Proniosome formulations for pulmonary drug delivery

By

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Declaration

I declare that while registered as a candidate for the research degree, for which this submission is made, I have not been a registered candidate or enrolled student for another award of the University or other academic or professional institution. No material in the thesis has been used in any other submission for an academic award.

Signed

Abstract

In this study niosomes were generated from proniosomes (Span 60: Cholesterol 1:1) using beclomethasone dipropionate (BDP, 5 mole %) as a model drug. When niosomes were processed via probe sonication, photon correlation spectroscopy study showed that niosomes generated from proniosomes had larger size than conventional niosomes, being 202 nm and 160 nm, respectively. Transmission electron microscopy (TEM) showed that these niosomes were highly aggregated. The zeta potential values of niosomes were negative regardless of the preparation method. However, a highly intense negative charge was detected for niosomes generated from proniosomes (-36 mV) compared to niosomes (-25 mV). The entrapment efficiency (EE) of BDP was higher in proniosomes-driven niosomes compared to conventional niosomes, being 35% and 27%, respectively.

Probe sonicated, niosomes manufactured using the proniosome technology were nebulised using either Pari LC Sprint (air-jet), Aeroneb Pro (actively vibrating-mesh) or Omron Micro Air NEU22 (passively vibrating-mesh) nebulisers. The Pari and Aeroneb Pro nebulisers generated higher aerosol and drug outputs compared with the Omron nebuliser. Whilst drug output was around 80% for the Pari and the Aeroneb Pro, it was around 70% for the Omron. Similarly, droplet size generated from the Omron was larger than that generated from the Pari and the Aeroneb Pro, being 4.86 µm, 3.06 µm and 3.32 µm respectively. Using laser diffraction for aerosol droplet size analysis, the Pari nebuliser generated aerosols with the highest fine particle fraction (FPF) (around 80%) followed by the Aeroneb Pro nebuliser (approximately 70%) and the Omron nebuliser (around 40%). The predicted alveolar deposition was negligible for the Omron and approximately 20% for the Aeroneb Pro and the Pari nebulisers.

Using niosomes generated from proniosomes, the high shear homogeniser (NanoDebee) has produced niosomes having a small size ($Z_{average}$ = 236.5 ±13 nm) which was comparable to probe sonicated niosomes ($Z_{average}$ =209.2 ±21.4 nm). The zeta potential was also similar, being, respectively -38 and -36 mV. Also similar EE values were found, being 36.4% ±2.8 and 29.65% ±4.04 for the probe sonicated vesicles and the Nano Debee-homogenised vesicles, respectively.

Probe sonicated niosomes generated from proniosomes were freeze-dried. Rehydration of freeze-dried niosomes caused a massive increase of the size (by 13 times) of niosomes, indicating instability of niosomes.

Overall, the study in thesis has demonstrated that niosomes generated from proniosomes were possible to be delivered via medical nebulisers with a high FPF using BDP as a model antiasthmatic drug. A large scale production method was introduced using high shear homogenisation. However, freeze-drying exerted a destabilising effect on niosomes, causing either massive aggregation or fusion of the vesicles.

Acknowledgment

I extend my appreciation and grateful thanks to Dr. Abdelbary Elhissi for guiding me through my MSc degree, giving me many excellent suggestions and supporting me in every aspect. I would like to thank the members of my supervisory team, Prof. Waqar Ahmed and Prof Jaipaul Singh for their valuable and kind advice throughout my research. My special thanks to Banu Abdallah and Maha Naser for their useful comments and mental support throughout the difficult times. I am indebted to many of my colleagues, Nozad Khurmaley, Seema Jaiswal, Samradhi Lal and Basel Arafat, who have provided their helping hand in my research. Lastly, I offer my regards and blessings to my husband Darya, my parents Nazifa and Fadhil and my siblings Robar, Mohamed and Midia for all their love and support.

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List of abbreviations

BDP	Beclomethasone Dipropionate
Span	sobritan monostearate
TEM	Transmission electron microscopy
EE	Entrapment Efficiency
W/O emulsion	Water in Oil emulsion
COPD	Chronic Obstructive Pulmonary Diseases
RDS	Respiratory Distress Syndrome
e.g.	exempli gratia/ for example
IgE	Immunoglobulin E
pMDIs	Pressurized metered dose inhalers
DPIs	Dry Powder Inhalers
CFC	Chloroflorocarbon
PBS	Phosphate Buffered Saline
PCS	Photon Correlation Spectroscopy
PI	Polydispersity Index
UV- Spectrophotometer	Ultra violet spectrophotometer
HPLC	High Liquid Chromatography
VMD	Volume median diameter
SD	Standard deviation
MLV	Multi Lamellar Vesicles
SUV	Single Unilamellar Vesicles
FPF	Fine Particle Fraction

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Chapter 1

Introduction

1.1 Liposomes and niosomes

In the recent years, continuous hard work has been done for development of drug carrier systems which are able to deliver active molecules specifically to the target organ, increase therapeutic efficacy and minimize adverse or side effects. Delivery systems may allow drug release to sustain for prolonged time in the targeted tissue, resulting in enhanced therapeutic efficacy, and minimised side effects due to the lower concentrations of the free drug in the blood (Lamprecht, 2009). Niosomes represent one of the promising drug delivery systems, with particular interest in delivery applications including nasal, pulmonary, transdermal and ophthalmic delivery. Liposomes and niosomes are able to entrap a wide range of therapeutic materials and release them at a slow rate to sustain the drug effect. Moreover, they can solubilise poorly soluble compounds, providing a tangible strategy to resolve formulation problems of many therapeutic molecules. Drug delivery to the pulmonary system comprises a strategy for treatment of local respiratory and systemic diseases. It has been previously demonstrated that pulmonary delivery of liposome-entrapped drugs can prolong drug residence in the respiratory tract which may reduce systemic side effects of the drug (Juliano and McCullough, 1980; Taylor et al., 1989). Unfortunately, liposomes in aqueous preparations are very unstable due to the presence of ester bond, and phospholipids may hydrolyse or oxidise during storage (Hunt and Tsang 1981; Kensil and Dennis 1981).

Therefore, research group of Dr Abdelbary Elhissi has investigated the potential of proliposome technologies as chemically stable alternatives to liposomes for pulmonary delivery via medical nebulisers (Elhissi and Taylor 2005; Elhissi et al., 2006). Other possible alternative involves formation of niosomes which are much more stable than liposomes (Baillie et al., 1985; Hu et al., 1999). Surprisingly, very little research has been conducted to investigate their potential in pulmonary delivery.

Niosomes are also known as non-ionic surfactant vesicles, and have been regarded as an alternative to liposomes. Niosomes can be prepared by hydration of synthetic non-ionic surfactants either with or without cholesterol (Barry, 2001; Vila et al., 1979). Vesicle formation is not spontaneous since it needs input of some kind of energy, for instance via extrusion, heating or shaking of the surfactant aqueous dispersions (Lasic, 1988). Niosomes have many beneficial characteristics over liposomes such as stability on storage and ease of handling. Niosomes are prepared from non-ionic surfactants which are not toxic, biodegradable, biocompatible and non- immunogenic. In fact, the non-ionic property of the surfactants used in preparation of niosomes is the reason behind the low toxicity of niosomal formulations (Yadav et al., 2011). Incorporation of surfactants within niosomes may also enhance the efficacy of the drug, possibly by facilitating its uptake by the target cells.

The materials (non-ionic surfactants and cholesterol) which are used to prepare niosomes are much cheaper than those used to manufacture liposomes namely phospholipids. Also the stability of niosomes is higher than that of liposomes. These properties may contribute to making niosomes attractive drug carrier alternatives to liposomes (Vyas et al., 2005; Nasseri et al., 2005).

Cholesterol is included in most niosome formulations in order to increase rigidity of the noisome membranes and subsequently improve stability of the vesicles and to reduce permeability of the encapsulated material (Nasseri et al., 2005; Guinedi et al., 2005). The *in vivo* behaviour of niosomes might be similar to that of liposomes, and can extend the circulation of entrapped drug in the blood, causing an alteration in organ distribution and metabolic stability of the encapsulated material (Ruckmani et al., 2000).

1.2 Niosomal preparation methods

There are several methods that are used to prepare niosomes. These methods are highly similar to those used to prepare liposomes. With regard to preparation, the main difference between liposomes and niosomes is that niosomes require higher energy input to be formed. The preparation methods influence the niosome characteristics, and hence the right method should be employed to manufacture niosomes of the

right characteristics. The most frequently used methods to prepare niosomes have been reported by Uchegbu and Vyas, (1998) and Yadav et al., (2011). These methods are listed below.

1. Thin-film hydration:

Surfactant and cholesterol are dissolved in organic solvent (e.g. chloroform) within a round bottomed flask followed by solvent evaporation using a rotary evaporator to leave a thin layer of the lipid phase on the inner wall of the flask. The thin film is then hydrated by aqueous phase at 0-60ºC with gentle shaking. This method is used to generate multilamellar niosomes (Baillie et al., 1986; Guinedi et al., 2005).

2. Ether injection:

This method depends on dissolving the surfactants in diethyl ether and injecting this solution into previously heated water (60ºC). Single layered vesicles are formed upon evaporation of ether. The disadvantage of this method is the possible residual ether left in the niosome preparation (Baillie et al., 1985; Carter et al., 1988).

3. Reverse phase evaporation:

This method is based on the formation of water in oil (w/o) emulsion, so that an aqueous solution is dispersed in an organic mixture of surfactant and cholesterol. Before emulsification, the drug is dissolved either in the aqueous or organic phase, depending on its solubility. The emulsion is

dried by evaporating the organic solvent using a rotary evaporator to disperse the niosomes in the aqueous phase. In other cases, the emulsion is dried to a semi-solid gel which needs to be hydrated to yield niosomes, which is employed to generate large unilamellar niosomes (Guinedi et al.2005; Naresh et al., 1994).

4. Proniosome technology:

This method comprises a stable free-flowing dry formulation of surfactant and cholesterol. Proniosomes are prepared by dissolving the surfactant/cholesterol in an organic solvent followed by spraying this mixture to coat a water-soluble carrier such as sucrose, sorbitol or other carbohydrates. After evaporation of the organic solvent a thin coat is left on the carbohydrate carrier particles; this formulation is referred to as proniosomes. The addition of an aqueous phase with shaking generates niosomes (Blazek-Walsh and Rhodes, 2001).

1.3 Materials used for preparation of niosomes

1.3.1 Cholesterol

Cholesterol ($C_{27}H_{45}OH$) is a material present in cell membranes of most animals. The chemical structure of cholesterol is shown in Fig. 1.1. Cholesterol influences membrane properties such as fluidity, enzymatic activity, ion permeability and elasticity as well as cell size and shape (Nasseri, 2005). Furthermore, cholesterol increases the stability and rigidity of niosome vesicles and reduces their aggregation. Cholesterol inclusion in niosomes as 1:1 mole/mole with non-ionic surfactants is the maximum concentration which can be beneficial for incorporation into niosomes in terms of vesicle stability (Nasseri, 2005; Leekumjorn, 2004; Uchegbu and Florence, 1995).

As mentioned in a previous study (Uchegbu and Florence, 1995), polyhedral noisome vesicles which are prepared with low amount of either cholesterol or with non-ionic surfactants without cholesterol seemed to lose their viscoelasticity after facing high shearing, for instance during extrusion. This may cause the niosomes to deform permanently into tubular structures. However, the spherical niosomes that include cholesterol may regain their original shape after releasing the pressure, suggesting that cholesterol may increase vesicle elasticity (Nasseri, 2005; Nasseri and Florence 2003)

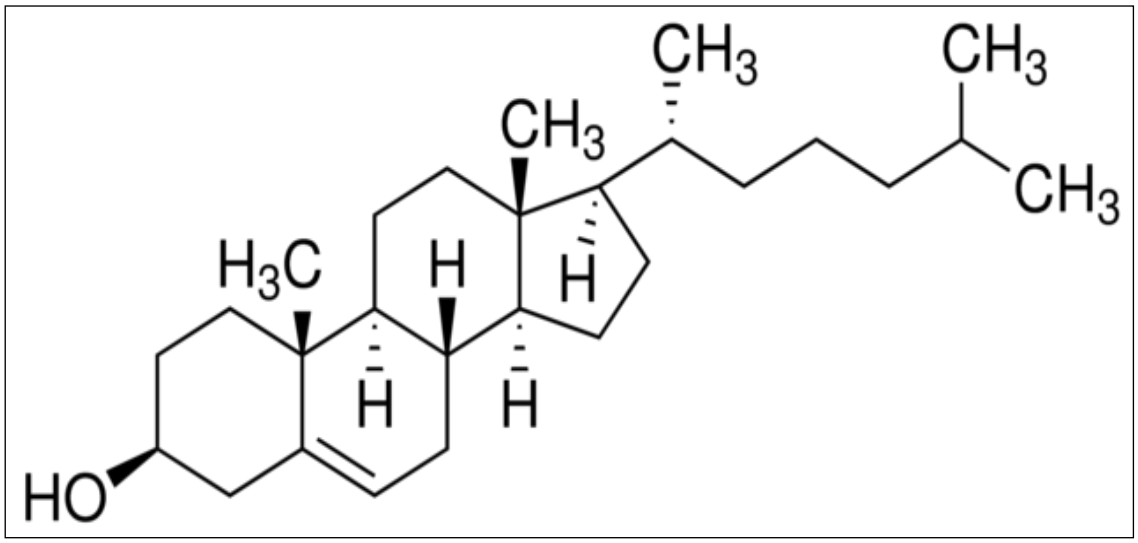


Fig 1.1: Structure of the cholesterol (adapted from: Sigma- Aldrich)

1.3.2 Non-ionic surfactants

Niosome formulations are made from non-ionic surfactants (amphiphilic structure) and aqueous solvents. Cholesterol is included in the noisome formulations in order to enhance vesicle stability and moreover, to reduce permeability of the encapsulated material. The aggregation of the vesicles can be decreased using molecules which stabilise the system and prevent or minimise the formation of aggregates by repulsive steric or electrostatic effects such as sorbitan monostearate (span 60). Electrostatic stabilisation could also be a charge inducer like dicetylphosphate. Different non-ionic surfactants are used in the literature to prepare niosomes. The surfactants used in niosome formulations must have both polar (hydrophilic) head group and non-polar (hydrophobic) tail. The non-polar tail can have one or two alkyl or perfluoroalkyl groups or a single steroidal group. The length of the alkyl group is usually 12-18 carbon atoms (Uchegbu and Vyas, 1998). The spans (sorbitan

monoesters) are surfactant molecules which can be used to prepare niosomes. Spans are a group of non-ionic surfactants that have shown very good commercial utilization (Namedo and Jain, 1999).

As described by Nasseri and Florence (2003), the nature of surfactant is also very important. For instance, ether-linked surfactants with dialkyl ether chain tail are less toxic than those having a single alkyl chain. In addition, ether-linked surfactants are chemically more stable than ester-linked surfactants. In addition, ester-linked surfactants are less toxic because esterases biodegrade to triglycerides and fatty acids. As mentioned previously, surfactants containing alkyl chain with length of 12-18 carbons are useful for preparation of niosomes (Nasseri and Florence, 2003).

1.4 Proniosome technology as an approach to generating niosomes

Niosomes of different characteristics can be prepared using different methods such as the conventional (thin film) method and the proniosome technology (Hu and Rhodes 1999). Proniosomes are surfactant-coated carbohydrate carrier particles (Fig. 1.2) that generate niosomes upon addition of aqueous phase (Hu and Rhodes 1999). The generated niosomes have been reported to be similar to conventional niosomes and more consistent in size. The advantage of using proniosomes is the

minimized physical instability such as aggregation and sedimentation of the vesicles and reduced drug leakage, and most importantly stability upon storage. Proniosomes are also easier to ship, and hence proniosome technology may be considered a stable and economical alternative to conventional niosomes (Yadav et al., 2011).

