy of the HT stored samples was notably lower than comparative entrapment efficiencies exhibited by the RT and FT stored samples. This trend was also demonstrated by the samples tested for month 6 (Figure 6.16), indicating that following the fourth month of storage, samples stored under HT conditions were no longer stable in terms of entrapment efficiency.

The lower entrapment values obtained for the HT stored samples at month 5 and 6 may be explain by potential degradation of the lipid phase. A potential degradation in the lipid phase would subsequently result in a lower concentration of liposome generation in the dispersion medium (D_2O) and as a consequence, a lower entrapment efficiency of the drug utilised, BDP. This is supported by Gupta et al., (2008) work in proliposome powders, who reported that lower lipid concentrations would result in lower entrapment efficiencies (Gupta et al., 2008). Moreover, temperatures above the phase transition temperature (T_m) of the lipid are believed to cause destabilization in the liposomes, resulting in drug leakage from the liposome vesicles and a consequently lower entrapment efficiency (Lasic, 1998).

In alternative study conducted by Kurakula et al., (2012) utilising prednisolone in proliposome powder formulation, it was found that samples stored at elevated temperatures as well as low

temperatures (i.e. freezing point) were found to insignificantly (*p>0.05*) effect the entrapment efficiency for up to a period of three months (Kurakula et al., 2012), which is mirrored in the present piece of work conducted. However, the present study conducted stability testing for a period of 6 months, in which after month 3, stability was found to decrease (i.e. lower drug entrapment) of the stored formulations was observed for the HT stored samples.

A further study conducted by Manjula D et al., (2014), examined proliposome powder formulation stored at varied temperatures (i.e. 8, 25, 37 and 45^{o}C) for 90 days. It was observed that following the 90 days period, the proliposome powders stored at 8 and 25^{o}C were stable in terms of physical appearance (e.g. colour) and also the entrapment of the utilised drug (Fenoprofen). Comparatively, proliposome colour was found to change and the entrapment efficiency dropped by 12% at the third month for the sample stored at 40^{o}C, with comparison to the initial sample. The elevated temperature may be potentially responsible for lipid degradation (Manjula et al., 2014). In the present research a change in colour of the sorbitol-based proliposome tablet formulation was observed at month 6 for the HT stored samples, this could potentially indicate degradation in the lipid component, which is also supported by lower entrapment values at this month tested (Figure 6.16).

In an additional study, proliposome powders formulated with naproxen as the API, showed that storage at elevated temperatures (40°C) for three months, resulted in a reduction of entrapment efficiency from 92.6% to 90.7%, further highlighting the deleterious effect of elevated temperatures upon proliposome formulations. In the study, it was proposed that drug leakage at the elevated temperature was responsible for the lower entrapment values observed, and that storage at 8°C may minimise this effect (Prasad et al., 2014).

The aforementioned research puts forward the suggestion that elevated temperatures are detrimental in the stability of proliposome formulations; a suggestion which is mirrored in this current piece of research. Additionally, lower storage temperatures (FT and RT conditions), are proposed as a means to maintain the stability of the formulations, preventing the stability in terms of entrapment efficiency from dropping.

