CHAPTER II

LITERATURE REVIEW

1. Delivery of Proteins and Peptides

Biotechnology advances have made it possible to use macromolecules such as peptides and proteins as therapeutic agents (Okamoto et al., 2002). At this time intravenous, intramuscular, and subcutaneous injections are the practical administration routes for such macromolecules. However, parenteral delivery has several drawbacks, including an invasive delivery method (often requiring medical professional administration), a sterile dosage form, systemic side effects, and rapid clearance (Conti et al., 2000). Peroral administration is the most convenient for patients, but the peroral bioavailability of macromolecules is extremely low due to their large molecular size and high susceptibility to enzymes in the gastrointestinal tract. Inhalation offers potential possibilities for the delivery of proteins and peptides for systemic and local activity.

2. Pulmonary Delivery of Proteins and Peptides

Pulmonary drug delivery involves the delivery of drugs providing direct access to the conducting zone (bronchi and bronchioles) of the lungs either for the treatment or prophylaxis of respiratory diseases, or access to the enormous surface area of the respiratory zone (alveoli) for treatment of systemic conditions (Figure 1) (Taylor, 2002; Daniher and Zhu, 2008). Advantages of pulmonary delivery of drugs to treat respiratory and systemic diseases are summarized in Table 1 (Patton and Platz, 1992; Agu et al., 2001; Okamoto et al., 2002; Labiris and Dolovich, 2003a; Smola, et al., 2008). Pulmonary delivery has received much attention as a non-invasive administration route for proteins and peptides. The transport of macromolecules across the absorptive area, the alveolar wall, occurs by transcytosis for proteins >22

kDa and through tight-junctional paracellular processes for smaller molecules (Patton and Platz, 1992; Patton, 1996; Patton, Fishburn, and Weers, 2004;). However, pulmonary delivery strategies of macromolecules present many difficulties, including protein denaturation during aerosolization, excessive loss of inhaled drug in the oropharyngeal cavity (often exceeding 80%), poor control over the site of deposition, lack of reproducibility of therapeutic results owing to variations in breathing patterns, the frequent too-rapid absorption of drug potentially resulting in local toxic effects, and phagocytosis by lung macrophages (Patton and Platz, 1992; Shoyele and Slowey, 2006).

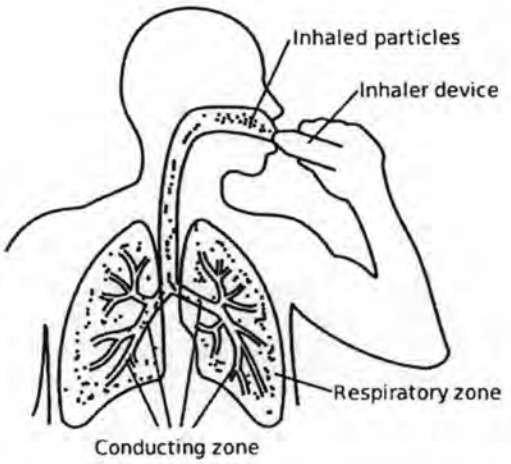

Figure 1 Schematic representative of pulmonary drug delivery (Daniher and Zhu, 2008)

2.1 Factors Affecting Therapeutic Effectiveness of Aerosolized Medications

For the lungs to be the target organ or a route of administration, the appropriate amount of drug must be deposited past the oropharyngeal region to achieve therapeutic effectiveness. The optimal site of deposition (central or peripheral airways) and the distribution of the inhaled drug may also play a role in an inhaled drug's effectiveness (Labiris and Dolovich, 2003a).

Table 1 Advantages of pulmonary delivery of drugs to treat respiratory and systemic diseases (Labiris and Dolovich, 2003a)

Treatment of respiratory diseases
- Delivery of high drug concentration directly to the disease site - Minimized risk of systemic side-effects - Rapid clinical response - Avoidance of the barriers to therapeutic efficacy, such as poor gastrointestinal absorption and first-pass metabolism in the liver - A similar or superior therapeutic effect at a fraction of the systemic dose. For example, oral salbutamol 2–4 mg is therapeutically equivalent to 100–200 μg by MDI.
Treatment of systemic diseases
- A noninvasive 'needle-free' delivery system - Suitability for a wide range of substances from small molecules to very large proteins - Enormous absorptive surface area (100 m^2) and a highly permeable membrane (0.2–0.7 μm thickness) in the alveolar region - Significantly improved absorption of large molecules with very low absorption rates; prolonged residency in the lung due to the slow mucociliary clearance in the lung periphery - A less harsh, low enzymatic environment that is devoid of hepatic first-pass metabolism - Reproducible absorption kinetics. Pulmonary delivery is independent of dietary complications, extracellular enzymes and interpatient metabolic differences that affect gastrointestinal absorption.