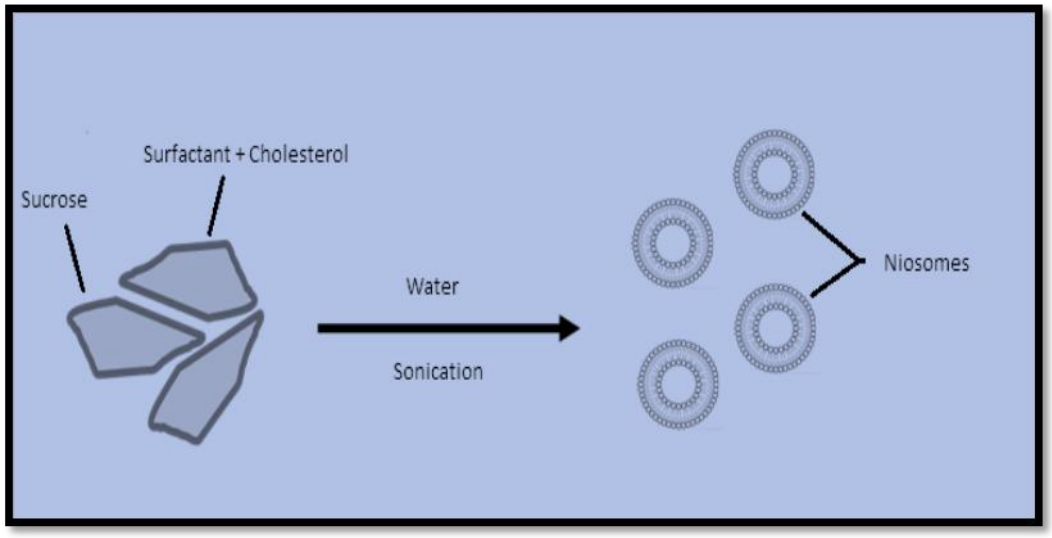


Fig 1.2: Proniosome derived niosomes. Proniosomes are carbohydrate (e.g. sucrose) carrier particles coated with surfactant and cholesterol. Niosomes are generated from proniosomes by addition of water and shaking. This can be followed by vesicle size reduction via sonication

1.5 Characterisation of the non-ionic surfactant vesicles

To be able to evaluate niosomes, a range of studies are required and commonly performed. Size and polydispersity of niosomes are commonly analysed using laser diffraction or photon correlation spectroscopy, depending on the method of preparation while zeta potential (surface

charge) of niosomes is analysed using laser Doppler velocimetry (Marianecci et al., 2010). The lamellarity and morphology of niosomes are studied using microscopic techniques such as Transmission Electron Microscopy (TEM). Since niosomes may provide real advantages by entrapping a wide range of therapeutic molecules, it is important to determine the entrapment efficiency of such molecules. The entrapment efficiency (EE) of a given drug can be calculated according to the following equation:

EE (%) = (amount of drug entrapped / total amount of the drug) × 100%

1.6 Respiratory tract

The respiratory system is divided into upper respiratory tract and lower respiratory tract. The upper respiratory tract includes the nose, nasopharynx, mouth, larynx, trachea and bronchi while the lower respiratory tract includes bronchioles and alveoli. The lungs are responsible for respiration and are positioned on the either side of the mediastinum bordered by the right and left pleural cavities.

The trachea is the top end of the lungs and is divided into the left and right bronchi. This is divided again to lead into narrower airways. The main bronchi are the place where the air enters and leaves continuously. This branching system will eventually end at the alveolar region (Fig. 1.3). Oxygen entering the blood through the blood vessels in the vicinity of the alveoli is carried to the rest of the body. The pulmonary arteries

transport blood with low oxygen levels from the right ventricle to be oxygenated. Blood containing oxygen circulates back to the left atrium through the pulmonary veins and then to the left ventricle to be pumped to the rest of the body (Drake et al., 2005).

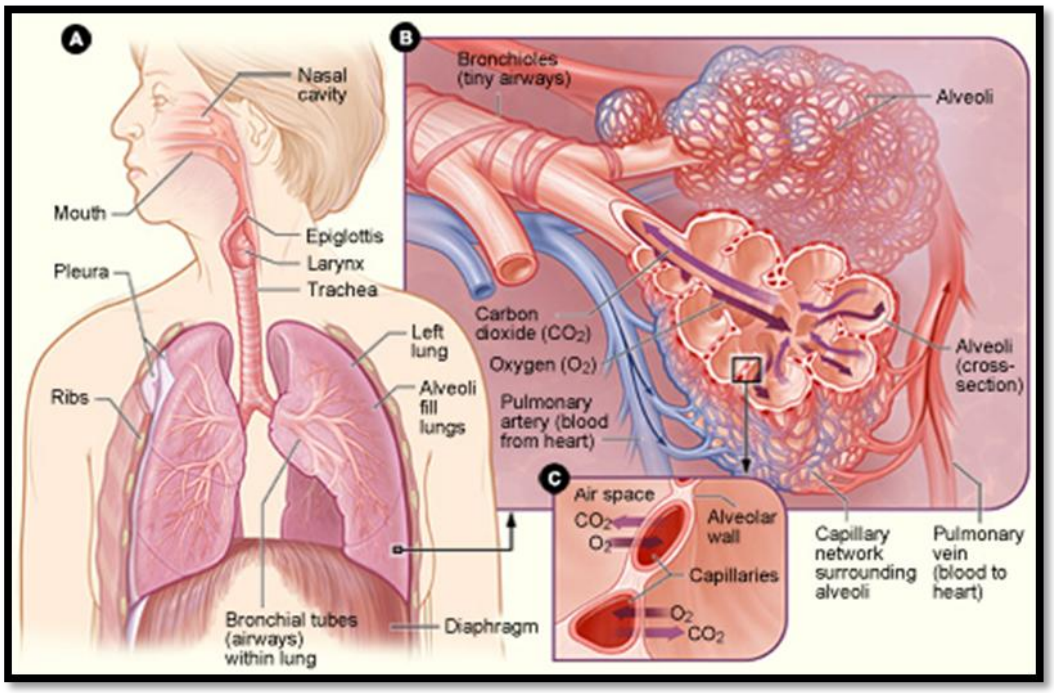


Fig 1.3: A detailed diagram of the respiratory system. The lungs are divided into upper respiratory tract and lower respiratory tract. The upper respiratory tract includes the nose, nasopharynx, mouth, larynx, trachea and bronchi. The lower respiratory tract consists of the bronchioles and alveoli. (Accredit by national heart lung and blood institute and adapted from: http://www.nhlbi.nih.gov/health/health-topics/topics/hlw/system.html)

1.7 Pulmonary drug delivery

Drug delivery to the respiratory system has been shown to be an important route for drug administration. The advantage of this route over alternative delivery routes is the suitability of this route for delivering low doses of the drug directly to the lung for treatment of local diseases within the respiratory tract (e.g. asthma) which may lead to elicit a rapid therapeutic response and potential avoidance of systemic side effects (Dolovich and Dhand, 2011). Therefore, inhalation of medical aerosols is a smart approach for treatment of local diseases in the lung such as chronic obstructive pulmonary diseases (COPD), pulmonary infectious diseases, respiratory distress syndrome (RDS), cystic fibrosis and asthma.

Pulmonary drug delivery may also be beneficial for delivering drugs that have systemic therapeutic effects. The large pulmonary surface area and thinness of the alveolar walls make systemic absorption highly convenient (Dolovich and Dhand, 2011; Marianecci et al., 2010).

It has been shown previously that aerosolisation of drug-loaded vesicles (e.g. liposomes) can reach the lower respiratory tract and extend the drug release. Also, this strategy may limit the distribution of the drug to the other tissues that are distant from the lung, leading to minimised systemic side effects. Liposome vesicles have stability problems (e.g. phospholipid oxidation or hydrolysis), and hence, finding

alternatives to liposomes is desirable. Niosomes are similar to liposomes in their function and are more chemically stable and cheaper to manufacture (Hao et al, 2002). This suggests that non-ionic surfactant vesicles (niosomes) should be studied for pulmonary drug delivery.

Many drugs are used to treat local diseases in the lung. However, antiasthma drugs (e.g. beclomethasone dipropionate) are still the most popular because of the large number of asthmatic patients. Glucocorticoids (e.g. beclomethasone dipropionate) are generally used via inhalation and systemic treatments for the inflammatory lung diseases (e.g. asthma).

1.8 Asthma

Asthma is defined as a reversible partial obstruction to airflow in the intrathoracic airways (Axford and O'Callaghan, 2004). In normal situations, the muscle around the bronchi is smooth, which causes a normal response to external or internal stimuli. People who have asthma have an abnormal increased reaction to stimuli. This phenomenon is termed as "bronchial hyper-reactivity" which may happen because of inflammatory-mediated mechanisms. Frequently stimuli can cause ongoing bronchial inflammation in susceptible individuals, and this may prolong bronchial hyper-reactivity.

There are two different types of stimuli to bronchus which are specific and non-specific. Specific stimuli include inhaled allergens such as house dust, mite and pollen. There are also occupational allergens like drugs (e.g. aspirin). Specific stimuli are more responsible to cause ongoing bronchial inflammation than non-specific bronchial stimuli and this can result in lasting bronchial hyper-reactivity. The non-specific bronchial stimuli include viral infections, cold air, exercise, emotional stress and inhaled pollutants such as perfumes. The difference between normal and obstructive respiratory airways is shown in Fig. 1.4.

Asthma may also be classified into allergic and non-allergic. People who have allergic asthma have higher levels of IgE (allergy antibody) circulating in their blood. In general these asthmatic individuals have positive skin prick tests to common allergens. The symptoms usually occur in childhood and there is a tendency for the symptoms to improve during later ages (e.g. adolescence). When these asthmatic patients avoid the allergens, the symptoms can improve and their quality of life may enhance. Non-allergic asthmatics may develop in middle age people, in general those people who are not atopic and the symptoms tend to be progressive and remitting (BNF, 2009; Drake et al., 2005).

Asthma is a disease that negatively affects the quality of life of asthmatic patients. However, importantly, severe asthma is life-threatening and can happen suddenly (acute severe asthma) or as the end stage of slowly deteriorating asthma (chronic worsening asthma).

Acute severe asthma can be precipitated by either emotional stress or a viral infection, but chronic worsening asthma usually develops over weeks with a slow increase in symptoms and progressive failure to respond to inhaled $ß_2$- agonists.

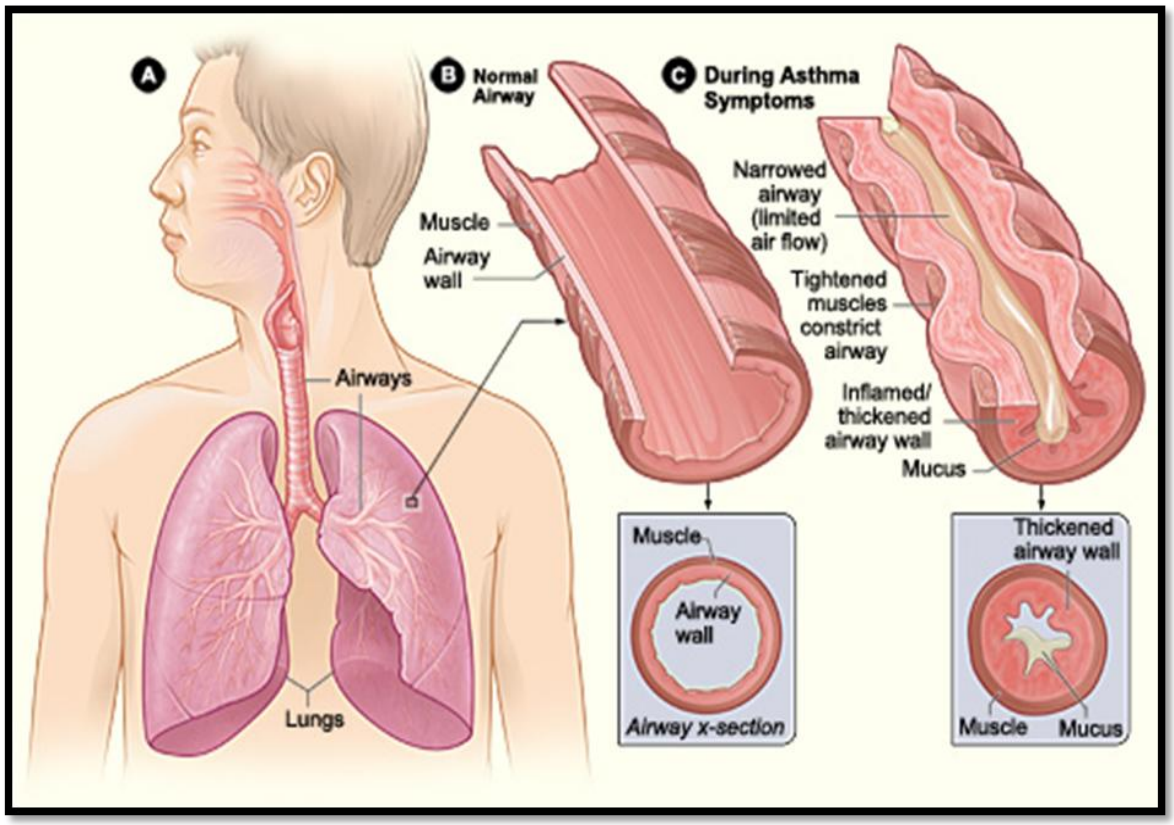


Fig 1.4: A diagram showing the location of the lungs and airways in the human body. B diagram showing normal airways versus airways during asthma symptoms (Accredit by national heart lung and blood institute and adapted from: http://www.nhlbi.nih.gov/health/health-topics/topics/asthma/)

Stages of asthma management

Asthma management should be stepwise in **children and adults** as instructed by the guidelines of the British National Formulary (BNF) (BNF, 2009):

1. **Occasional relief bronchodilators**

Inhaled short acting ß$_2$-agonist (e.g. salbutamol) when required. **Regular inhaled prophylactic therapy** - inhaled short acting ß$_2$-agonist as required with regular standard dose of inhaled corticosteroid such as beclomethasone dipropionate (BDP).

2. **Inhaled corticosteroids and long acting ß$_2$-agonist** - inhaled short acting ß$_2$- agonist when required plus regular inhalation of corticosteroid and regular inhalation of long acting ß$_2$-agonist (e.g. salmeterol or formeterol) if asthma is still uncontrolled. The dose of inhaled corticosteroid should be increased to upper end of standard dose and either stop long acting ß$_2$-agonist if no benefit or continued if of some benefit. If the asthma is still not controlled and long acting ß$_2$-agonist stopped, the leukotriene receptor antagonist should be added to the therapy plan. Modified release oral theophylline and modified release oral ß$_2$-agonist may also be used.

3. **High dose inhaled corticosteroid plus regular bronchodilators** - inhaled short-acting ß$_2$-agonist with regular high dose corticosteroid plus one or more long-acting bronchodilators may be given. In adults, 6 week trial of one or more of these following drugs may be given they include: leukotriene receptor antagonist, modified release oral thoephylline or modified release ß$_2$-agonist.

Regular corticosteroid tablets – It is recommended that the patients inhale short acting ß$_2$-agonist with regular high dose inhaled corticosteroids and one or more long acting bronchodilators plus regular oral corticosteroids as a single daily dose (e.g. prednisolone tablets) are recommended. In addition to oral prednisolone, the patient should continue to use a regular high dose corticosteroid.

1.9 Mechanisms of particle deposition

The ability of particles to reach the lower pulmonary airways is dependent on their size, since the anatomy and physiology of the respiratory system can produce defence against the deposition of airborne particles in the lower respiratory regions.

This must be recommended when taking particle size as the prime determinant of particle deposition in the airways, there are three important mechanisms: inertial impaction, Brownian diffusion and

sedimentation. There are also two other mechanisms which are less important namely interception which is dependent on particle shape and electrostatic precipitation which is dependent on particle charge. The dominant mechanism of particle deposition in the respiratory tract is inertial impaction deposition for particles larger than 5 μm. However, particles larger than 12 μm will be highly likely to deposit in the extrathoracic region, and hence particles > 12 μm are not likely to deposit in the respirable region of the lungs (bronchioles and alveolar region) (Heyder, 1982).

Particles with either high velocity or density are expected to impact in the upper regions of the respiratory tract depending also on particle size. For example light (low density) particles are more expected to move with air streamlines and travel deeper into the lung for effective deposition in the lower airways (Maa et al., 1999). As mentioned before, Brownian diffusion is also a deposition mechanism. This deposition mechanism is more suitable for particles <1 μm (Tsuda et al., 1994). Random bombardment of the gas molecules with the particles may cause particle deposition on the respiratory airway walls. This happens in the alveolar region where airflow velocity is negligible. Sedimentation could also be a deposition mechanism. The confrontation to the airflow which is increased in the lower airways especially in the alveolar region results in reducing the velocity of the inhaled gas. As a result of prolonged time of residence in the airways, large mass particles might deposit by sedimentation. The sedimentation mechanism might deposit

particle with the size between 1-5 μm in the effective region of the airways (Martonen and Yang, 1996).

In principle, particles size smaller than 5- 6 μm is required for possible deposition in the lower airways to have a therapeutic effect. Particles with a size < 2 μm are preferred for alveolar deposition, which is a highly necessary target in the anti-inflammatory therapy of various diseases. Even though the percentage fraction of particles smaller than 5-6 μm (i.e. the “fine particle fraction” (FPF)), deposition of particles in the lower airways is not guaranteed as it might be dependent on other pathological and physiological factors as well as other physical factors of the particles (Newman et al., 1982; O’Callaghan and Barry, 1997; Terzano et al., 2007).