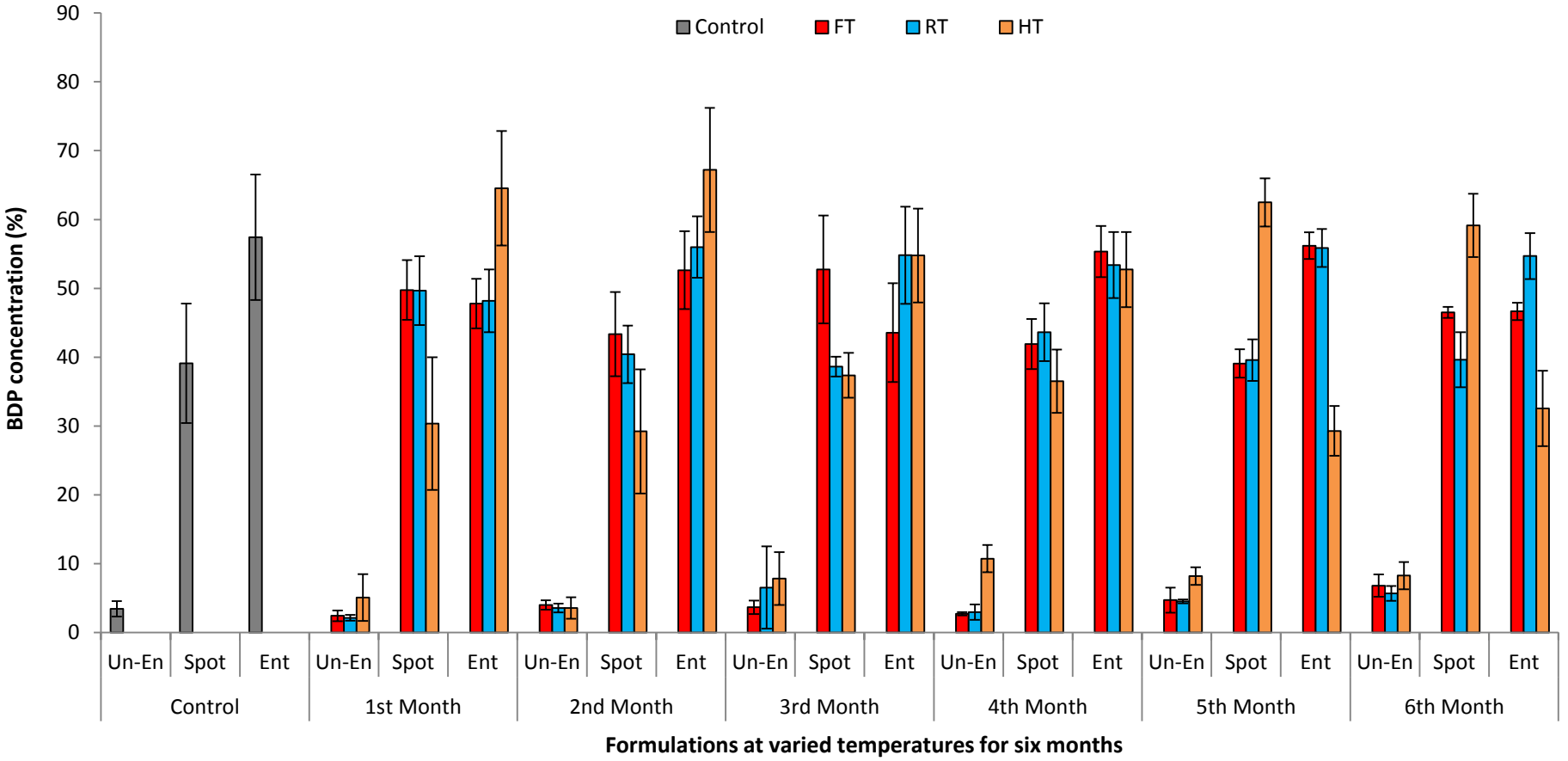


Figure 6.16: BDP-entrapped in liposomes, the unentraped fraction and free BDP crystals of sorbitol-based proliposome tablets with (1:15 w/w lipid to carrier ratio), when control proliposome generated liposomes were compared with the proliposome tables stored in FT, RT and HT conditions for the six months of stability. Data are mean ± SD, n=3; $p<0.05$