Aerosol particle size is one of the most important variables in defining the dose deposited and the distribution of drug aerosol in the lung. Mass median diameter of an aerosol refers to the particle diameter that has 50% of the aerosol mass residing above and 50% of its mass below it. The aerodynamic diameter relates the particle to the diameter of a sphere of unit density that has the same settling velocity as the particle of interest regardless of its shape or density.

Particles can be deposited by inertial impaction, gravitational sedimentation or diffusion (Brownian motion) depending on their size (Figure 2) (Lourenco and Cotromanes, 1982). Most particles > 10 μm are deposited in the oropharyngeal region with a large amount impacting on the larynx. Deposition by gravitational sedimentation predominates in smaller bronchi and bronchioles. Particles in the alveolar region have a longer residence time and are deposited by both sedimentation and diffusion. Particles not deposited during inhalation are exhaled. In general, aerosols with a MMAD of 5–10 μm are mainly deposited in the large conducting airways and oropharyngeal region. Particles 1–5 μm in diameter are deposited in the small airways and alveoli with > 50% of the 3 μm diameter particles being deposited in the alveolar region. For systemic effect, aerosols with a small particle size would be required to ensure peripheral penetration of the drug. Particles < 3 μm have an approximately 80% chance of reaching the lower airways with 50–60% being deposited in the alveoli.

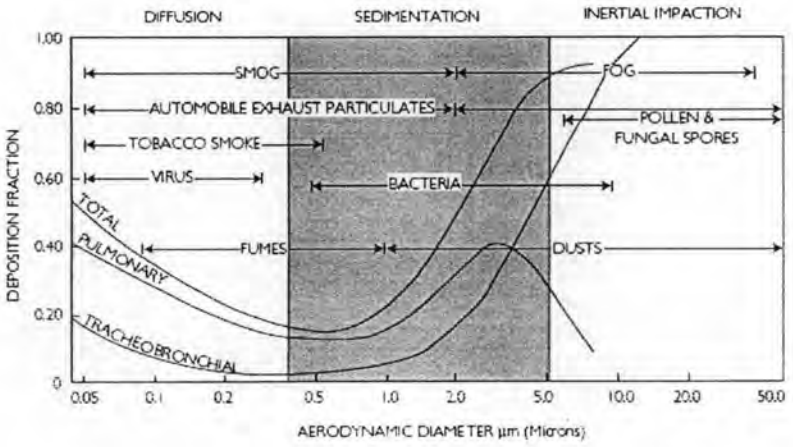

Figure 2 Relationship between particle size and lung depositions (Labiris and Dolovich, 2003a)

Figure 3 shows the barriers to macromolecule absorption and sustained delivery in the respiratory tract (Koushik and Kompella, 2004). Independent of the method used to produce the aerosol, before reaching the deep lung, inhaled particles must overcome certain obstacles and lung defense mechanisms, essentially the effect of the airways structure and the mucus layer, which protects the epithelium in the tracheobronchial region. Particles targeted to the deep lung should be small enough to pass through the mouth, throat and conducting airways and reach the deep lung, but not so small that they fail to deposit and are breathed out again. Even so, a certain number of particles will be transported away from the lung by mucocilliary clearance (Agu et al., 2001). In addition, particles will have to face at least two other defense mechanisms: the alveolar macrophages and the enzymatic activity. The alveolar surface is covered by a thin layer of fluid with suspended macrophages, which play an important role in lung defense. With the capacity of moving freely in the surface, they are able to engulf "foreign" substances from the airway surface. Uptake of particles by the alveolar macrophages has been found to be size dependent (Kompella and Lee, 2001). It has been reported that the phagocytic activity is maximum for particles of 1-2 μm, decreasing for both smaller and larger particles out of this range (Akhtar and Lewis, 1997; Ahsan et al., 2002; Makino et al., 2003). However, if the objective of drug delivery was to target the alveolar macrophages, particles of mass median diameter of around 1-2 μm would be the ideal particles to target. The enzymatic degradation within the lungs, although less severe compared to the gastrointestinal tract, represents an important metabolic barrier limiting the absorption of drugs through the lungs (Koushik and Kompella, 2004). Some therapeutic peptides that are degraded in the lungs are salmon calcitonin, vasoactive intestinal peptide, Substance P, Met- and Leu-Enkephalin, and leutinizing hormone releasing hormone (LHRH) and its analogs.