1.10 Inhalation devices

There are three different types of inhalation devices. These are pressurised metered dose inhalers (pMDIs), dry power inhalers (DPIs) and nebulisers. The pMDI contains a pressurised propellant gas with the medicinal ingredient within a metallic canister. Upon actuation of the canister by pressing on the top of it, a metered dose of the propellant is released carrying a specific amount of the drug. The propellant evaporates because of its very high vapour pressure, leaving very small particles of the drug for inhalation. The propellant gas in the inhaler is

chlorofluorocarbon (CFC) and recent environmental regulations have restricted the use of such propellants due to their damaging effects on the ozone layer. Hydrofluoroalkanes are the most recently used propellants, but their inability to dissolve some amphipathic materials may limit their use for solubilising phospholipids and surfactants (Elhissi and Ahmed, 2011). The drug is stored within the spacer until the valve of the spacer opens by the intake of breath and then closes on exhalation.

Dry powder inhalers (DPIs) are alternatives to pMDIs. These are powdered formulations of micronised drug adsorbed onto lactose carrier particles. The formulation is loaded into specially designed devices, and upon inhalation the micronised drug is detached from the carrier particles and inhaled whilst the carrier deposits in the upper regions of the pulmonary airways (Elhissi and Ahmed, 2011).

The third type of inhalation devices are nebulisers. These devices generate aerosols of the drug dissolved or dispersed in an isotonic aqueous phase. The aerosol cloud is effectively inhaled via a face mask or a mouthpiece. This device is commonly used in hospitals for severe asthma attacks or when large drug doses are required at homes, and the drug is delivered by normal breathing (Elhissi and Ahmed, 2011).

1.11 Nebulisers

Nebulisers have been widely investigated for their efficiency in delivery of regular solutions, suspensions and novel drug delivery systems to the respiratory tract (Elhissi and Ahmed, 2011). Nebulisers are highly convenient to use and can aerosolise large volumes of therapeutic aerosols to deep lung. Unlike DPIs and MDIs, nebulisers can aerosolise solutions or dispersions which do not require complicated procedures to prepare (O'Callaghan and Barry, 1997).

In a retrospective-cohort study, Marcus et al (2006) indicated that old patients who are using nebulisers to inhale corticosteroids persistently have shown reduced visits to the emergency department and less use of the systemic corticosteroids compared to before nebulisation. These positive outcomes did not increase health care costs, suggesting that nebulisers are preferred as the inhalation device by many patients.

Delivery of aerosols using nebulisers is dependent on the nebuliser's operating principle, design and aerosol characteristics. The most important critical issue is the size of aerosol droplets and drug output of the nebuliser (Terzano et al., 2007). The required size of the particles should be less than 5-6 μm to be able to deliver the drug to the lower respiratory tract. However, to target some other specific deeper regions in the lung (e.g. the alveoli) smaller size of droplets is required (Bridges and Taylor, 2000a).

In the market, there are three different types of nebulisers: air-jet nebulisers, ultrasonic nebulisers and vibrating-mesh nebulisers. Ultrasonic nebulisers are being increasingly replaced with the new generation of vibrating-mesh nebulisers. Therefore, ultrasonic nebulisers will not be discussed in this review.

Air-jet nebulisers

Air-jet nebulisers are also named jet or pneumatic nebulisers. Sometimes, they are called compressor nebulisers since they are attached to air compressors (Elhissi and Ahmed, 2011). The first medical inhalation device known for production of aerosols was the jet nebuliser. Air-jet nebulisers have been extensively studied for the treatment of asthma in delivery of the anti-asthma formulations to the respiratory tract (Meurs, 1997; O'Callaghan and Barry, 1997). Modern jet nebulisers convert liquid formulations into aerosol droplets by using a high velocity gas passing through a narrow nozzle. An area of negative pressure is formed on top of the medical fluid. This creates filaments of the liquid that collapse into droplets because of liquid's surface tension (McCallion et al., 1996a; O'Callaghan and Barry, 1997; Elhissi et al., 2006).

A small amount of aerosols are suitable to be released for inhalation, while the largest amount of aerosol droplets generated from the liquid are too large (15-500 μm). Therefore, this fraction will be

deflected and recycled by the baffles within the nebuliser for further fragmentation into inhalable aerosols (McCallion et al., 1996a; O'Callaghan and Barry, 1997and Vecellio, 2006). Fig. 1.5 shows a schematic presentation of an air-jet nebuliser.

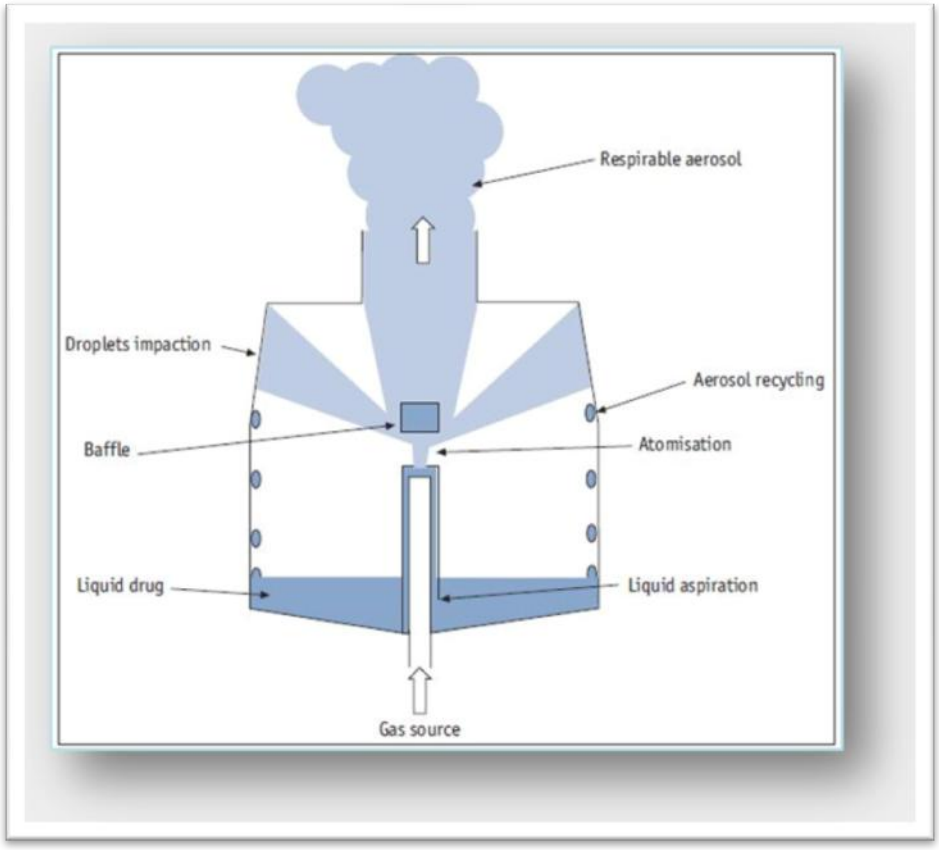


Fig 1.5: A schematic presentation of an air-jet nebuliser (Source: Vecellio, 2006)

The different design of the air-jet nebulisers results in different aerosol characteristics (output, size of the droplets, etc.) (Clay et al., 1983; Hurley and Smye, 1994; Niven and Brain, 1994; Hess et al., 1996; O'Callaghan and Barry, 1997).

Air-jet nebulisers may be sub-classified into different types which are defined by the output during the inhalation. Firstly, *Conventional jet nebulisers* can generate a fixed flow rate of the gas containing aerosols. This nebuliser has a constant aerosol output during inhalation and exhalation times by the patient which results in wasting of a large proportion of the generated aerosols (Vecellio, 2006).

Secondly, *Open vent nebulisers*. This type of nebuliser works constantly like the conventional jet nebulisers, but incorporates an extra air vent which deals with the negative pressure in the nebuliser. The fluid from the feeding tubes and air will be sucked, resulting in an improved airflow through the nebuliser which drives smaller aerosol droplets for inhalation over a certain length of time. Consequently, the time for nebulisation to be completed is shorter (O'Callaghan and Barry, 1997).

Thirdly, *Breath-enhanced (assisted) nebulisers.* A common example of this type is the Pari LC nebuliser (Fig. 1.6). This nebuliser is designed to generate high aerosol output during inhalation and lower aerosol output during exhalation. This is attributed to the presence of a valve on the top of the device, which opens during inhalation to permit extra air to be sucked through the nebuliser. The valve closes during exhalation, resulting in decreased air flow through the nebuliser. However, there is another vent which is present at the top of the mouthpiece and opens during exhalation. This type of nebuliser

increases the amount of the drug inspired (O'Callaghan and Barry, 1997; Vecellio, 2006).

Finally, *Breath-actuated or Dosimetric nebulisers* have been commercialised. This type of nebuliser generates aerosols only during inhalation, and hence aerosol wastage is minimised during exhalation (Vecellio, 2006).

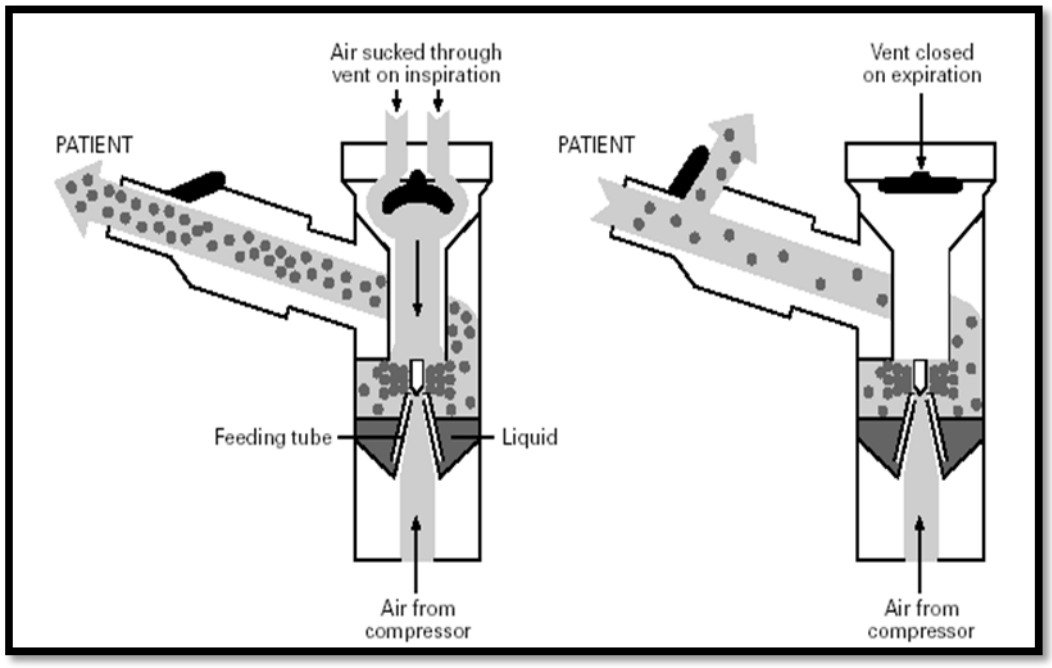


Fig 1.6: A breath-enhanced open vent air-jet nebuliser. The schematic diagram shows how the vent system operates during inhalation (left) and exhalation (right) (Source: O'Callaghan and Barry, 1997)

Vibrating-mesh nebulisers

This group of nebulisers is based on the vibrating-mesh technology (Dhand, 2002). To generate aerosols all the nebulisers of this type employ vibrating mesh or plates with multiple apertures. These nebulisers are hand held, easy to carry and can operate using batteries (Dhand, 2002; Vecellio, 2006). According to the mechanism of nebuliser operation, these devices are divided into passively vibrating and actively vibrating-mesh nebulisers (Elhissi and Ahmed, 2011).

Passively vibrating-mesh nebulisers

After applying an electrical current, the incorporated piezoelectric crystal vibrates at high frequency. The crystal vibrations are transmitted to a transducer horn which is in contact with the medical fluid. This produces “passive” vibrations (upward and downward) movements of the mesh, which extrudes the fluid through the mesh apertures (around 6,000 apertures) to generate aerosol. The flow rate and particle size of the aerosols are determined by the diameter of the mesh aperture which is around 3 µm for the Omron NE-U22 (Dhand, 2002; Vecellio, 2006; Ghazanfari et al., 2007; Elhissi and Ahmed, 2011) (Fig. 1.7).

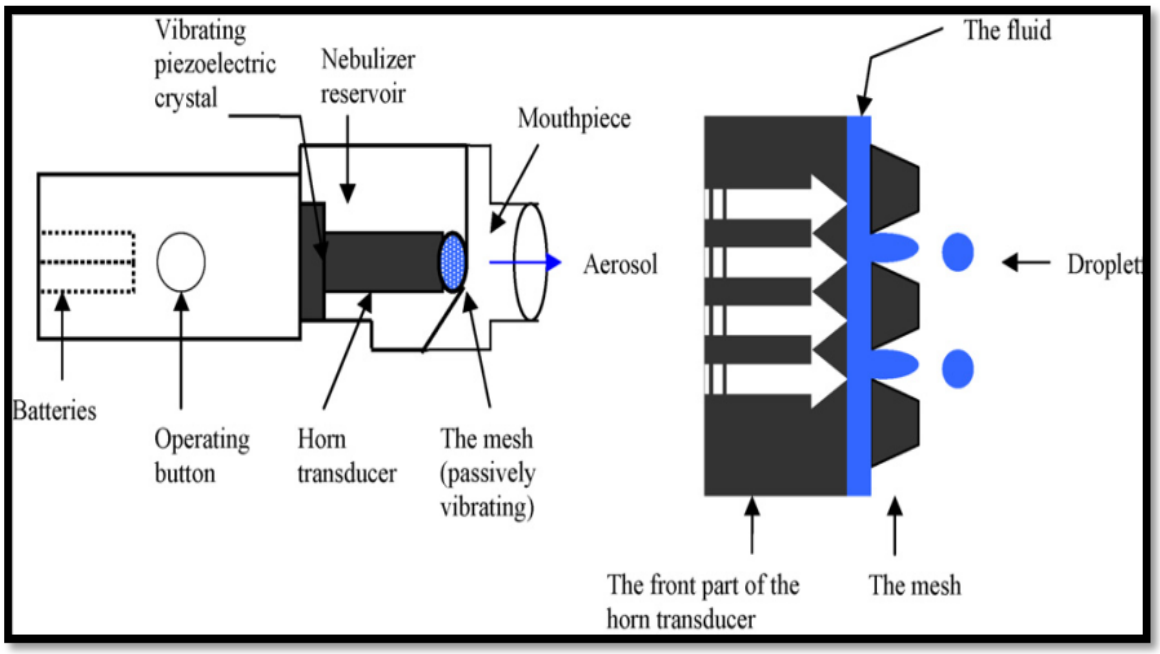


Fig 1.7: The design of the Omron MicroAir NE-U22 nebuliser (left). The vibrations transmitted via the horn transducer from the piezoelectric crystal causes "passive" vibrations of the perforated plate, resulting in aerosol generation (right) (Source: Ghazanfari et al, 2007).

Actively vibrating-mesh nebulisers

This type of nebulisers generates aerosols by using a “micropump” system, which employs an aerosol generator that includes around 1,000 electroformed holes surrounded by a vibrational element. This causes an upward and downward movements of the mesh when an electrical current is applied to generate the aerosol (Fink et al., 2001a; Gopalakrishnan and Uster, 2001; Dhand, 2002; Elhissi and Ahmed, 2011). An example of that is the Aeroneb Pro nebuliser (Fig. 1.8).