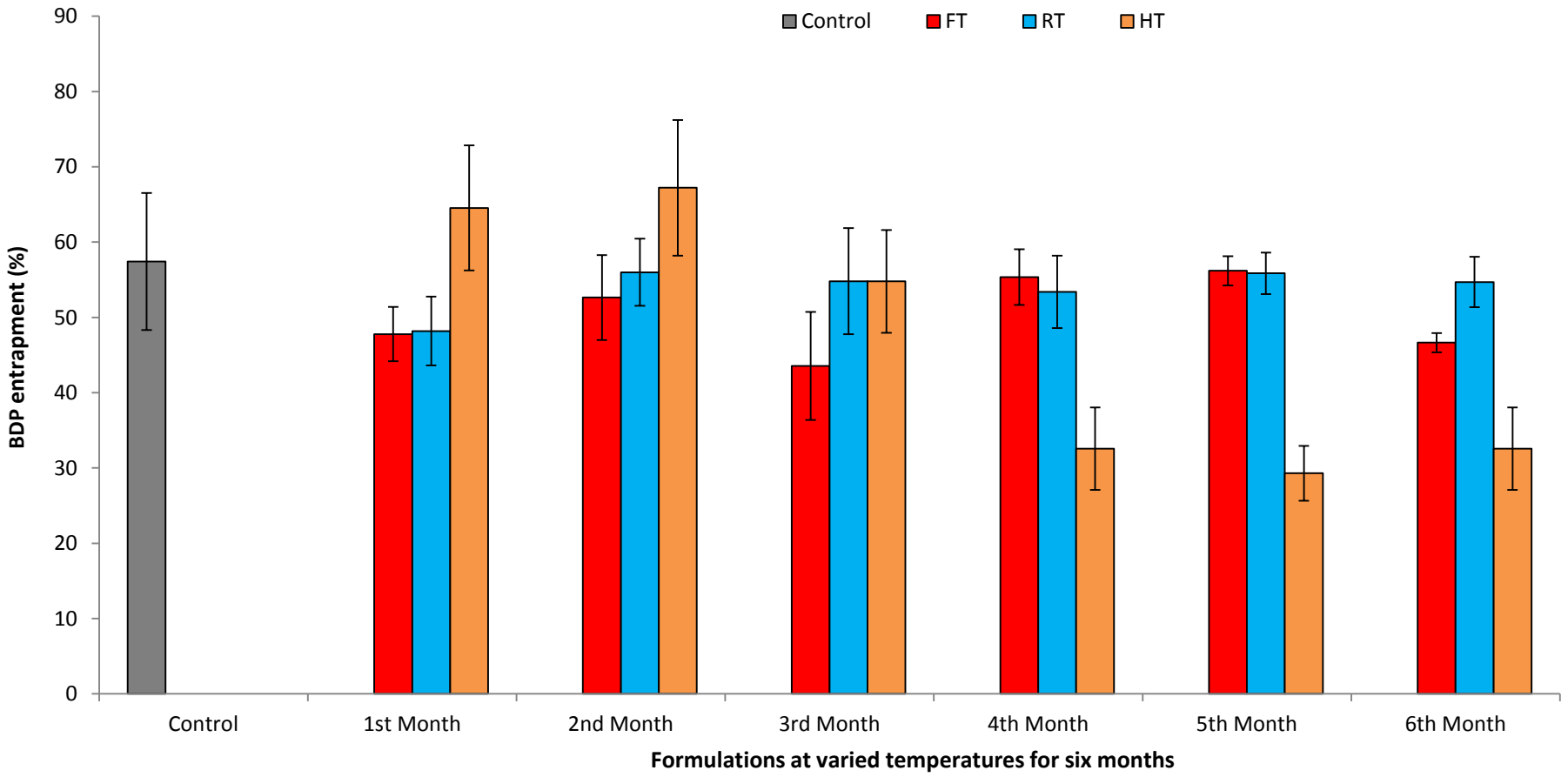


Figure 6.17: Entrapment efficiency of BDP-entrapped in liposomes after centrifugation in D_2O when liposomes were generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio); control proliposome generated liposomes were compared with the proliposome tables stored in FT, RT and HT conditions for the six months of stability. Data are mean $\pm$ SD, n=3; $p<0.05$

TEM studies were performed upon liposomes generated from sorbitol-based proliposomes in a 1:15 w/w lipid to carrier ration. It was observed that the majority of liposomes generated (freshly prepared) were oligolamellar in nature (Figure 6.18).