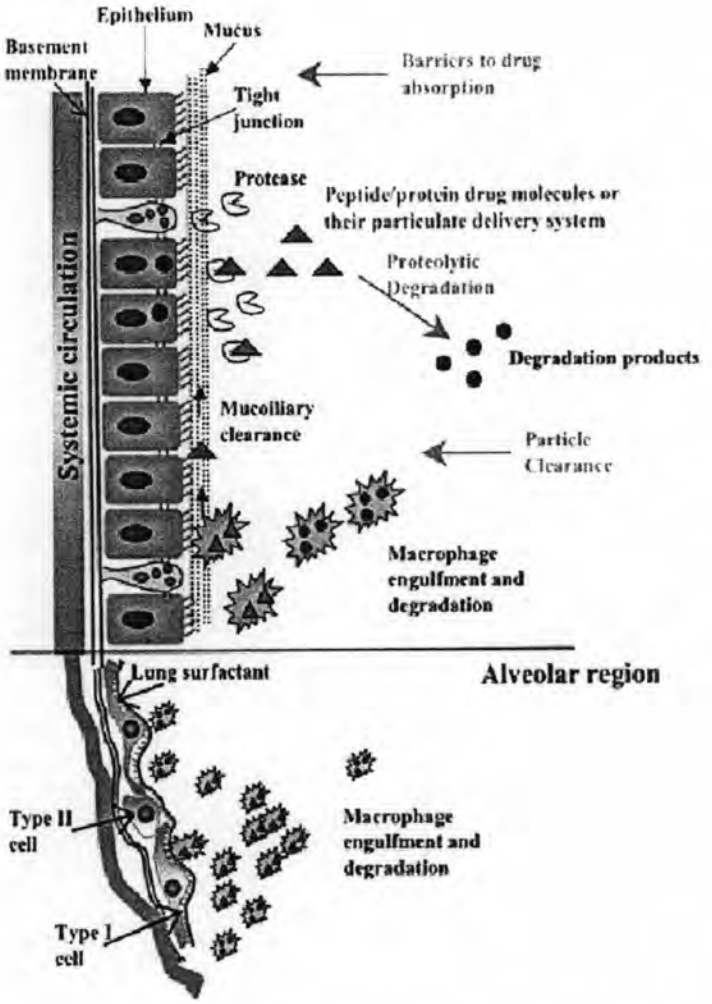

Figure 3 Barriers to macromolecule absorption and sustained delivery in the respiratory tract (Koushik and Kompella, 2004)

2.2 Inhalant Delivery Devices

The delivery device plays a major role in the efficiency of pulmonary delivery (Labiris and Dolovich, 2003; Cryan, 2005). The devices most commonly used for respiratory delivery, including nebulizers, metered-dose inhalers (MDIs), and dry powder inhalers (DPIs), can all be adapted for use with protein/peptide drugs. Table 2 summarizes advantages and disadvantages of inhalation devices. The choice of device will depend on the drug, the formulation, the site of action, and the pathophysiology of the lungs. For example, liposomes do not form in conventional MDIs and would therefore be better suited for nebulization or drying to form a DPI.

Table 2 Advantages and disadvantages of inhalation devices (Labiris and Dolovich, 2003b)

Inhalation device	*Advantages*	*Disadvantages*
Nebulizers (jet, ultrasonic)	No specific inhalation technique or co-ordination required	Time consuming
	Aerosolizes most drug solutions	Bulky
	Delivers large doses	Nonportable
	Suitable for infants and people too sick or physically unable to use other devices	Contents easily contaminated
		Relatively expensive
		Poor delivery efficiency
		Drug wastage
		Wide performance variation between different models and operating conditions
Pressurized metered dose inhalers (pMDI)	Compact	
	Portable	Inhalation technique and patient co-ordination required
	Multidose (approximately 200 doses)	High oral deposition
	Inexpensive	Maximum dose of 5 mg
	Sealed environment (no degradation of drug)	Limited range of drugs available
	Reproducible dosing	Respirable dose dependent on inspiratory flow rate
Dry powder inhalers (DPI)	Compact	Humidity may cause powders to aggregate and capsules to soften
	Portable	Dose lost if patient inadvertently exhales into the DPI
	Breath actuated	Most DPIs contain lactose
	Easy to use	
	No hand-mouth co-ordination required	