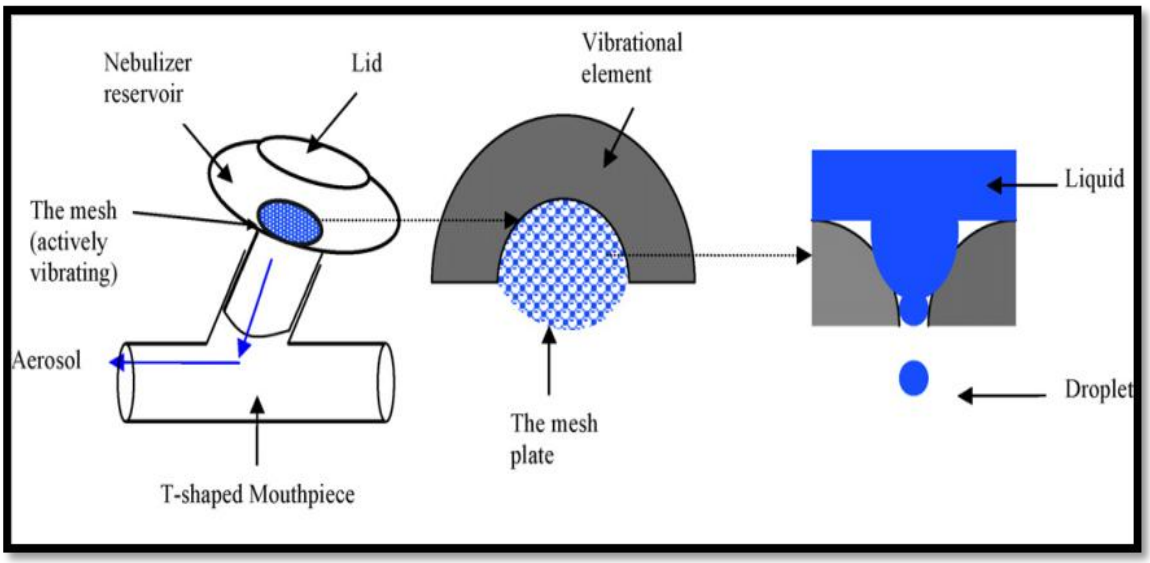


Fig 1.8: The design of the Aeroneb Pro vibrating-mesh nebuliser (left). A perforated plate is surrounded by a vibrational element that is attached to an electrical current (middle). This causes the mesh to vibrate and extrudes the medical fluid through the apertures to generate aerosols (right) Source: Ghazanfari et al, 2007).

1.12 Asthma therapy using glucocorticoids (Beclomthasone dipropionate)

The delivery of glucocorticoids to the respiratory tract has been extensively studied for local treatment of inflammatory lung diseases (e.g. asthma) (Marianecci et al., 2010). Currently, inhaled glucocorticoids are the first line therapy for prophylactic treatment of asthma (Leflein et al., 1995).

Glucocorticoids act by reducing the synthesis of arachidonic acid, blocking the expression of COX-2 and enhancing the sensitivity of β-adrenoceptors. All these actions happen when glucorticoids bind to specific intracellular receptors (Trevor and Katzung, 2008).

Beclomethasone dipropionate (BDP) is a hydrophobic antiasthma drug (Fig. 1.9). Because of beclomethasone dipropionate hydrophobicity, its formulations are made as suspensions which might be used in nebuliser therapy (Marianecci et al., 2010).

BDP has an inactive prodrug structure with low receptor binding affinity. BDP requires to be changed to its active form 17-beclomethasone monopropionate with high binding affinity to the receptors. The activation of the prodrug (BDP) is caused by esterases in the respiratory tract. Furthermore, the active metabolite 17-

beclomethasone monopropionate is metabolised to beclomethasone (Winkler et al., 2004).

The administration of glucocordicoids directly to the respiratory tract may cause localised side-effects such as candidiasis, since a fraction of the drug can be deposited in the mouth and oropharynx. The cause of such side-effects can be reduced using the prodrug form by inhalation, since the fraction deposited in the mouth and oropharynx will be consumed before the activation happens (Winkler et al., 2004).

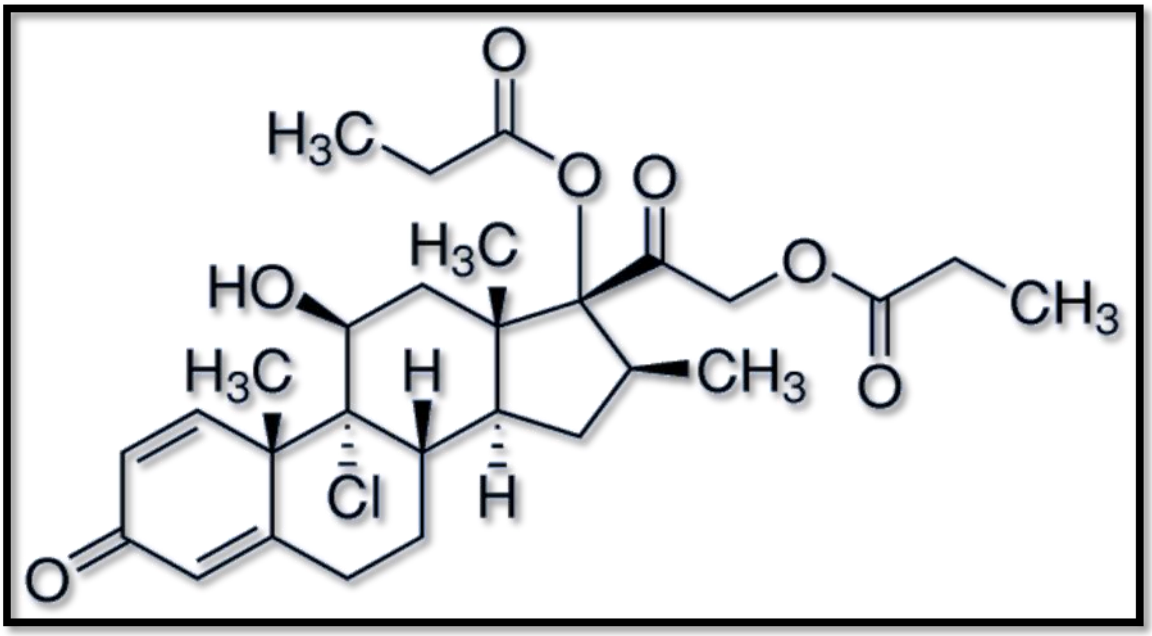


Fig 1.9: Chemical structure of Beclomethasone dipropionate (Adapted from Sigma- Aldrich)

1.13 Working Hypothesis

Is it possible to develop BDP proniosome formulations that would generate niosomes for drug delivery via medical nebulisers to the respiratory tract?

1.14 Aims of the study

1. To formulate niosomes which are potentially applicable in pulmonary delivery of antiasthma steroids using medical nebulisers.

2. To investigate a large scale manufacturing approach to hydrating proniosomes and generating noisomes by using high pressure homogenisation.

3. To study the effect of freeze-drying on the stability of niosomes.

4. To generate niosomal aerosols using medical nebulisers and analyse the aerosol properties.

5. To quantify the entrapment of the steroid before and after nebulisation.

6. To analyse the data and write up the MSc thesis by research.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals and consumables used in this study for preparation and analysis of niosomes and proniosomes were purchased from Sigma Aldrich, UK or Fisher Scientific, UK. All chemicals and consumables (Table 2.1) are analytical or pharmaceutical grade.

Table 2.1: Materials used in the preparation and analysis of proniosomes and niosomes

Material	Supplier
Sorbitan monostearate (Span 60)	Sigma Aldrich, UK
Cholesterol (99%)	Sigma Aldrich, UK
Sucrose	Fisher Scientific, UK
Methanol	Fisher Scientific, UK
Chloroform	Fisher Scientific, UK
Phosphate buffered saline (PBS) tablets	Sigma Aldrich, UK
Beclomethasone dipropionate (BDP, ≥99%)	Sigma Aldrich, UK

2.2. Methods

2.2.1. Preparation of niosomes using thin film hydration method

Niosomes were prepared from two different components, namely the non-ionic surfactant Span 60 and cholesterol (1:1) to constitute a quantity of 150 µmols. This was achieved by using Span 60 (32.2 mg) and cholesterol (29 mg). The mixture was placed into a 100 ml round bottom flask and dissolved in chloroform (3 ml). The organic solvent (chloroform) was removed under vacuum using a rotary evaporator (Buchi Rotavapor R-114, Buchi, Swizerland) at a rotation speed of 150 rpm with the flask being partially immersed in a water bath previously adjusted to 60^{o}C. After 1 hr, the rotary evaporator was switched off, the negative pressure released and the flask was detached. A thin film of dry surfactant/cholesterol mixture was seen on the inner surfaces of the flask. The dry film was hydrated with an isotonic solution of PBS (10 ml; 35-40°C) followed by hand-shaking for 10 minutes. The niosomal suspension was left overnight to ensure complete hydration of surfactant molecules and proper formation of niosomes. The same formulation was made using BDP (5 mole %) within the lipid phase (i.e. surfactant and cholesterol). The hydration procedure took place as for drug-free thin film. After preparation of noisome formulations (including or excluding BDP), samples were taken for size and zeta potential analysis of the vesicles using the Zeta sizer instrument (Malvern Instruments, UK). Other samples were taken to study the morphology of niosomes using transmission electron microscopy (TEM).

2.2.2. Bath sonication of niosomes

Niosomes prepared by thin film hydration (section 2.2.1) were sonicated for 15 min using a bath sonicator to reduce the size of the vesicles. This was followed by size, zeta potential and microscopic morphology studies.

2.2.3. Probe sonication of niosomes

Niosomes prepared by thin film hydration (section 2.2.1) followed by bath sonication (2.2.1) were probe sonicated for 3 min with intermittent cooling of the preparation every 30 sec for 2 min in order to avoid overheating of the formulation. Characterisation studies were conducted for niosomes employing either thin film or bath sonication.

2.2.4. Manufacture of niosomes using the proniosome method

As for conventional niosomes (i.e. vesicles made by thin film method), the lipid phase (surfactant/cholesterol mixture (1:1) with or without 5 mole% BDP) was dissolved in chloroform and sprayed onto sucrose carrier particles (300-500 µm) within a round bottomed flask using the rotary evaporator with a feed-line tube (Fig. 2.1). Spraying the chloroformic solution was performed in portions with each comprising 1 ml in order to coat the carbohydrate particles uniformly and permit complete evaporation of the chloroform. This was allowed by rotary evaporation at 150 rpm and 60°C for approx. 1 hr, the rotary evaporator

was switched off thus, releasing the negative pressure and detaching the flask. The resultant powdered formulation (i.e. the granules) was referred to as proniosomes that comprised a ratio of 5:1 (w/w) sucrose to lipid phase mixture. The proniosomes were collected and hydrated by addition of deionised water (approx. 40°C) and shaking to generate niosomes. The vesicles were reduced in size using bath sonication and further by using a probe sonicator as performed for the conventional niosomes. Alternatively, a high shear homogeniser Nano Debee (B.E.E International, INC) was used to generate niosomes from proniosomes, as explained in section 2.2.14.

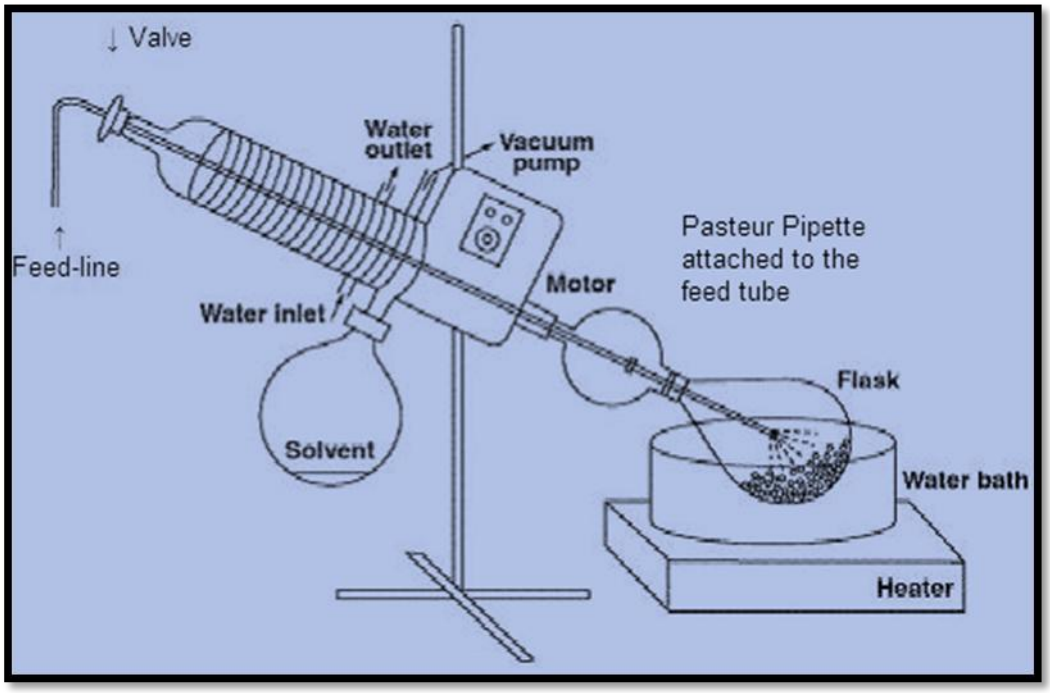


Fig 2.1: A modified rotary evaporator with a feed-line spray system for manufacturing proniosomes (Adapted from Song et al., 2002)

2.2.5 Size analysis of niosomes

Size analysis of niosome samples was performed by using the Malvern's Zetasizer (Malvern Instruments Ltd., UK) which employed Photon Correlation Spectroscopy (PCS) and performed measurements of particles in the range between 3 nm and 6 µm. Size analysis was conducted by adding niosomes (1 ml) into a Malvern's disposable vial (Malvern Instruments Ltd., UK). The measurements were conducted three times and the Zeta average ($Z_{average}$) and polydispersity index (PI) were recorded to represent the size and size distribution, respectively.

2.2.6 Zeta potential (surface charge) analysis

Zeta potential of niosomes was measured using Laser Doppler velocimetry, again by employing the Zetasizer (Malvern Instruments Ltd., UK). A zeta cell was washed several times with deionised water. Niosome formulation (700 µl) was added to the zeta cell using an automatic pipette. This was done carefully in order to avoid formation of air bubbles within the cell. The zeta potential was measured in triplicate and the resultant value was presented as the mean (± standard deviation).

2.2.7 Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was performed to investigate the morphology of niosomes manufactured using the conventional or proniosome methods. This took place by placing a drop of noisome suspension onto carbon-coated copper grids (400 mesh pores). The sample was negatively stained with (1% Au) uranyl acetate. The resultant mixture was left for 2 min to allow its absorption on the carbon film. This was followed by viewing the vesicles and taking photographs to investigate their morphology using the Philips CM-120 BioTwin transmission electron microscope (Philips Electron Optics BV, The Netherlands).

2.2.8 UV analysis of BDP

BDP (10 mg) was weighed in a 100 ml volumetric flask and made up to the volume with HPLC-grade methanol. The solution was gently mixed to allow complete dissolution of BDP. A series of dilutions with methanol in volumetric flasks (5 ml each) was performed to obtain drug concentrations of 0, 10, 12.5, 20, 25, 30, 40 and 100 µg / ml. The UV-spectrophotometer was used to measure the absorbance of the samples at a UV wavelength of 238 nm (Batavia et al. 2001). This was followed by constructing a calibration curve.

2.2.9 Drug entrapment studies

BDP was included within the lipid phase (surfactant and cholesterol) to be entrapped in the niosomal membranes upon hydration of the thin lipid film or proniosomes. This procedure was applied only after probe sonication of the niosome dispersions. Thus, after probe sonication, the unentrapped fraction of the steroid was separated using 0.45 µm sterile cellulose acetate membrane syringe filters (Sigma-Aldrich). To quantify the amount of BDP in the vesicles, methanol was used to disrupt the niosomes, dissolve the components of the lipid phase and hence convert these components into a lipid solution that contains BDP. The amount of BDP was quantified using UV spectrophotometer against a blank of lipid phase without BDP. The entrapment efficiency (EE) of the steroid was calculated as follows:

EE (%) = (amount of drug entrapped / total drug amount in the formulation) X 100%.

Below is the detailed procedure of the EE study:

1. After homogenisation of the particles (niosomes or proniosomes), 5 ml was filtered through 0.45 µm filters.
2. The filtered fraction (entrapped fraction) was diluted with methanol. A volume of 1 ml of the entrapped fraction was taken using a pipette into 10ml volumetric flask which was then diluted up to volume with HPLC grade methanol. This step was repeated again to dilute the sample. The diluted sample was measured for UV absorption.

3. The UV-reading was placed in the equation of the calibration curve and calculated as the amount of the drug in mg.

2.2.10 Aerosol droplet size analysis

In this study three nebulisers were investigated:

1. Pari LC Sprint (Air-jet) nebuliser attached to Pari TurboBoy S compressor (Pari GmbH, Germany).
2. Aeroneb Pro (Vibrating-mesh) nebuliser (Aerogen, Ireland).
3. Omron MicroAir NEU22 (Vibrating-mesh) nebuliser (Omron Healthcare, Japan) and purchased from EverGreen, UK.

Niosomes generated from proniosomes (2.5 ml) were pipetted into the nebuliser which was previously directed with its mouthpiece perpendicular to the laser beam of the Malvern's Spraytec laser diffraction instrument (Malvern Instruments Ltd., UK) with a distance of approx 3 cm from the beam (Fig. 2.2). The nebuliser was operated to commence aerosolisation of the niosomes to "dryness" (i.e. until aerosol generation completely ceased). The volume median diameter (VMD, 50% undersize) of the droplets and the Span value were recorded to represent the size and size distribution of the droplets respectively. Span = (90% undersize – 10% undersize) / VMD.