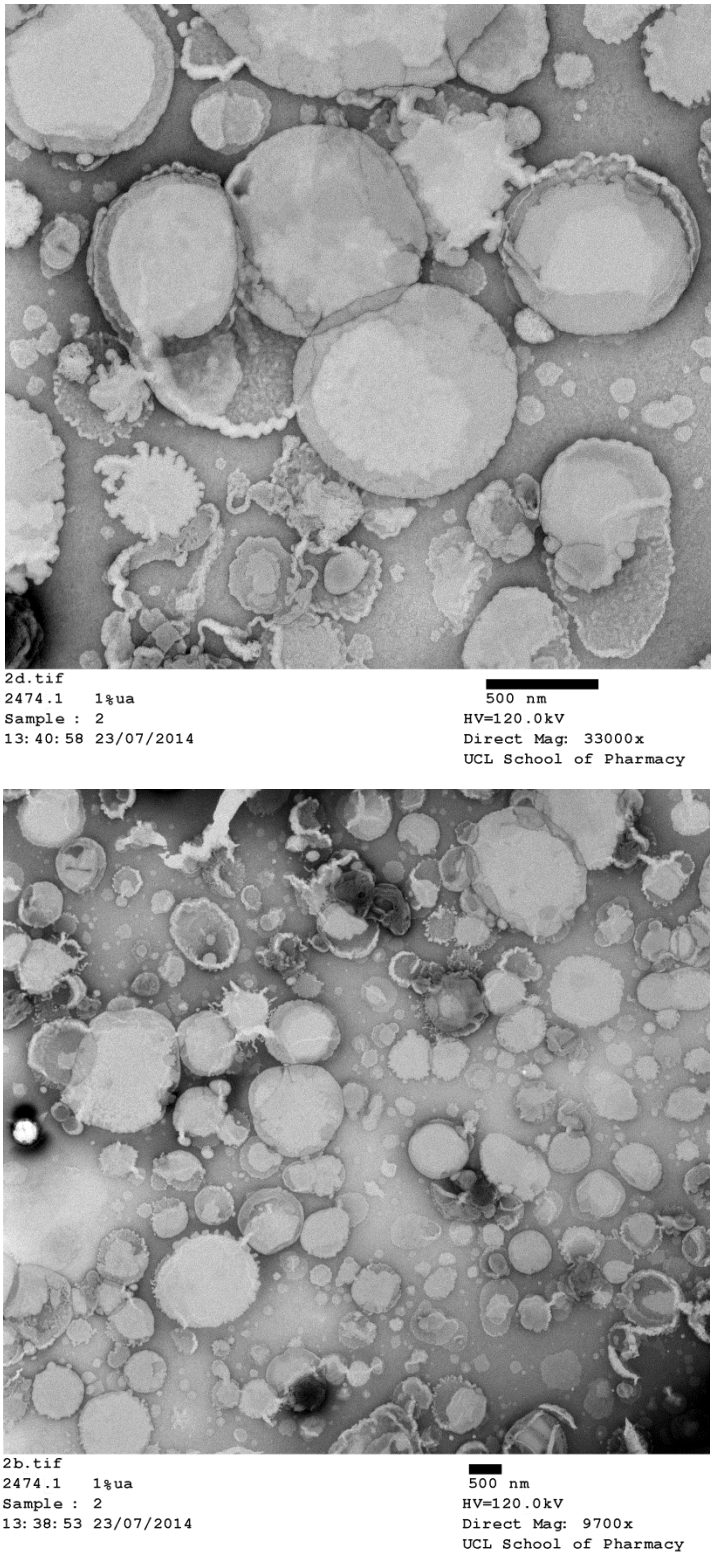


Figure 6.18: TEM photographs of liposomes generated from sorbitol-based proliposome tablets (1:15 w/w lipid to carrier ratio) with magnification of X33000 and X9700

6.4. Conclusions

Various parameters have been examined in this research in the assessment of the stability of the proliposome tablets formulated, including: Physical morphology, liposome size, size distribution, zeta potential of liposomes, weight variation of the tablets, disintegration time, tablet hardness, friability and of crucial importance, the entrapment efficiency.