The selection of device for proteins delivery to the lungs is an important factor in the formulation design (Cryan, 2005). This relates to fundamental choices of the state of the protein (solution or dry powder) to be used, the method and state of storage, the choice of additives, and the interactions between the formulation and the device (adsorption). If the drug is being targeted to a specific region of the lungs, then a device capable of generating and delivering droplets/particles with the requisite aerodynamic diameter will be required. MDIs have some limitations because on actuation, the first propellant droplets exit at a high velocity. Consequently, much of the drug is lost through impaction of these droplets in oropharyngeal areas. The stability of proteins and peptides on nebulization is a potential limitation. Many biopharmaceuticals are unstable in aqueous solutions, and lung penetration can occur due to the thermal and surface effects during nebulization (Shoyele and Slowey, 2006). DPIs present advantages over nebulizers and MDIs for delivery of proteins/peptides to the lungs because DPIs are easy to operate (breath-actuated), inexpensive, propellant-free and show improved stability of the formulation as a result of the dry state.

2.3 Dry Powder Inhalers (DPIs)

The success of inhalation therapy with dry powders is determined by the active ingredient's biological aspects, by the physicochemical aspects of formulation, and by inhaler performance (Figure 4) (Okamoto, 2002). Dry powder inhaler devices are classified by dose type into single-unit dose, multi-dose reservoirs, and multi-unit dose (Daniher and Zhu, 2008). In a single-unit dose device, the drug is formulated as a micronized drug powder and carrier system and supplied in individual gelatin capsules, which are then inserted into the inhaler for a single dose and removed and discarded after use. The multi-dose reservoir device stores the formulation in bulk, and has a built in mechanism to meter individual doses from the bulk upon actuation. The multi-unit dose device uses factory metered and sealed doses packaged in a manner that the device can hold multiple doses without having to reload.

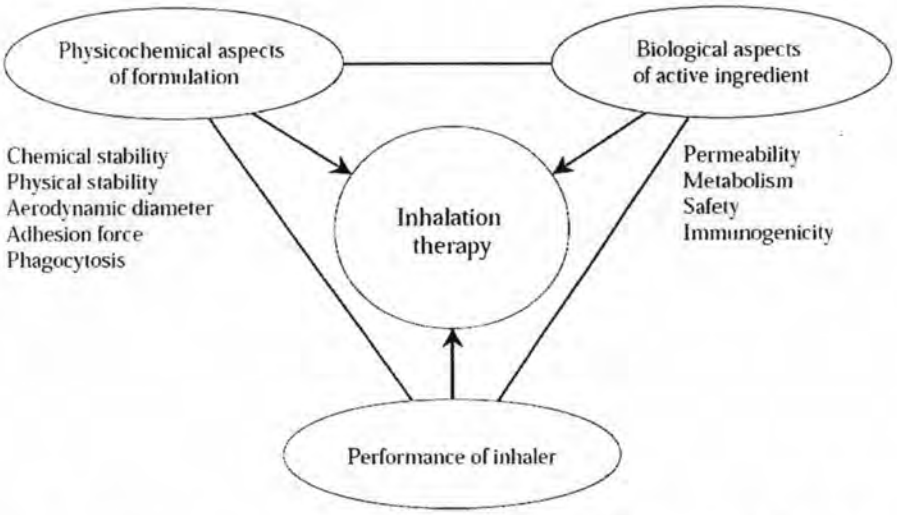

Figure 4 Factors determining successful inhalation therapy with dry powders (Okamoto, 2002)

Drug formulation plays an important role in producing an effective inhalable medication (Labiris and Dolovich, 2003). An active drug must be efficiently delivered into the lungs to the appropriate site of action and remain in the lungs until the desired pharmacological effect occurs. A drug designed to treat a systemic disease, such as insulin, must be deposited in the lung periphery to ensure maximum systemic bioavailability. For gene therapy or antibiotic treatment in cystic fibrosis, prolonged drug residence in the lungs may be required to obtain the optimal therapeutic effect. Thus, a formulation that is retained in the lungs for the desired length of time and avoids the clearance mechanisms of the lung may be necessary.