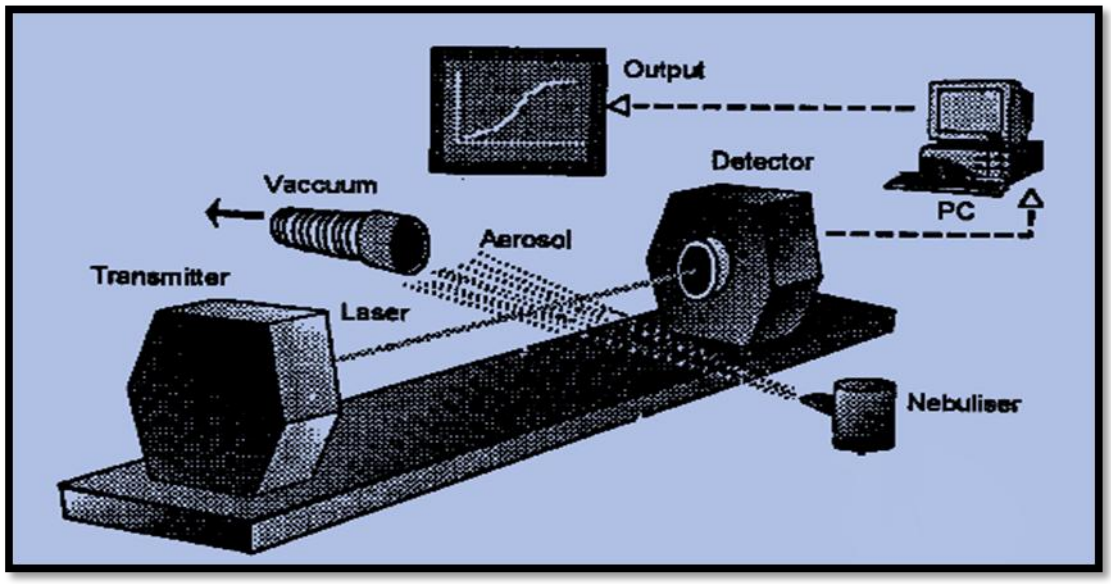


Fig 2.2: A schematic presentation of a laser diffraction aerosol particle size analyser

2.2.11 Determination of nebuliser output

As a matter of fact, nebulisers do not deliver all of the medical fluid and always a fraction of the fluid originally placed in the nebuliser reservoir remains as a “residual volume” (Clay et al., 1983). The amount of fluid delivered from nebuliser is referred to as the aerosol output. However, most important is the drug output which is the percentage amount of the drug successfully delivered as aerosols out of the total amount of drug originally placed in the nebuliser. For each nebuliser, niosomes (2.5 ml) were placed in the nebuliser and nebulisation was commenced and continued to “dryness”. Thus aerosol mass output was determined gravimetrically by weighing the nebulisers before and after nebulisation. Drug output was determined using UV spectrophotometer to measure the quantity of BDP in the residual volume. The amount of BDP delivered

was calculated by subtracting the amount of BDP in the residual fluid from the total amount of BDP originally placed in the nebuliser.

2.2.12 Estimation of “fine particle fraction”, prediction of alveolar deposition and extrathoracic deposition

Using the Spraytec laser diffraction instrument, the percentage of drug delivered in droplets, having a size below 5.4 µm, below 2.1 µm and greater than 11.6 µm, was measured to represent the “fine particle fraction” of the aerosols, the predicted alveolar deposition and the extrathoracic deposition, respectively.

2.2.13 Freeze-drying of niosomes generated from proniosomes

Proniosomes (560 mg) were hydrated according to the procedure described in section (2.2.3) using 30 ml of deionised water. Niosomes (4 ml) were freezed at -20 °C overnight followed by drying using a freeze dryer (Alpha 2-4 LD Plus, Christ, Germany) to yield a lyophilized “cake” of niosomes. The lyophilisate was rehydrated with deionised water (4 ml) with vortex mixing for 4 min. A sample (1 ml) was taken for size analysis using photon correlation spectroscopy (section 2.2.5) and zeta potential analysis using laser Doppler velocimetry (section 2.2.6). Further experiments were conducted to calculate the EE of BDP by filtration of the niosome dispersion through microfilters (0.45 µm). The filtered liquid (0.5 ml) was taken for UV spectrophotometric analysis to calculate the EE as previously described.

2.2.14 High shear homogenisation of proniosomes

This experiment was conducted to investigate the possibility of generating niosomes on a large scale from proniosomes. This was conducted using the Nano Debee homogeniser (Bee International Inc., UK). The Nano Debee homogeniser was washed 5 times to avoid cross contamination. Proniosomes were prepared in excess amount. The samples used in the Nano Debee homogeniser were 60 ml proniosome suspension. The proniosome suspension was homogenised via the Nano-Debee for 12 circles to reduce the size of the vesicles. Size and zeta potential studies were conducted by following the procedures previously described.

2.3.1 Statistical analysis

All experiments were prepared three times and the results were expressed as the mean ± standard deviation (SD). One way analysis of variance (ANOVA) followed by Tukey Kramer test were performed using Graphpad instat® to check the significance between various groups. A difference was considered to be significant when the p-value was less than or equal 0.05.

Chapter 3

Characterisation of conventional niosomes and niosomes generated from proniosmes and performance of nebulisation studies

3.1 Introduction

Previously, proniosomes have been introduced as a stable powder alternative to the liquid formulations of niosomes, offering advantages in terms of formulation stability and shipping expenses. Characterisation studies on the properties of niosomes and proniosomes have been performed to investigate whether niosomes generated by using the proniosome method would be advantageous to the conventional method. The conventional niosomes were prepared by using the thin film hydration method and proniosomes were prepared by spraying a chloroformic solution of the lipid phase onto the carbohydrate carrier particles (e.g. sucrose). Particle size analysis, zeta potential (surface charge) measurements, microscopic morphology investigations and drug entrapment efficiency studies were conducted. Furthermore, the effect of drug loading on particle size, zeta potential and vesicle morphology was evaluated.

3.2 Particle size analysis

Table 3.1 shows the mean particle size of niosomes generated using the thin film method and those prepared from proniosomes. Using the thin film method or the proniosome technology, three different homogenisation techniques were employed to generate niosomes. The techniques performed were hand-shaking, bath sonication or probe

sonication. It has been found that the size of vesicles generated from proniosomes was significantly larger ($P<0.05$) than that of conventional niosomes, regardless of the homogenisation technique.

Amongst the three homogenisation techniques, hand shaking has generated niosomes that are markedly larger than the vesicles prepared using sonication (Table 3.1). This is attributed to the higher energy input by sonication which caused the niosomes to fragment into smaller vesicles. Similar findings have been reported for liposomes, and probe sonication is well established as a technique that converts large multilamellar liposomes (MLVs) into small unilamellar vesicles (SUVs) having a size of 100 nm or smaller (Elhissi et al., 2010). The large size of niosomes generated from proniosomes might be attributed to possible aggregation of the vesicles, especially when no sonication was applied (Table 3.1).

Apparently, niosomes prepared by hand shaking were not adequately dispersed in the aqueous phase, resulting in aggregation of the vesicles. Therefore, manual shaking alone was not enough to produce a homogenised suspension of niosomes. In addition, the larger size of niosomes produced by hand shaking was accompanied by a measured polydispersity index (PI) larger than 0.6, confirming the aggregation behaviour. Thus, probe sonication was necessary to generate small niosomes in the range of 150-250 nm. It has been previously demonstrated that niosome agglomerates are broken up by

probe sonication (Jiang and Oberdòrster, 2009), which agrees with our finding that employed BDP proniosomes.

Table 3.1: Size analysis of niosomes produced by conventional method and proniosome technology (Data are mean ±SD, n=3,*= P < 0.05)

Method	Size of niosomes (nm)		
	Hand shaking	**Bath sonication**	**Probe sonication**
Thin film	1847.0 ±359.40	684.6 ±2551	156.9 ±8.0
Proniosome	5312.3±1310.6*	1019.9 ±288.8*	236.5 ±15.3*

3.3 Zeta potential analysis

Table 3.2 shows the zeta potential measurements for niosomes generated from proniosome and conventional methods. Regardless of the preparation method, the surface charge of niosomes was negative. However, the intensity of the negative charge was higher ($P<0.05$) for the proniosome formulations (Table 3.2). It is possible that an interaction between sucrose molecules and vesicle membranes has occurred, causing a difference in charge distribution on vesicle surfaces. Further studies are required to investigate the validity of this hypothesis.

Table 3.2: Zeta potential analysis of niosomes produced by conventional method and proniosome technology (Data are mean ±SD, n=3, *= P < 0.05)

Method	Zeta potential of niosomes (mV)		
	Hand shaking	**Bath sonication**	**Probe sonication**
Thin film	-22.4 ±1.6	-16.8 ±2.4	-22.0 ±2.1
Proniosome	-54.5 ±3.9*	-24.1 ±4.9*	-35.7 ±3.7*

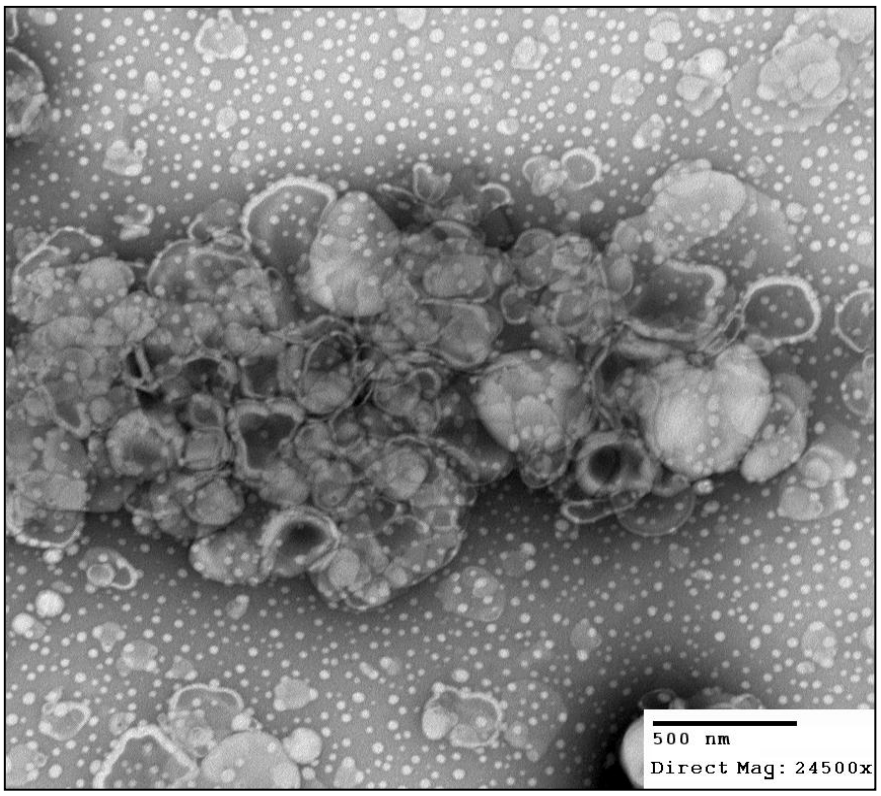
The higher charge intensity on niosomes derived from proniosomes possibly indicates higher storage stability of the liquid dispersions of these vesicles as compared to the conventional niosomes (Klang et al., 2010). The presence of similar charge on vesicles may enhance the repulsion between them and moreover, can minimize the possible aggregation or fusion. It has been previously reported that a decrease in electrophoretic mobility of particles may increase the negativity of the surface charge of the particle (Junyaprasert et al., 2008). It is likely that the larger size of niosomes generated from proniosomes (Table 3.1) has slowed the electrophoretic mobility of the vesicles, causing the surface charge to be highly negative. A long term stability study is needed in the future to investigate the differences in stability between vesicles generated using different methods.

3.4 Morphology of niosomes

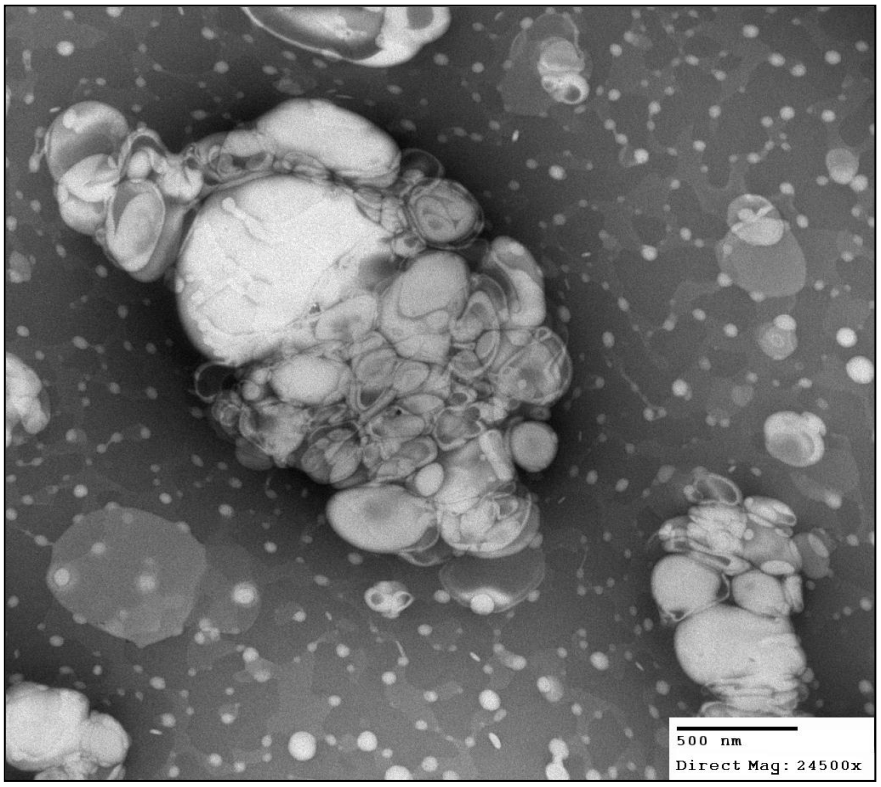
TEM studies showed that both conventional and proniosome methods generated highly spherical noisomes (Fig. 3.1 and Fig. 3.2). Fig. 3.1b shows that conventional niosomes were aggregated even after bath sonication for 5 min. The size of these vesicles was large (approx. 700 nm) (Fig. 3.1.b), agreeing with the size analysis findings (Table 3.1) and

indicating that the energy input by bath sonication was not sufficient for producing mono-dispersed small vesicles.

Hand shaking of proniosomes for 10 min has generated very large and highly aggregated niosomes (Fig. 3.2a). However, the size observed by TEM (Fig. 3.2) was obviously smaller than that measured using photon correlation spectroscopy (Table 3.1). This might be attributed to the negative pressure applied while conducting the TEM experiments, resulting in damaging the larger vesicles and subsequently only the smaller niosomes were viewed. Fig. 3.2 also demonstrates that the extent of agglomeration of niosomes generated from proniosomes was much higher than that of niosomes produced using the thin film method (Fig. 3.1). The inclusion of BDP does not seem to have any effect on the morphology and aggregation of niosomes (Fig. 3.2). For the TEM studies, it was necessary to get pictures of the samples at different magnifications to get more detailed shape of the particles.