A number of the investigated parameters clearly showed the formulated tablets retained stability, irrespective of storage condition or length. Some of which include: weight variation of tablets, tablet hardness and friability, all of which exhibited no statistically significant difference ($p>0.05$) across each temperature condition and across the six month duration. This would indicate that in terms of manual handling of the tablets, the formulated tablets were sufficiently stable to withstand physical handling (indicating possible suitability for packing and transport), for a period of up to six months.

Contrastingly, there were parameters which showed a difference in terms of the proliposome tablet stability depending on storage condition and storage duration. Firstly, tablet morphology was found to differ when placed under HT storage conditions after a period of three months, here the stored tablets were found to discolour, transforming from a brilliant white to off-white yellowish colour. Prior to additional testing, this was the first indication that storage at elevated temperatures may be detrimental upon the formulation. The explanation suggested for this discolouration was degradation of the lipid component, as sorbitol is not known to discolour under elevated temperatures.

On examination of liposome size generated from these stored formulations, the majority of the tested formulations showed little statistically significant difference. However, in support of the suggested degradation at elevated temperatures, liposome size was found to dramatically increase in the latter months (months 4, 5 and 6) of the HT stored samples, and size distribution correspondingly to a much lesser extent. All other stored formulation showed very little difference in size.

In terms of zeta potential, the trend exhibited by the HT stored samples was mirrored to the size of liposome i.e. it increased over time. In the third month of storage zeta potential showed a significant increase with comparison to the former months and comparatively stored formulations. This difference was observed to amplify as the six month duration continued, again indicating a change in the stability of the formulation. Additionally whilst samples stored under FT conditions continued to show no variance, samples stored under RT conditions also showed some

increase in zeta potential in the latter months. Overall, this suggested that the FT conditions were favourable thus far in maintaining the stability of the proliposome tablets.

Additionally wide variances in disintegration time were observed depending upon the storage conditions. For HT stored samples disintegration time was found to decrease notably over the six month period whereas for RT stored samples this trend was less exaggerated. Contrastingly, FT stored samples exhibited increasing disintegration time over the six month duration. Though variances in disintegration time were noted, all stored formulations tested fell within the pass time range as outlined by the BP. Overall, disintegration time trends, indicated a temperature dependency, this can yet again be attributed potentially to the lipid component, however further work would be required to establish and elaborate upon this trend.

Finally entrapment efficiency was examined for each of the stored formulations under each storage condition. For HT stored samples, this drastically decreased following the month 3 of testing, suggesting that the formulation was only stable for three months in terms of entrapment efficiency. FT and RT stored samples exhibited small variance in entrapment efficiency over the six month period and by comparison to each other.

Comparisons of each of the parameters investigated for the stored formulations outlined in this chapter are tangible for selection of the appropriate storage conditions for proliposome. High variance in entrapment efficiency, liposome size and also zeta potential in the latter months of the HT stored samples suggests that the proliposome tablet formulation is unable to withstand HT storage conditions and these are not suitable for use, particularly after three months. FT and RT samples showed much greater stability than HT stores samples, in terms of the parameters investigated, however aside for a large increase in disintegration time (which still passed BP guidelines) FT storage conditions for proliposomes tablets exhibited the most superior stability which may potentially carried forward for further stability, testing and product development.

CHAPTER 7: CONCLUSIONS AND FUTURE WORK

"As long as you keep learning from defeats, you will keep coming back and get stronger all the time"

- Imran Khan -

7.1. Research Overview

Pulmonary drug delivery remains a heavy area of interest for research. Developments within this field; and the high number cases of pulmonary diseases per year globally, necessitate the pursuit of novel and viable pulmonary drug delivery formulations.