Formulating DPIs involves either micronization via jet milling, precipitation, freeze-drying or spray-drying using various excipients, such as lipids and polymers, or carrier systems like lactose. Each method offers advantages as well as disadvantages, depending on the therapeutic agent being formulated (Labiris and Dolovich, 2003b). The main difficulty associated with inhalation drug powders is the strong interparticle forces which make the cohesive bulk powder agglomerate (Daniher and Zhu, 2008). There are three types of interparticle forces, the van der Waals force, the capillary force and the electrostatic forces. Figure 5 shows formulation strategies for modifying surface morphology or surface chemistry to

reduce interparticle force (Daniher and Zhu, 2008). Carrier-free formulation of a single compound, multi-compound composite or encapsulated particles can be prepared using spray drying, supercritical fluid processing and sonocrystallization (Figure 5a). Uniform coating of drug particles with the additives is an effective means to improve the flowability of certain cohesive powders (Figure 5b). Formulation of DPIs consists of coarse carrier lactose for enhancing the powder flow of the formulations and increasing the powder bulk for capsule filling (Figure 5c). An additional particle type (such as nanoparticles and fine particles) may be added to the drug-carrier system to function as a physical spacer or possibly by occupying high-energy sites in the carrier surface (Figure 5d). In addition, the performance of DPIs was improved significantly through particle engineering by lowering the aerodynamic diameters of the particles using small geometric diameter of the particles, lowering particle density (by increasing porosity of particles), altering shape (elongated particles) and by creating rough surface (to increase the air drag force) (Chougule et al., 2007).

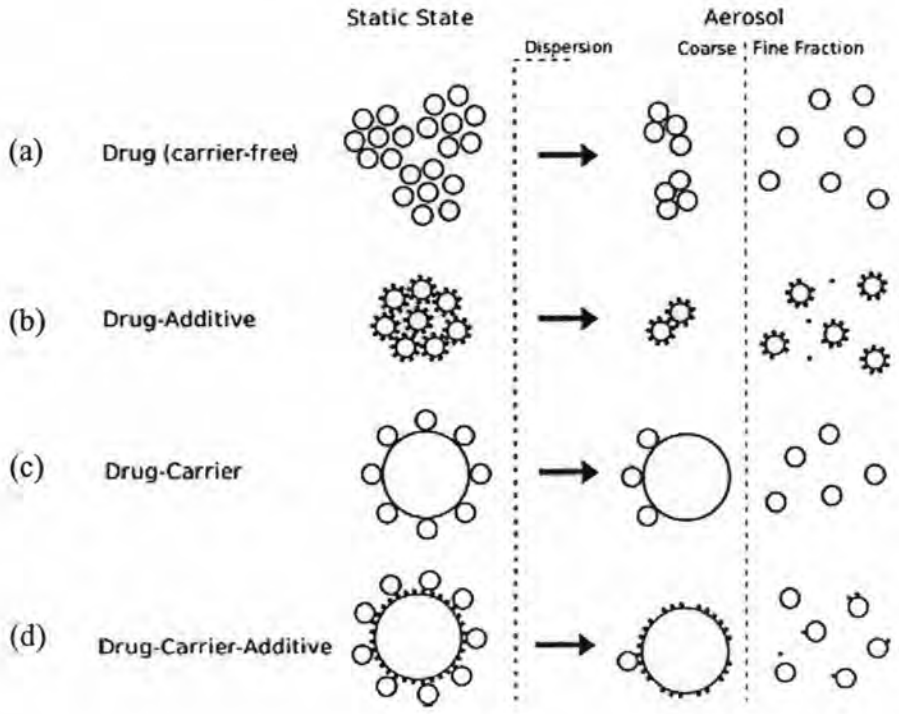

Figure 5 Illustration of the different types of formulation strategies for powders intended for pulmonary drug delivery (Daniher and Zhu, 2008)

3. Liposomes

Liposomes are simply vesicles in which an aqueous volume is entirely enclosed by a membrane composed of lipid molecules (usually phospholipids). They form spontaneously when these lipids are dispersed in aqueous media with input of external energy, giving rise to a population of vesicles which may range in size from tens of nanometers to tens of microns in diameter (New, 1989). Liposomes are classified by their diameter of the vesicles and number of bilayers into four classes: multilamellar vesicles (MLVs; several bilayers, size 100 nm-20 μm); large unilamellar vesicles (LUVs; single bilayer, size 100-1000 nm); small unilamellar vesicles (SUVs; single bilayer, 10-100 nm); and multivesicular vesicles (MVVs; size 100 nm-20 μm) (Figure 6). The mean size, the lamellarity and the physical stability of the vesicles not only depend on the chemical structure of the amphiphiles used, but in general particularly on the method of vesicle preparation (Walde and Ichikawa, 2001).