(a)



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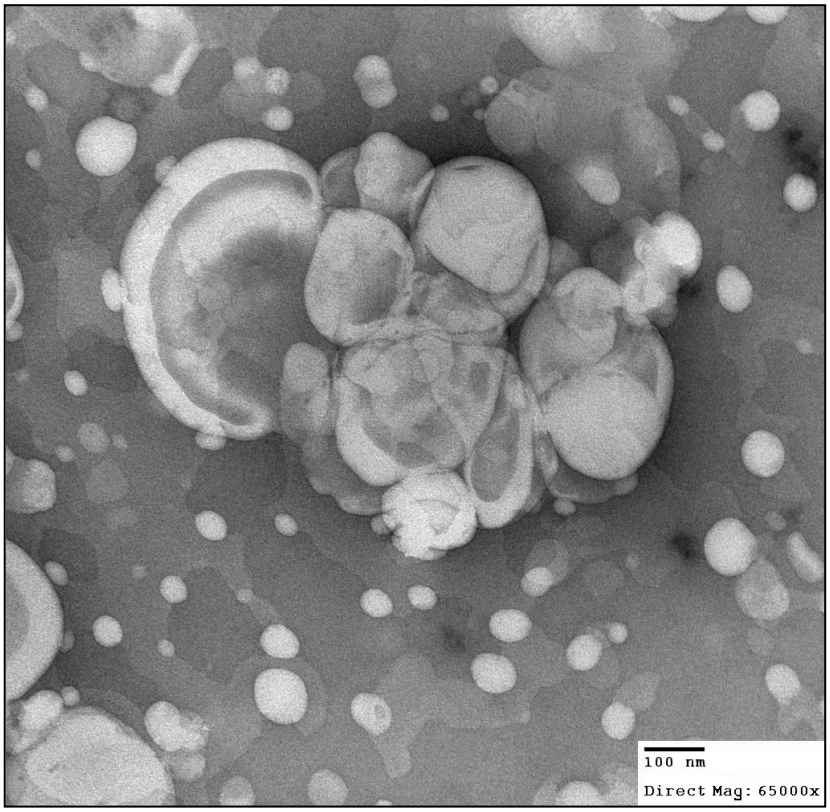

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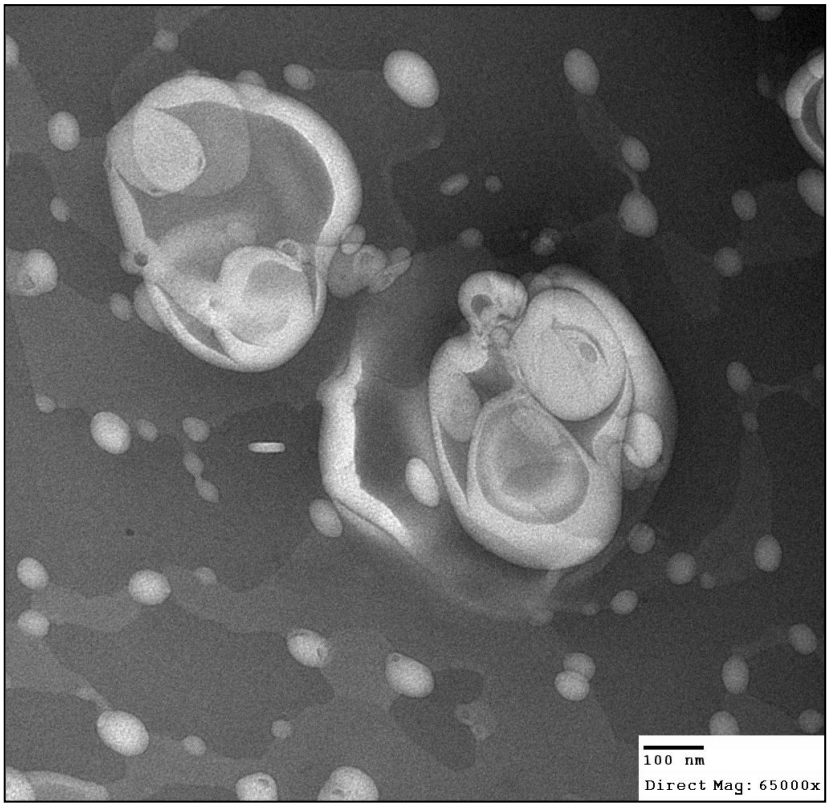
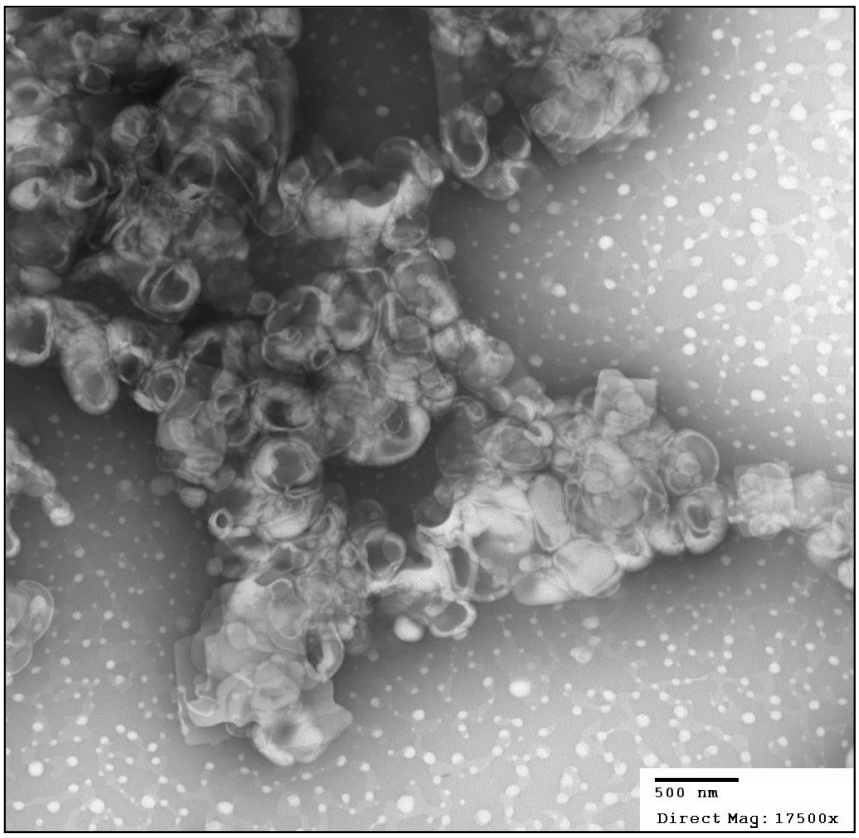
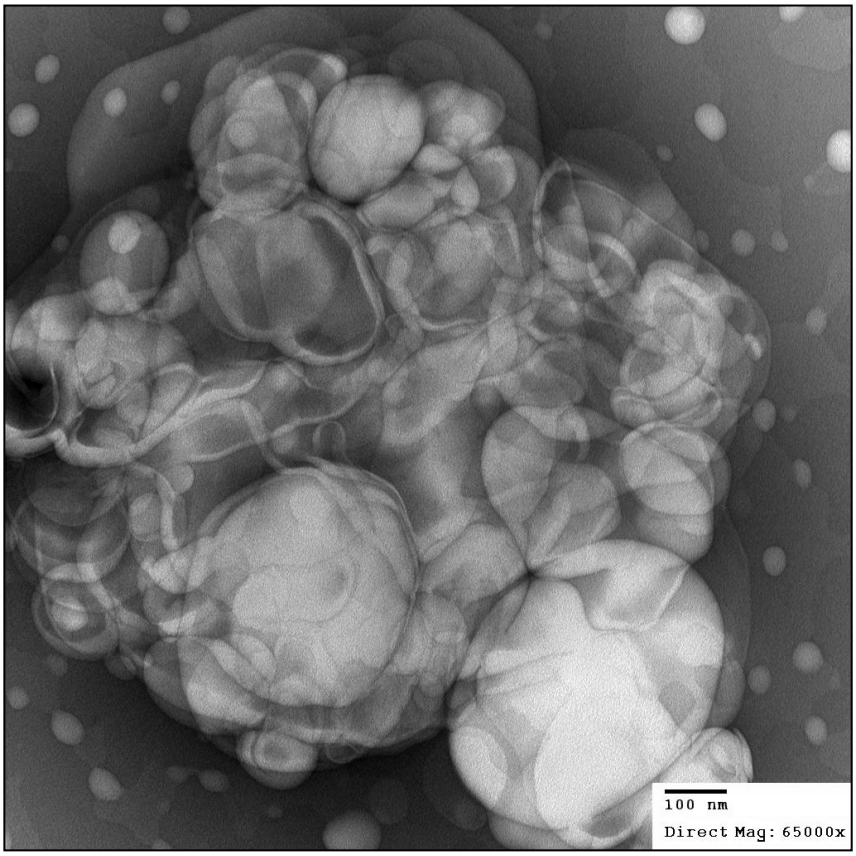


(d)

Fig 3.1: Transmission electron photomicrographs of BDP-loaded conventional niosomes using different homogenisation techniques a) Handshaking b) Bath sonication at magnification: 24500X c) Bath sonication at 65000X d) Probe sonicated particles. (n=3)



(a)



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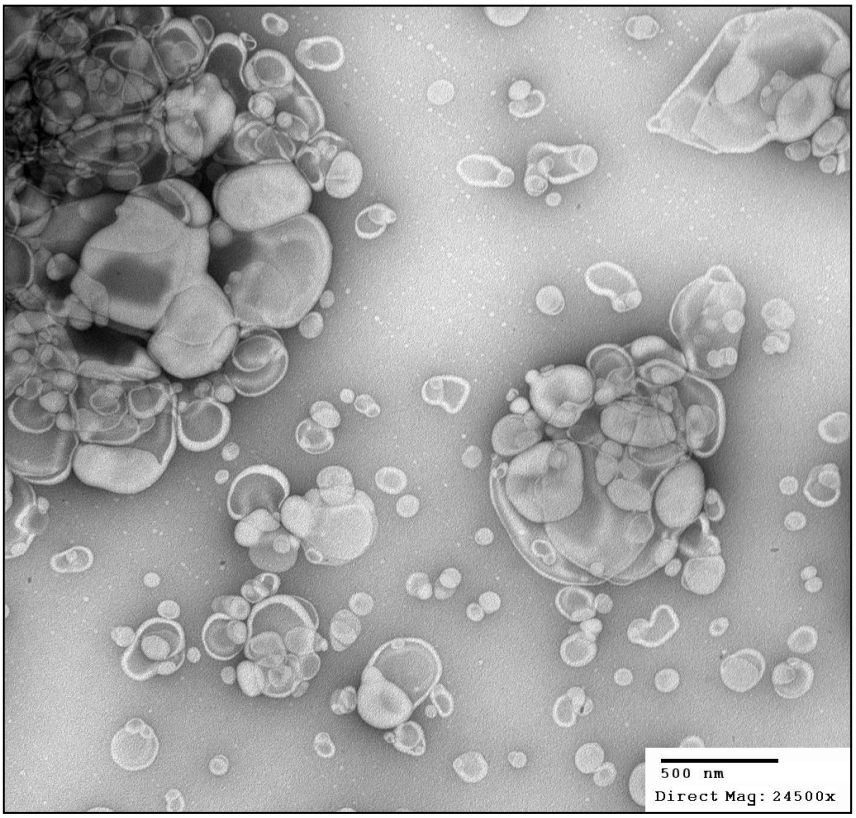

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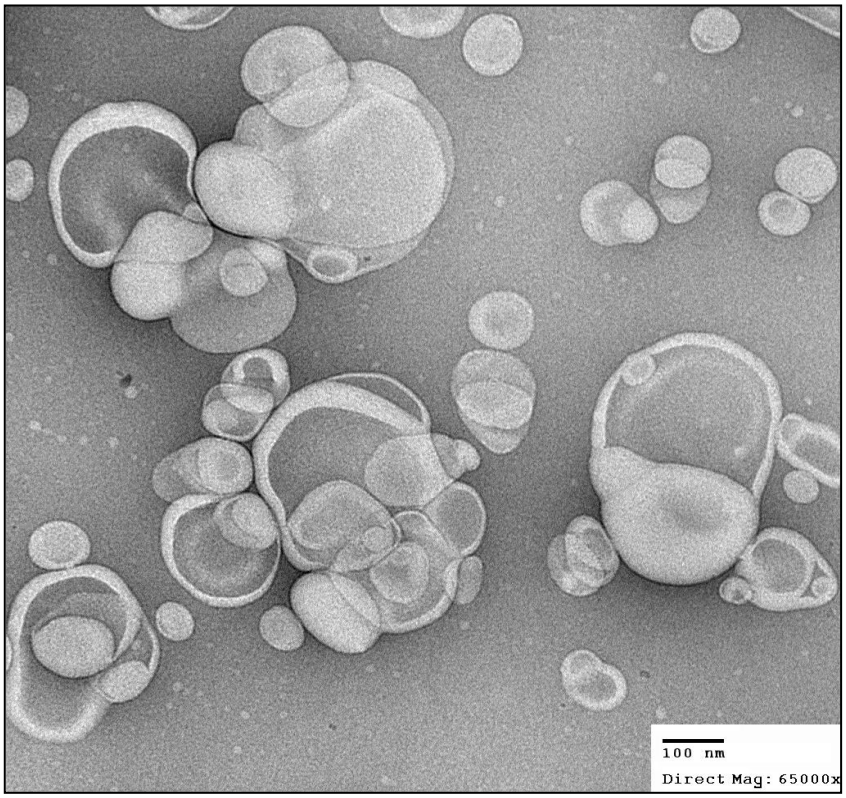

(d)

Fig 3.2: Transmission electron photomicrographs of empty and BDP-loaded niosomes derived from proniosomes. The empty niosomes are shown using different homogenisation techniques at different magnifications a) handshaking b) Bath sonication, whilst BDP-loaded niosomes are shown at different magnifications using c) bath sonication and d) probe sonication. (n=3)

3.5 Size and zeta potential analysis of BDP containing niosomes

Liposomes in the micrometer size range are highly unstable during nebulisation. This was demonstrated by the massive losses of the entrapped materials (Niven et al., 1991; Elhissi et al., 2007). Moreover, large liposomes are highly resistant to atomisation within nebulisers, which may result in poor output and prolonged nebulisation time (Elhissi et al., 2006). Since niosomes prepared by hand shaking without sonication are very large, they are expected to be highly resistant to delivery from the nebulisers. Earlier investigations of this project have shown that probe sonicated niosomes were the smallest and hence nebulisation studies were carried out using niosomes generated by the probe sonication technique.

Table 3.3 shows the size and zeta potential of BDP-loaded niosomes prepared by the conventional method and those generated from proniosomes. Niosomes generated from proniosomes had larger size ($P<0.05$) and more negative surface charge ($P<0.05$) than vesicles prepared using the conventional thin film method (Table 3.3). When the size of BDP-included niosomes (Table 3.3) was compared to that of the corresponding BDP-free vesicles (Table 3.1), there was only a slight trend of increase in vesicle size upon inclusion of the BDP. Also, no effect ($P>0.05$) on the zeta potential was observed upon inclusion of the drug.

Table 3.3: Size and zeta potential of probe sonicated BDP containing niosomes using the conventional and proniosome methods
(Data are mean ±SD, n=3, * = P< 0.05)

Method	Size and zeta potential of niosomes	
	Size (nm)	**Zeta potential (mV)**
Thin film	160.7 ±4.1	-25.2 ±1.8
Proniosome	202.5 ±9.5*	-36.2 ±3.3*

3.6 Entrapment efficiency (EE) of BDP in niosomes

Table 3.4 and Fig. 3.3 show a linear relationship between the concentration and absorbance of BDP. Using a personal computer, a regression line of the data (Table 3.4) was calculated for the different concentrations in HPLC-grade methanol at 239 nm. Thus, UV-spectrophotometer is a suitable technique to quantify BDP.

Table 3.4: Relationship between concentration and absorbance for BDP in methanol at a wavelength of 239 nm

BDP concentration (µg/ml)	Absorbance
0	0
10	0.28
12.5	0.353
20	0.565
25	0.723
30	0.844
37.5	1.124
40	1.143

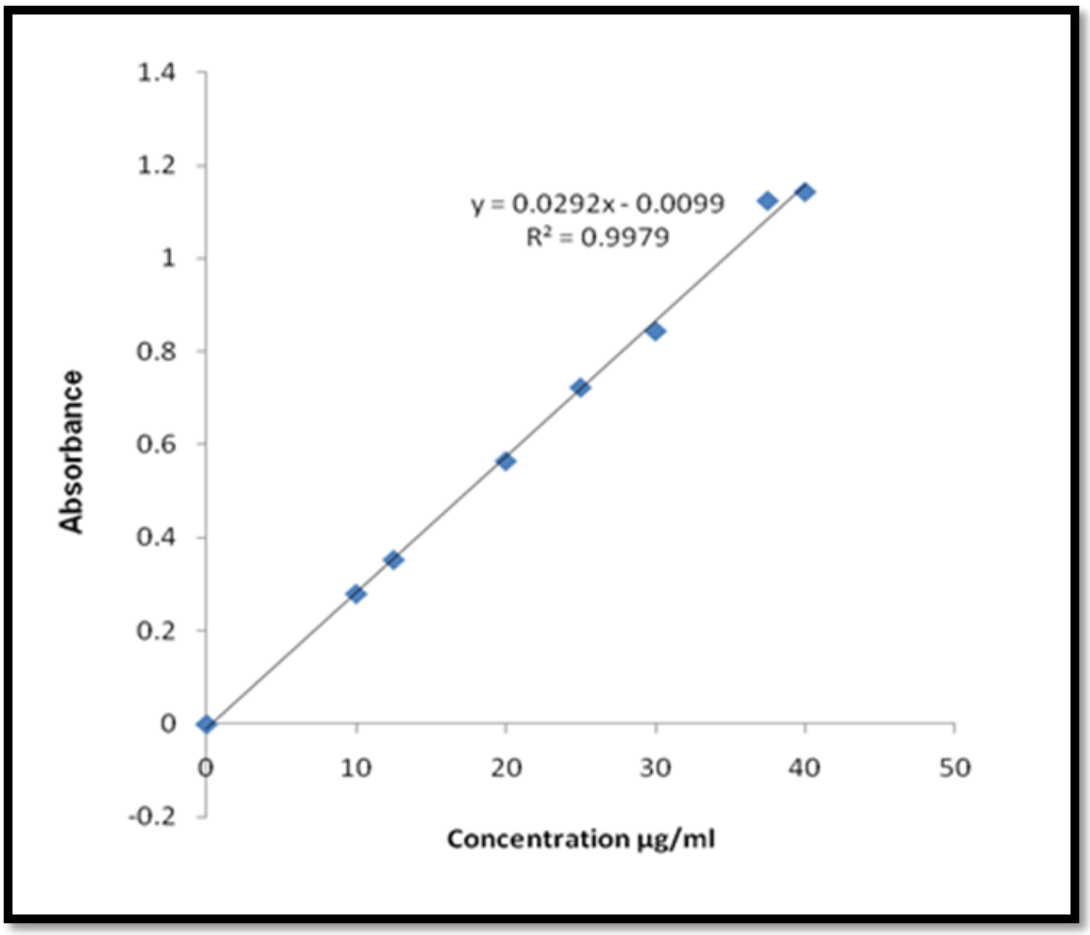

Fig 3.3: Calibration curve of BDP in methanol at a wavelength of 239 nm

Using only the probe sonicated formulations, compared to the conventional niosomes, vesicles generated from proniosomes have demonstrated higher EE ($P<0.05$) for the BDP steroid (Fig. 3.4). The results revealed that the EE of BDP in proniosome-derived niosomes was 36.4% compared to an EE of 27.5% offered by the conventional niosomes. This demonstrates that the proniosome method does not only provide a means for higher stability but also could be employed to enhance the entrapment of this antiasthma steroid.