- Liposomes offer a viable drug delivery system for the delivery of API to the pulmonary system, providing sustained release and low toxicity. Stability, however remains a problem, including: oxidation and hydrolysis; liposome fusion and aggregation; and leakage of drug. Proliposome preparations have been introduced; however, manufacturing methods suffer from pitfalls (e.g. uneven lipid coating over carbohydrate carriers).

- Entrapment efficiency is a crucial parameter, as this essentially dictates the quantity of drug which reaches the patient. The usage of DW, as a dispersion medium for the separation of BDP-entrapped liposomes from the unentraped BDP, is widespread. However, whilst reported entrapment values in this medium are in excess of 80%, there remains conflict amongst studies in terms of entrapment efficiency reported for BDP. The analysis of the entrapment efficiency process is paramount in understanding these wide ranging values.

- Though proliposomes offer stability over liposomes, the bulky nature of the formulated powders present difficulty with regards to packaging (i.e. blistering) and transport; necessitating the development of compact solid dosage forms.

- Size and size distribution of liposomes, is a crucial parameter which dictates the degree of drug delivery to pulmonary regions. Smaller liposomes with low size distribution are readily nebulized, and reach the various pulmonary regions with ease. Whereas, larger sized liposomes typically stay as dead volume (in the nebuliser reservoir). The development of formulations which have minimal associated dead volumes during nebulization, is therefore an area of high interest.

- With respect to the stability of proliposome formulations, studies are ordinarily conducted for a maximum period of three months (across literature). As the majority of

solid dosage forms available on the market have relatively long shelf-lives, this period may be deemed as insufficient, to wholly analyse stability.

7.2. Research Contribution

The purpose of this research was to address the aforementioned issues, particularly the development of a novel proliposome tablet formulation, for pulmonary drug delivery. Through this process, a number of key developments have been made in this research area, these include:

7.2.1. Slurry method

The loss of formulation associated with current proliposome manufacture methods; instigated the development of the novel "slurry" proliposome manufacture method, which was used in this research. Essentially, an ethanolic solution (containing drug and lipid) was poured onto the chosen carbohydrate carrier, to form a “slurry”. After which, drying was achieved using a rotary evaporator, to yield proliposomes with uniform coating of lipid and high yield.

7.2.2. Separation methods and entrapment efficiency

The disparity in entrapment values for BDP when DW was utilised as a dispersion medium, is widespread across literature. These conflicting findings formed the basis of the conceptualization and development of a novel separation technique, using a higher density medium, D_2O. The raised density of D_2O allowed for more complete separation of BDP-entrapped liposomes from unentrapped components; allowing for greater accuracy in the determination of entrapment efficiency. The results indicated that the presence of BDP crystals, ineffectively separated when DW was used as a dispersion medium, gave rise to false entrapment values; which were clarified using the techniques outlined in chapter 3.

7.2.4. Proliposome tablets

Novel proliposome tablets were manufactured and characterised using official Quality Control (QC) tests, specified by British and United States Pharmacopeia. Via analysis, sorbitol-based proliposome powders at a 1:15 w/w lipid to carrier ratio, were found to be the most ideal formulation. This was in terms of their ease for proliposome tablet manufacturing, via the automated function of the Minipress machine. The overall formulation characteristics, and capability of automated tableting, identify this formulation as most suitable for upscaling to industrial manufacture, as oppose to powder formulations.

7.2.5. Nebulization of proliposome powder and tablets

The proposed formulations were designed with intent for nebulization. Upon research, sorbitol-based proliposome tablets in a 1:15 w/w lipid to carrier ratio, were found to possess shorter nebulization times; and higher output rates of aerosol, when compared to the remaining tested formulations. The utilisation of a TSI during this process, allowed for examination of the degree of pulmonary aerosol deposition. Liposomes sized around and below 4.4 μm, were observed to be most suitable for alveolar targeting; a desired goal of pulmonary drug delivery.