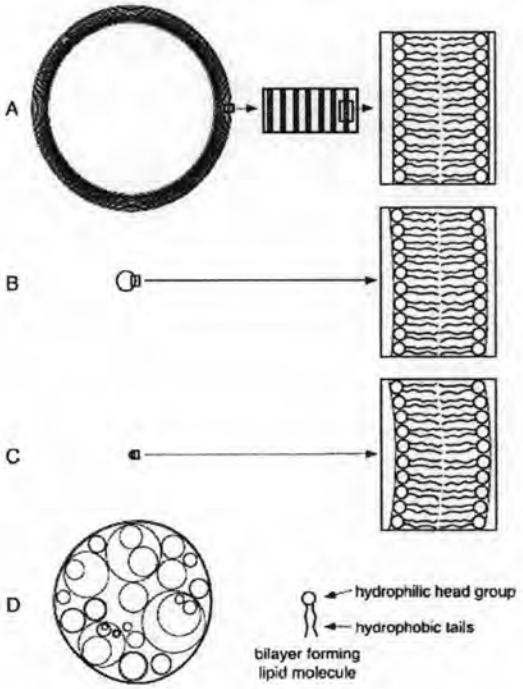

Figure 6 Schematic representation of different types of liposomes: (A) multilamellar vesicles, MLV; (B) large unilamellar vesicles, LUV; (C) small unilamellar vesicles, SUV; (D) multivesicular vesicles, MVV (Walde and Ichikawa, 2001)

3.1 Materials used in the Preparation of Liposomes (New, 1989)

Phospholipids

Phospholipids are the major structural components of biological membranes. Representative structures of phospholipids are depicted in Figure 7. The most common phospholipids are phosphatidylcholine molecules (PC)–amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains, with a hydrophilic polar headgroup (phosphocholine). Molecules of PC are not soluble in aqueous media but they align themselves closely in planar bilayer sheets in order to minimize the unfavorable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets fold on themselves to form closed sealed vesicles.

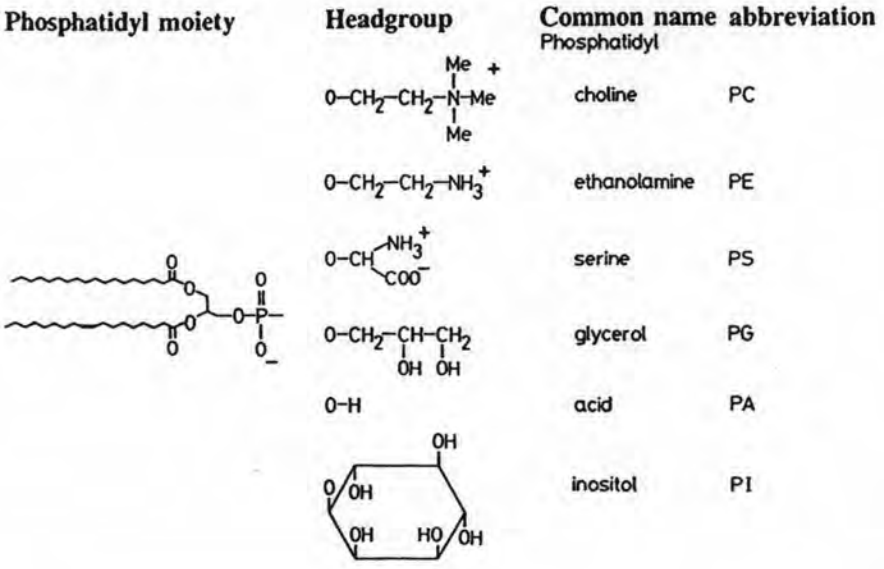

Figure 7 Chemical structure of some common naturally-occurring phosphatidyl phospholipids (New, 1989)

Phosphatidylcholine (leithin) can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean, but less readily from bovine heart and spinal cord. They are often used as the principal phospholipid

in liposomes for a wide range of applications due to their low cost relative to other phospholipids, and their neutral charge and chemical inertness. Lecithin from natural sources is a mixture of phosphatidylcholines with chains of different lengths and various degrees of unsaturation.