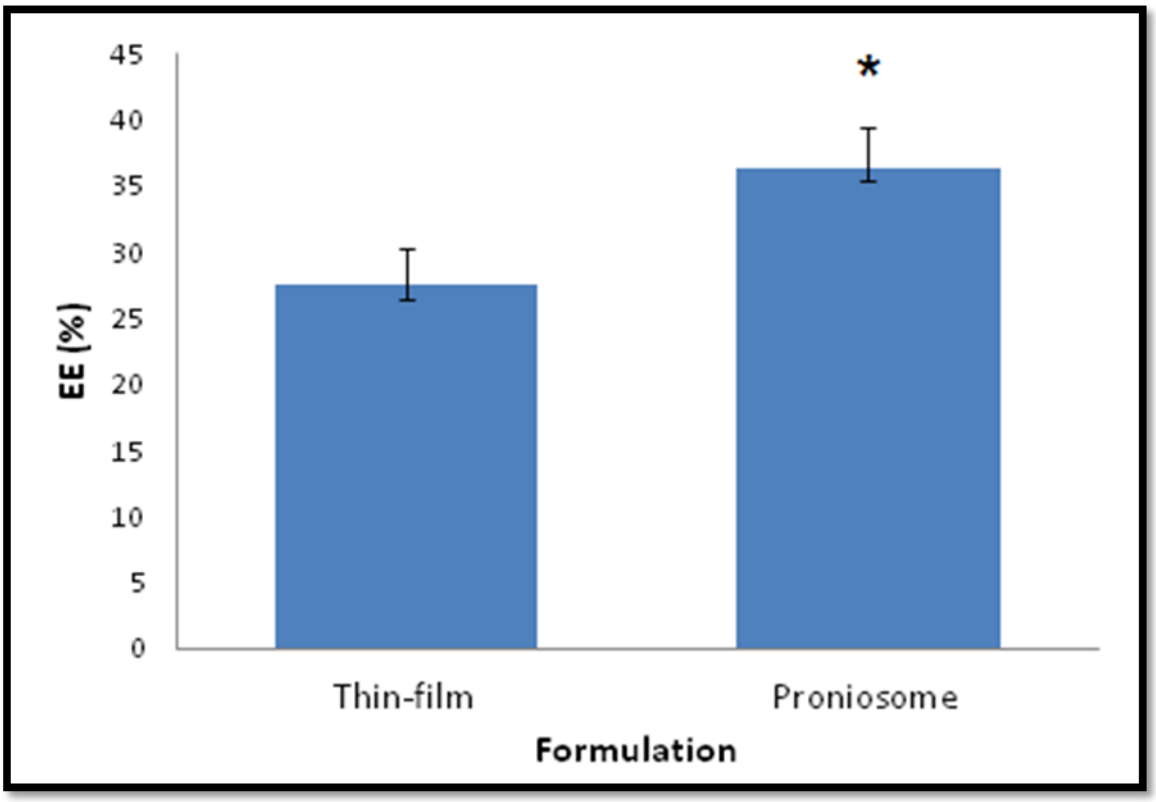

Fig 3.4: Charts showing EE (%) of BDP in conventional niosomes manufactured by using the thin film method and in niosomes generated from proniosomes

(Data are mean± SD, n=3, * = P< 0.05)

For liposomes, it has been shown previously that the larger the liposomes the greater the EE of beclomethasone (Darwis and Kellaway, 2001). Thus, the higher EE using the proniosomes might be attributed to the larger size of the vesicles. It is also possible that sucrose molecules in proniosome formulations have interacted with the niosome membranes, preventing the leakage of the drug. To investigate the validity of this hypothesis, further studies are needed. Additionally, from a technical perspective, the reason of higher drug entrapment in

proniosome-derivate niosomes is that coating of lipid onto sucrose particles has caused more efficient hydration and subsequent generation of vesicles. In contrast for the conventional method, the lipid film was highly sticky to the inner flask surfaces, causing difficulty in hydration and lower EE of the drug. Abd-Elbary et al (2008) have shown that hydration of surfactants was optimal upon the use of the proniosome method**.**

3.7 Aerosol mass and drug output study

Nebulisers are capable of delivering large volumes of aerosol from simply prepared solutions or dispersions (Terzano et al., 2007), justifying their use in this study. The aim of this study was to investigate the nebulisation of BDP in niosomal formulations using the proniosome method in terms of aerosol mass output and drug output. This study was conducted using three nebulisers operating at different mechanisms, namely the Pari LC Sprint (air-jet), the Aeroneb Pro (actively vibrating-mesh) and the Omron MicroAir (passively vibrating-mesh) nebulisers. The use of the proniosome method is justified by the higher EE of the steroid in niosomes (Fig. 3.4).

Aerosol mass and drug output of niosomes were calculated for the three different nebulisers used in this study (Fig. 3.5). After nebulisation to “dryness” none of the devices delivered 100% of the formulation, which is consistent with the previous findings using liposomes (Elhissi et

al., 2006; 2007). On completed nebulisation, a “dead” or “residual volume” is left within the nebuliser reservoir. The quantity of the “residual” volume is dependent on both nebuliser type and formulation characteristics (Ghazanfari et al., 2007).

For the Pari (jet) nebuliser, aerosol output was slightly but significantly ($P<0.05$) higher than drug output (Fig. 3.5). By contrast, the Aeroneb Pro (mesh) device demonstrated a trend of higher aerosol output than drug output but that was not statistically significant ($P>0.05$) (Fig. 3.5). This higher aerosol output trend indicates a trend for accumulation of some BDP within the Pari and Aeroneb Pro nebulisers at the end of nebulisation. For the jet nebuliser, this might be attributed to solvent evaporation from the aerosols during jet nebulisation, causing the drug to concentrate within the nebuliser (O’Callaghand & Barry, 1997). For the Aeroneb Pro device, it is possible that some mesh pores were blocked by the niosomes or that niosomes were aggregated within nebuliser, resulting in a trend of higher mass output than drug output.

The Omron (mesh) nebuliser behaved differently as the drug output was significantly ($P<0.05$) higher than aerosol output (Fig. 3.5), indicating preferential delivery of BDP compared to the other components of the niosome formulations. Previous investigations have shown this nebuliser to be highly dependent on formulation physicochemical properties such as viscosity, surface tension and presence of electrolytes within formulation (Ghazanfari et al., 2007).

When the Pari (jet) and the Aeroneb Pro (mesh) were compared, the mass output for both devices was similar and so was the drug output, with relatively high output values (Fig. 3.5), clearly indicating higher delivery performance of niosomes when either of these devices was employed. By contrast, the aerosol and drug outputs demonstrated by the Omron (mesh) were significantly lower ($P<0.05$) than outputs from the other two nebulisers (Fig. 3.5).

Although, the Omron (mesh) nebuliser represents the lowest outputs, it is important to report that drug output from this device was approximately 70% (Fig. 3.5), suggesting that this device is suitable for delivery of niosomes generated from proniosomes. Literature findings reveal that both air-jet and vibrating-mesh nebulisers are far more efficient than ultrasonic nebulisers in delivery of vesicle-based dispersions (e.g. liposomes) (Elhissi and Taylor, 2005), which explains the reason behind excluding ultrasonic nebulisers from this study.

Noteworthy, it was observed that the Pari (jet) nebuliser has completed nebulisation in approximately 10 minutes whilst the vibrating-mesh devices required at least 20 minutes. This indicates that the jet nebuliser is more efficient and may enhance patient compliance to nebulisation therapy when this formulation is used.

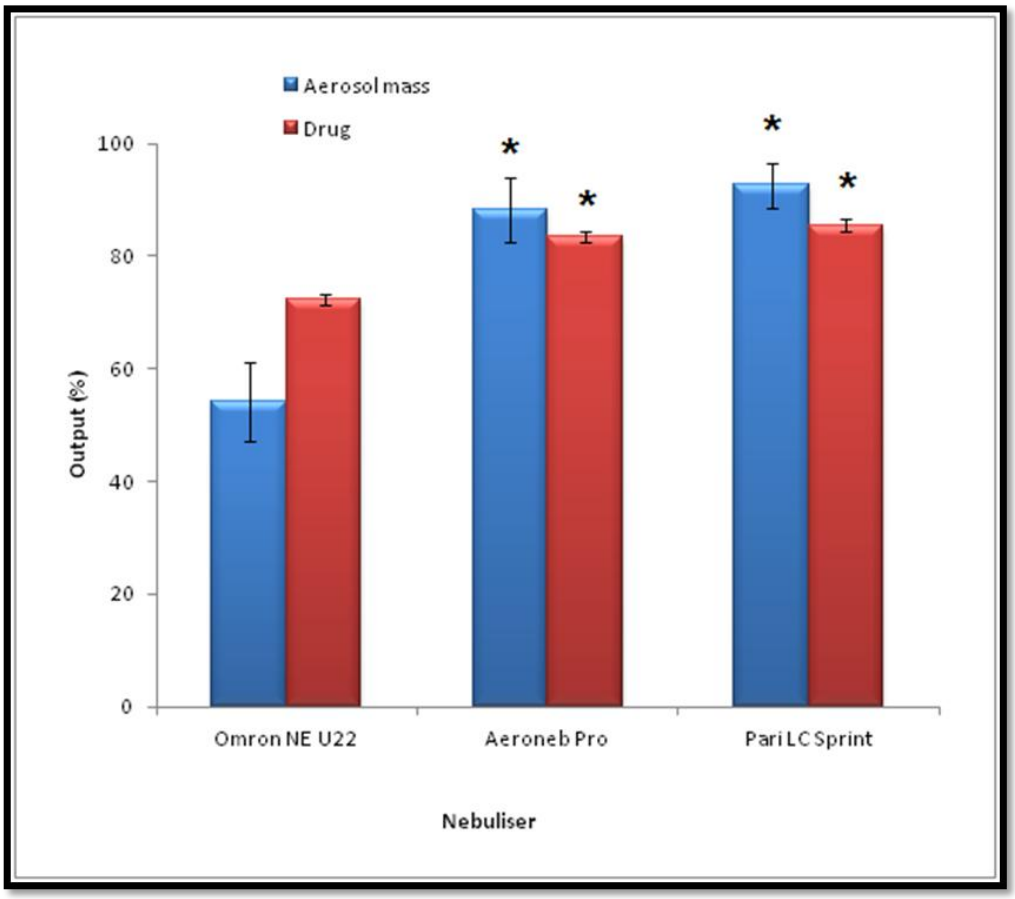

Fig 3.5: Charts showing aerosol and drug outputs of niosomes generated from proniosomes using Omron MicroAir NE U22 (passively vibrating-mesh), Aeroneb Pro (actively vibrating-mesh) and Pari LC Sprint (air-jet) nebulisers (Data are mean ± SD, n=3, * = p< 0.05)

3.8 Aerosol droplet size analysis and predicted pulmonary deposition

In this study, size and size distribution of the aerosol droplets generated from the nebulisers were measured using laser diffraction and presented as the median particle size based on a volumetric particle size distribution (Dv50) and Span respectively (Table 3.5). Moreover, the fraction of the delivered drug in droplets having a size below 5.4 µm,

below 2.1 μm and above 11.6 μm were recorded to represent the “fine particle fraction” (FPF), the predicted alveolar deposition and the predicted extrathoracic deposition respectively (Fig. 3.6).

The Dv50 of aerosol droplets generated from the Omron (mesh) nebuliser were significantly larger ($P<0.05$) than the droplets generated from the Aeroneb Pro (mesh) and the Pari (air-jet) nebulisers (Table 3.5). However, no significant ($P>0.05$) difference was found between the Dv50 for the jet and aeroneb Pro devices ($P>0.05$) (Table 3.5).

Similarly, the Span of the aerosol droplets were not different ($P>0.05$) when the Pari (jet) and the Aeroneb Pro (mesh) nebulisers were compared (Table 3.5). The Dv50 of aerosols generated from the Omron was the highest ($P<0.05$), indicating larger size of the aerosols (Table 3.5).

It is possible that the passive vibrations of the Omron’s mesh means that liquid is broken up into large droplets whilst the active vibrations of the mesh in the Aeroneb Pro device ensures an adequate conversion of the nebuliser fluid into smaller droplets that are equivalent in size to the droplets generated from the Pari (jet) nebuliser.

Table 3.5: Median size (Dv50) and Span of aerosol droplets generated from niosomes using different types of nebulisers (Data are mean ± SD, n=3, * = P< 0.05)

Nebuliser	Dv50 (μm)	Span
Omron NEU22 (mesh)	4.86 ± 0.16*	1.35 ± 0.33
Aeroneb Pro (mesh)	3.32 ± 0.17	1.52 ± 0.09
Pari LC Sprint (jet)	3.06 ± 0.15	1.33 ± 0.12

It has been previously reported that the suspended particles within nebuliser should be smaller than 2 μm in order to be easily incorporated in droplets having a diameter of 3 μm (Dahlback, 1994). Thus the size of droplets in this study indicates the suitability of the three nebulisers for delivery of niosomes generated from proniosomes, since the vesicles had much smaller size (Table 3.3) that would permit them to be incorporated into the generated aerosol droplets. In addition, the devices investigated in this work demonstrated that the generated aerosols are likely to deposit in high FPF.

There are many factors that influence deposition of particles in the respiratory tract. However, principally for particles to be considered therapeutically useful or in “FPF” (i.e. suitable for deposition in bronchioles and alveolar region) they should have a size below 5 - 6 µm, with particles below 2 µm being more likely to deposit in the alveolar region (O’Callaghan and Barry, 1997; Terzano et al., 2007). Particles that are larger than 12 µm may deposit in the extrathoracic region (Heyder, 1982), indicating their unsuitability for pulmonary delivery. Fig. 3.6. demonstrates the predicted regional distribution of the aerosol droplets in the respiratory system based on laser diffraction analysis. Droplets of size less than 5.4 µm, less than 2.1 µm and greater than 11.6 µm were recorded to represent the FPF, predicted deposition in the alveolar region and predicted deposition in the extra-thoracic region, respectively.