7.2.6. Stability studies of proliposome tablets

The stability testing of developed formulations, for a period in excess of three months was considered crucial for this research. Tablets were stored at three storage conditions, including: Room Temperature (22 $\pm$ 1°C), Fridge Temperature (6 $\pm$ 1°C) and Hot Temperature (40 $\pm$ 1°C); to investigate the formulation stability, for a duration of six months. Up until three months, all formulations demonstrated ample stability (i.e. no significant difference was observed in QC tests conducted). Post three months, the size of liposomes generated was found to increase, significantly, when stored at the hot temperature. Moreover, under hot temperature storage, entrapment efficiency was noted to drop to half of the initial recorded values. With the exception of the hot condition, marginal changes in formulation stability were observed over the six month period.

7.3. Summary

This research aimed to successfully develop a proliposome tablet formulation for pulmonary drug delivery. Through this process two novel methods; one for proliposome powder manufacture, and the other for accurate determination of entrapment efficiency, were developed. The selected proliposome tablets manufactured, showed sufficient capability for nebulization, indicating potentially good treatment outcomes. With added stability over a six month period, this highlighted the formulation's potential as a viable, marketable pharmaceutical product.

7.4. Future work

Research conducted throughout this study, is by no means exhaustive; a number of proposed continued research ideas were put forward; in order to further confirm and elaborate upon research findings in this study, these include:

- The usage of various combinations of phospholipids and carbohydrates carriers (i.e. with the exception of those utilised in this research), to ascertain whether the trends observed in this research are generic, or restricted to the carriers and formulations ratios investigated.
- To further investigate the trend of decreasing entrapment efficiency, with increasing lipid content. This again would be partially achieved using different carriers and phospholipids. However, analysis of the composition of the BDP crystal spot, following separation, via TEM, XRD and DSC would be proposed to establish this.
- A multitude of medical nebulizers are available in the market, which may be employed for further assessment of aerosol deposition and subsequent deposition of API in the lungs.
- In order to enhance disintegration time, superdisintegrants may be incorporated. This would reduce the time between nebulizer formulation preparation (i.e. dissolution of tablet) and patient administration.
- It is highly desirable for these formulations to be tested *in vivo,* or at the minimum, tested upon cell lines; to establish their potential for sustained release and targeting.

CHAPTER 8: REFERENCES

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National Anthem of Pakistan

قومی ترانہ (Urdu)	Transliteration	Translation (English)
پاک سرزمین شاد باد:	pāk sarzamīn shād bād	Blessed be the sacred land
کشور حسین شاد باد	kishwar-e-hasīn shād bād	Happy be the bounteous realm
تو نشان عزم علیشان	tū nishān-e`azm-e-`alīshān	Thou symbol of high resolve
ارض پاکستان !	arz-e-pākistān!	O Land of Pakistan!
مرکز یقین شاد باد	markaz-e-yaqīn shād bād	Blessed be the citadel of faith
پاک سرزمین کا نظام:	pāk sarzamīn kā nizām	The order of this sacred land
قوت اخوت عوام	qūwat-e-ukhūwat-e`awām	Is the might of the brotherhood of the people
قوم ، ملک ، سلطنت	qaum, mulk, sultanat	May the nation, the country, and the state
پائندہ تابندہ باد !	pā'inda tābinda bād!	Shine in glory everlasting!
شاد باد منزل مراد	shād bād manzil-e-murād	Blessed be the goal of our ambition
پرچم ستارہ و ہلال:	parcham-e-sitāra-o hilāl	The flag of the crescent and star
رہبر ترقی و کمال	rahbar-e-taraqqī-o kamāl	Leads the way to progress and perfection
ترجمان ماضی شان حال	tarjumān-e-māzī, shān-e-hāl	Interpreter of our past, glory of our present
جان استقبال !	jān-e-istiqbāl!	Inspiration for our future!
سایۂ خدائے ذوالجلال	sāyah-e-khudā-e-zu-l-jalāl	Shadow of God, the Glorious and Mighty