At different temperatures, lecithin membranes can exist in different phases, and transitions from one phase to another can be detected by physical techniques as the temperature is increased. The most widely used method for determining the phase transition temperature (T_c) is microcalorimetry. The gel-to-liquid crystalline phase transition temperature (T_c) of the membranes is also called the main lamellar chain-melting phase transition temperature (T_m). A typical DSC trace for a diacylphosphatidylcholine is shown in Figure 8 (Torchilin and Weissig, 2003). At temperature below T_m, fully hydrated, long chain phosphatidylcholines are in the ordered, condensed crystalline subgel (L_c) state, in which the hydrocarbon chains are in the fully extended, all *trans* conformation, and the polar head groups are relatively immobile at the water interface. On heating, L_c state phospholipids undergo a subtransition to the $L_{\beta'}$ state, in which there is increased head group mobility and water penetration into the interfacial region of the bilayer. The subtransition usually occurs approximately 30 °C below the T_m. On heating, $L_{\beta'}$ gel state phospholipids undergo the pretransition to the "rippled" gel ($P_{\beta'}$) state. The pretransition usually occurs between 5-10 °C below the T_m, with a smaller enthalpy, and may be due to rotation of the polar head groups or co-operative movement of the hydrocarbon chains, prior to melting. The pretransition of DMPC and DPPC has been explained in terms of structural changes in the lamellar lattice, with the bilayer reorganizing from a one-dimensional lamellar to a two-dimensional monoclinic lattice consisting of lipid lamellae distorted by periodic "ripples". Heating $P_{\beta'}$ state phospholipids results in co-operative "melting" of the hydrocarbon chains (the main gel to liquid-crystalline phase transition) to give the L_α state. The orientation of the carbon-carbon single bonds changes from *trans* to a situation where *gauche* configurations are present. The intermolecular distance between molecules is approximately 2 nm, consequently rotation in one molecule, impacts on adjacent molecules, such that this transition is a

co-operative event. The membrane passes from a tightly ordered 'gel' or 'solid' phase, to a liquid-crystal phase at raised temperatures where the freedom of movement of individual molecules is higher. In general, increasing the hydrocarbon chain length, or increasing the saturation of the chains increases the transition temperature. The phase behavior of a liposome membrane determines such properties as permeability, fusion, aggregation, and protein binding, all of which can markedly affect the stability of liposomes, and their behavior in biological systems.

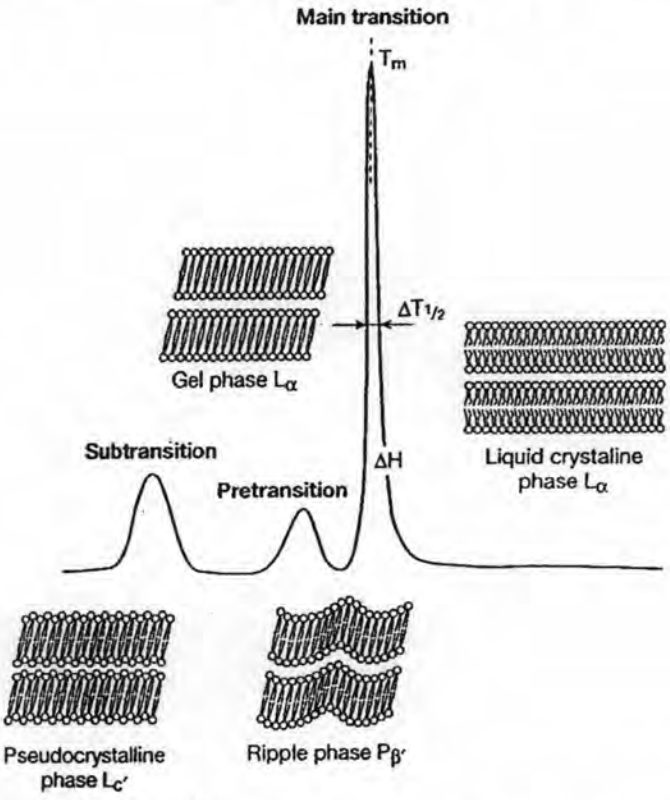

Figure 8 Schematic representation of DSC trace and the lipid arrangement in a planar bilayer below and above the main lamellar chain-melting phase transition temperature (T_m) (Torchilin and Weissig, 2003)