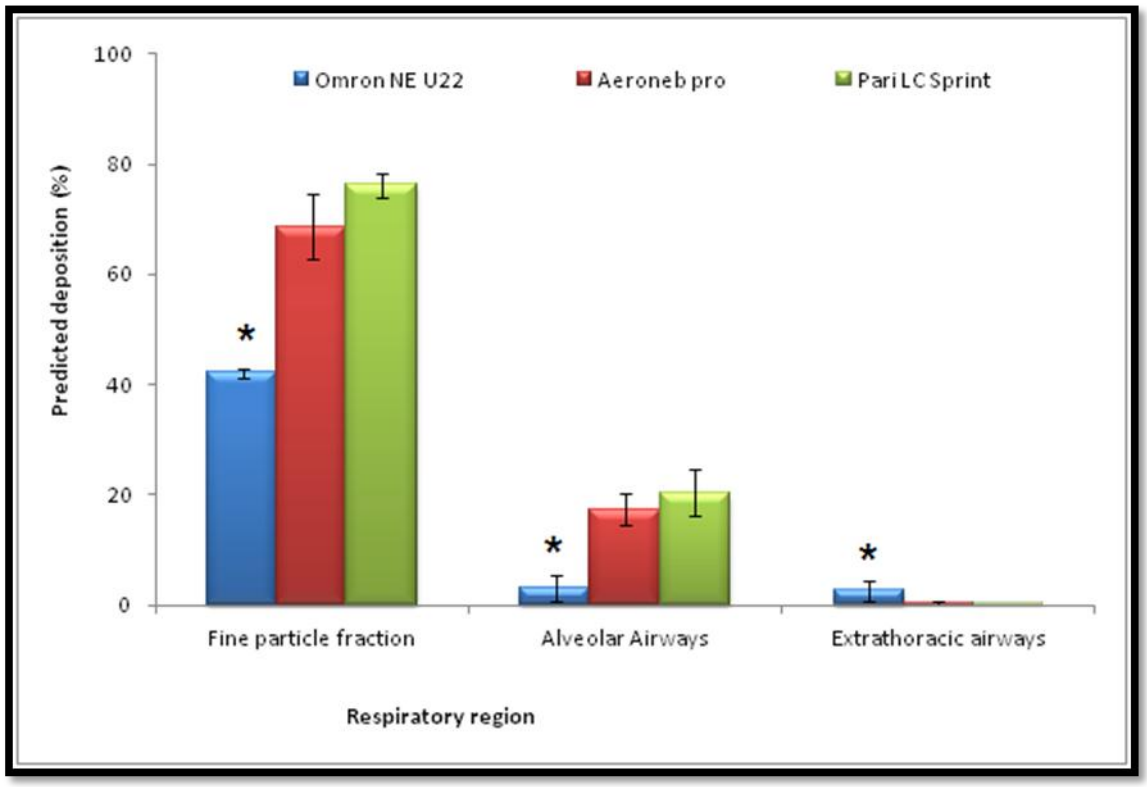

Fig 3.6: Charts showing predicted regional deposition of the nebulised BDP using the three nebulisers

(Data are mean ± SD, n=3* = P < 0.05)

Similar to Dv50 and Span findings, the Aeroneb Pro (mesh) and Pari (jet) nebulisers were similar in generating droplets with high FPF and predicted alveolar deposition, whilst the Omron device generated droplets with much lower FPF ($P<0.05$) and negligible alveolar deposition (Fig. 3.6). This again demonstrates that the Pari and the Aeroneb Pro nebulisers operate by mechanisms that are more appropriate for delivery of niosomes to the deep lung. The predicted extrathoracic deposition was negligible for all devices (Fig. 3.6). Noteworthy, although the Omron seemed to be the least appropriate for delivery of niosomes, it generated

droplets with 42% FPF (Fig. 3.6), indicating its suitability for delivering BDP-niosomes.

Thus, the proniosome technology represents a successful approach to delivering BDP in niosomes with high FPF when air-jet or vibrating-mesh nebulisers are used.

3.9 Freeze-drying of niosomes generated from proniosomes

Freeze-drying is a widely used technology in pharmaceutical industry to stabilise therapeutic agents or enhance their solubility or dispersion in aqueous environments. Freeze-drying has been used to stabilise liposomes (van Bommel and Crommelin, 1984). Delivery of freeze-dried liposomes via jet nebulisers has been shown to be achievable using beclomethasone as a model antiasthma steroid (Darwis and Kellaway, 2001). However, to avoid aggregation and fusion of freeze-dried liposomes upon rehydration, cryoprotectants (e.g. carbohydrates) are commonly added prior to freeze-drying (van Bommel and Crommelin, 1984).

In this study, niosomes generated from proniosomes were freeze-dried and rehydrated in order to investigate the possibility of manufacturing freeze-dried niosomes. No cryoprotectant was added since sucrose was one of the proniosome components.

Unfortunately, rehydration of niosomes caused a massive increase (by more than 13 times) in the measured particle size (Table 3.6). Freeze-drying of cryoprotected small unilamellar BDP-liposomes did not massively increase the size upon rehydration (Elhissi et al., 2011), contrary to our observation for niosomes which demonstrated an increase from the nanometer range to higher than 3 µm (Table 3.6). This suggests different behaviour of niosome membranes compared to liposome membranes. Further investigations are needed to provide a better understanding of the aggregation behaviour of niosomes upon freeze-drying and rehydration.

Table 3.6: Size, zeta potential and drug EE using niosomes generated from pronisomes before freeze-drying and upon rehydration of freeze-dried formulation (Data are mean ± SD, n=3,* = P < 0.05

Formulation	Size (nm)	Zeta potential (mV)	EE (%)
Niosomes	236.5 ±15.3	-35.7 ±3.7	36.4 ±2.81
Freeze-dried niosomes	3125.3 ±667.4*	-28.27 ±7.47	24.85 ±10.66*

Zeta potential had a trend to become less negative as a result of freeze-drying and rehydration, however, the change in zeta potential was not statistically significant (Table 3.6). This trend for change in the surface charge might be attributed to the massive increment in the measured size of vesicle (Table 3.6). If no cryoprotectant (e.g. sucrose) was included, small liposomes tend to considerably aggregate or fuse (Elhissi et al., 2010). However, for niosomes generated from proniosomes, although the cryoprotectant (i.e. sucrose) was included prior to freeze-drying the measured size increased dramatically, suggesting niosomes may require different cryoprotection mechanism.

Compared to unfreeze dried niosomes, the EE of BDP tended to decrease upon freeze-drying and rehydration, however, the entrapment values were inconsistent, with no statistically significant change being observed ($P>0.05$), possibly reflecting instability of the niosome formulation (Table 3.6). The trend for decreased EE of BDP might be attributed to drug leakage from niosomes as a result of vesicle aggregation or fusion. BDP is a hydrophobic drug and its association with the niosome membranes may have minimised its loss upon aggregation or fusion of vesicles. By contrast, previous findings using hydrophilic drugs have shown that leakage is significant upon aggregation or fusion of freeze-dried liposomes (Van Winden, 2003). It is suggested that the increased size of niosomes may make nebulisation less efficient or even unsuitable. Further work is needed to investigate this.

3.10 Niosmes generated from proniosomes using high shear homogenisation

This study aimed to introduce a method that might be employed for large scale manufacturing of niosomes. Proniosomes were manufactured and hydrated by hand shaking to generate niosomes. This was followed by size reduction in a Nano Debee homogeniser. This approach has shown to be successful since niosomes had a small size after homogenisation which is similar to that using the probe sonication technique (Table 3.7). Probe sonication may heat formulation and probes made of titanium may cause contamination of the preparation, resulting in limited use of this technique. In addition, heating might damage thermolabile drugs and thus, enhance the rate of oxidation for liable materials. By contrast, homogenisation using the Nano Debee homogeniser provides the advantage of safety, absence of titanium contamination and similar efficiency in vesicle size reduction without heating the preparation.

Table 3.7 revealed no significant difference ($P>0.05$) in particles size and surface charge (zeta-potential) between the two different methods of homogenisation namely the probe sonication and the Nano Debee homogenisation. The only difference was that the PdI of the particle size using Nano Debee homogeniser was around 0.5 whilst as

low as 0.3 or less by using the probe sonication, which indicates that probe sonication is more capable of reducing the vesicle polydispersity.

From a practical perspective, this might not negatively affect the applicability of the Nano Debee homogenisation method. Nano Debee offered the possibility of homogenisation much larger volumes of niosomes compared to probe sonication.

Table 3.7: Size, zeta potential and drug EE for niosomes generated from pronisomes using probe sonication or Nano Debee homogenisation
(Data are mean ± SD, n=3)

Production method	Size (nm)	Zeta potential (mV)	EE (%)
Probe sonication	236.5 ±15.3	-35.7 ±3.7	36.4 ±2.81
Nano Debee homogenisation	209.17 ±21.4	-38.0 ±6.42	29.65 ±4.04

It has been observed that the EE of BDP in niosomes homogenised by the Nano Debee homogeniser was not significantly different ($P>0.05$) from that offered by probe sonication (Table 3.7), again confirming that the use of Nano Debee homogeniser to homogenise niosomes in a relatively large scale was successful with only minimal compromisation of the quality of formulation. Further studies are required to investigate the behaviour of Nano Debee homogenised vesicles during nebulisation and the regional deposition of BDP in the respiratory tract.

Chapter 4

Conclusions and future work

4.1 Introduction

This chapter summarises the findings of this research and draws a map for the future investigations by our research group. The main idea behind using niosomes instead of liposomes was to avoid the instability problems of liposomes. Phospholipids in liposomes as are liable to hydrolysis or oxidation and liposomes as liquid dispersions may aggregate or fuse during storage with a subsequent leakage of the entrapped material. Initially proliposomes have been postulated as a potential resolution for the instability manifestations of liposomes. One type of proliposomes is the particulate-based type which represents carbohydrate carrier particles coated with phospholipids, followed by storage at low temperature (e.g. -20°C). Once liposomes are needed, an aqueous phase is added to the proliposomes with shaking to generate liposomes. The storage of proliposomes at this very low temperature aims to protect the phospholipids from hydrolysis and oxidation. In developed countries this may not be a difficult requirement, however, developing countries which have problems with electricity supply may find it very inconvenient to store proliposomes at -20°C. For this reason, particulate-based proniosomes have been suggested as an alternative to liposomes and proliposomes in our study.

Proniosomes are carbohydrate carrier particles coated with surfactants and cholesterol to generate niosomes upon addition of aqueous phase.

Since carbohydrates and surfactants are possible to store at room temperature, the storage requirement will not represent a problem for developing countries.

4.2 Experimental design

This study attempted to develop novel proniosome formulations containing the antiasthma steroid beclomethasone dipropionate (BDP). It was known previously that niosomes could be derived from the proniosome formulations. In addition, the properties of niosomes manufactured by the conventional thin film method (i.e. conventional niosomes) were compared to niosomes generated from proniosomes in terms of particle size, zeta potential (surface charge), morphology, aggregation behaviour and entrapment efficiency (EE) of BDP.

Moreover, niosomes generated from proniosomes were homogenised (i.e. reduced in size) by different techniques including hand shaking alone, hand shaking followed by bath sonication or hand shaking followed by bath sonication and probe sonication. The resultant vesicles were studies in terms of size, zeta potential and EE of BDP.

Probe sonicated niosomes generated from proniosomes were nebulised using three types of nebulisers: Pari LC Sprint (air-jet), Aeroneb Pro (actively vibrating-mesh) and Omron MicroAir NEU22

(passively vibrating-mesh) nebulisers. The performance of nebulisation using each nebuliser was evaluated in terms of aerosol mass output, drug output, median droplet size (Dv50) and droplet size distribution (Span). Span = (90% undersize – 10% undersize)/ Dv50. Span here does not refer to the well-known group of non-ionic surfactants. It is rather a term introduced by Malvern Instruments Ltd., UK (the supplier of the instrument used in this study) to express the polydispersity of particles.

In another experiment, the potential of manufacturing niosomes on a large scale was explored. High pressure homogenisation using the Nano Debee homogeniser was employed to process niosomes that were freshly prepared from proniosomes. The resultant vesicles were compared to probe sonicated vesicles in terms of particle size, zeta potential and EE of BDP.

Finally, the possibility of manufacturing freeze-dried niosomes generated from proniosomes by probe sonication was investigated. The particle size, zeta potential and EE of BDP were compared between vesicles before freeze-drying and after freeze-drying and rehydration. In these experiments, the cryoprotectant used was the sugar originally included within proniosomes (i.e. no external cryoprotectant was used).

4.3 General conclusions

4.3.1 Comparison between conventional niosomes and niosomes generated from proniosomes

Using either hand shaking, hand shaking and bath sonication or hand shaking with bath and probe sonication, niosomes generated from proniosomes were larger than conventional niosomes and had a wider size distribution. Morphology studies using transmission electron microscopy (TEM) confirmed that niosomes generated from proniosomes were aggregated. There was no observed effect of BDP inclusion on the aggregation of the niosomes generated from proniosomes.

Moreover, proniosome-generated niosomes had a more intense negative zeta potential when compared to the conventional vesicles. This was attributed either to the larger size of niosomes or to the presence of sucrose in niosomes generated from proniosomes. Taking the probe sonicated vesicles as an example, the zeta potential measurements were approximately - 25 mV and - 36 mV for the conventional vesicles and niosomes generated from proniosomes, respectively.

The EE of BDP was higher in proniosome-derived niosomes than in conventional niosomes being around 35% and 27%, respectively suggesting the superiority of niosomes manufactured by the proniosome technology.

Amongst the homogenisation techniques investigated, and using thin film method or proniosome technology, probe sonication was found to be the most efficient technique in reducing the size and polydispersity of niosomes. Thus, using probe sonication, the measured size ($Z_{average}$) was 160 nm and 202 nm for conventional niosomes and niosomes generated from proniosomes respectively.

4.3.2 Nebulisation output studies

Using the probe sonicated vesicles generated from proniosomes, the Pari (jet) and Aeroneb Pro (mesh) nebulisers generated aerosols with higher mass output than drug output, contrary to what was found when the Omron (mesh) was used.

The output from the Pari and the Aeroneb Pro devices were similar (approximately 80% for the drug output and 85% for the mass output) and higher than the values found with the Omron nebuliser (drug output was around 70%). Overall, all nebulisers were suitable for generating niosome aerosols with high aerosol and drug outputs.

4.3.3 Aerosol droplet size analysis and predicted pulmonary deposition

This study was conducted using laser diffraction. The Dv50 of droplets generated from the Pari and Aeroneb Pro nebulisers were around 3µm compared to a Dv50 value of around 5µm generated by the Omron

nebuliser. However, the size distribution (Span) presented by the Aeroneb Pro device was slightly higher than that for the Omron or the Pari nebulisers.

The study was conducted in order to predict the pulmonary deposition of BDP in niosomal formulations. The results have revealed higher “fine particle fraction” of the droplets generated from the Pari or the Aeroneb Pro nebulisers, being 70% or higher compared to FPF values of around 40% for the Omron nebuliser.

Moreover, the predicted alveolar deposition for the Pari or Aeroneb Pro was around 20% compared to 1 or 2% using the Omron device. This may suggest that the Omron is unsuitable for targeting the alveolar region. All nebulisers generated aerosols with negligible predicted extrathoracic deposition.

Overall, the findings of this study suggest that, if the niosomes are to target the bronchioles all nebulisers are potentially suitable, whilst the Omron may not be recommended for targeting the alveolar region.

4.3.4 Homogenisation using high pressure homogeniser

Using the proniosome technology, vesicle size reduction using the Nano Debee homogeniser has provided a successful means of producing small niosomes ($Z_{average}$ = 236.5 ±13 nm) which was comparable to niosomes processed via the probe sonicator ($Z_{average}$= 209.2 ±21.4 nm) with no statistical difference being detected (P>0.05).

Moreover, no significant difference (P>0.05) was found in the zeta potential of probe sonicated vesicles and niosomes homogenised by the Nano Debee homogeniser, being approximately -36 mV and -38 mV, respectively.

Similarly, the EE of BDP was not statistically different (P>0.05) between the two formulations, being 36.4% ±2.8 and 29.65% ±4.04 for the probe sonicated vesicles and the Nano Debee homogenised vesicles, respectively.

From a health and safety viewpoint, the Nano Debee homogeniser seems better since probe sonication might leach titanium in the niosome dispersions. Incomplete removal of that by centrifugation may cause undesirable effects *in vivo*. In addition, with the homogeniser, larger volumes can be processed. Overall, these findings indicate that homogenisation using the Nano Debee homogeniser is more desirable than the traditional method of probe sonication.

4.3.5 Freeze-drying of niosomes

In order to optimise an approach that is suitable for large scale production of niosomes that are appropriate for nebulisation, probe sonicated niosomes were freeze-dried.

Unfortunately, freeze-drying followed by rehydration has caused a marked increase (by more than 13 times) in the measured particle size and a trend for inconsistent reduction in the EE of BDP (EE = 24.85% ±10.66) when compared to vesicles that were probe sonicated without freeze-drying (EE= 36.4% ±2.81). However, the zeta potential was similar for both formulations.

4.4 Scope for future studies

Due to time limitations many studies have not been conducted. For instance, it would have been useful to study the interaction between sucrose and the niosome membranes using calorimetric techniques such as high sensitivity differential scanning calorimetry. This might possibly explain the reasons behind the aggregation behaviour of niosomes generated from proniosomes.

In addition, optimisation of the freeze-drying conditions is required in order to produce niosomes that are stable as a freeze-dried formulation. This would include future investigations of using cryoprotectants other than sucrose or sucrose in different concentrations.

It would be very interesting to study the in *vivo* behaviour of niosomes generated from proniosomes and compare the findings to those of the well established liposome formulations.

Chapter 5

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