In addition to phosphatidylcholine, neutral lipid bilayers may be composed of sphingomyelin, or alkyl ether lecithin analogues which substitute entirely for lecithin in the membranes. Replacement of ester groupings by ether linkages increases the resistance of such lipids to hydrolysis, while apparently not greatly affecting the physical properties of the membranes. In sphingomyelin, the presence of the amide linkage and hydroxyl groups (in the region corresponding to the glycerol backbone of

lecithin), gives rise to hydrogen bond interactions which may explain the more highly ordered gel phase relative to phosphatidylcholine. The other neutral phospholipid found commonly in natural membrane is phosphatidylethanolamine (PE). Possessing an unsubstituted quaternary ammonium group protonated at neutral pH, this lipid differs from lecithin in two respects: first, its headgroup is smaller than the bulky phosphocholine of lecithin; second, it is able to take part in hydrogen bonding interactions with its neighbors in the membrane.

In negatively charged (acidic) phospholipids (e.g. cardiolipin, phosphatidylserine (PS), and phosphatidylglycerol (PG)), all three possible forces regulating headgroup interaction of bilayer membranes come into play, namely steric hindrance, hydrogen bonding, and electrostatic charge. Membranes composed of acid phospholipids can bind strongly to cations particularly divalent cations such as calcium and magnesium. The reduction in electrostatic charge of the headgroups as a result of binding causes the bilayer to condense, increasing the packing density in the gel phase, and as expected, raising the transition temperature. Thus, at the appropriate ambient temperature, addition of cations can induce a phase change from liquid-crystalline to gel phase.

Cholesterol

Cholesterol is a major component of natural membranes, and its incorporation into liposome bilayers causes major changes in the properties of vesicles. Cholesterol does not, by itself, form bilayer structures, but it can be incorporated into phospholipid membranes at high concentrations. In natural membranes, the molar ratio varies from 0.1-1, depending on the anatomical and cellular location. Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and with the aliphatic chain aligned parallel to the acyl chains in the center of the bilayer (Figure 9). The 3β-hydroxyl group is positioned level with the carboxyl residues of the ester linkages in the phospholipids, with very little vertical freedom of movement. The presence of the rigid steroid nucleus alongside the first ten or so carbons of the phospholipid chain has the effect

of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining carbons towards the terminal end of the chain.

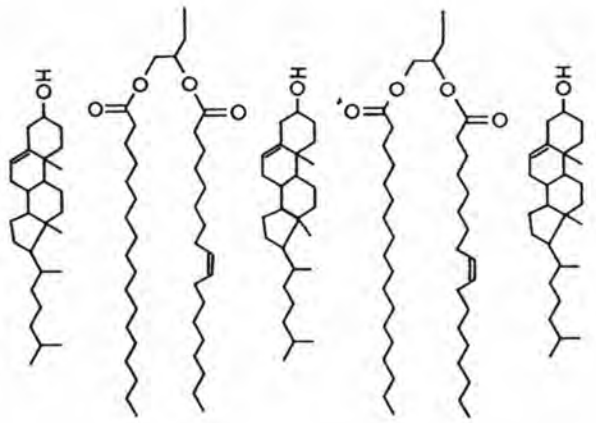

Figure 9 Position occupied by cholesterol in the membrane bilayer (New, 1989)

Other substances

Diacylglyceryl, stearylamine and dicetylphosphate have been incorporated into liposomes in order to impart either a negative or positive surface charge to these structure. In liposomal preparation, a charged species may be added (normally at 5-20 %w/w) to prevent aggregation. Small amounts of antioxidants such as α–tocopherol or BHT are also included when polyunsaturated natural lipids are used.

3.2 Preparation of Liposome by One-step Method (Brandl et al., 1993)

The one-step liposome preparation method is based on using the machine for homogenization not only of preformed liposomes, but also for mixtures of dry lipid(s) and drug solutions. In high pressure homogenization process, solid lipid particles or crystals are broken up and lipids undergo what we call "forced hydration" and self-aggregation into vesicles. Dry, solid lipids mixed with water or hydrophilic solvents can be transformed into homogeneous dispersions of SUVs in one single step, avoiding any adjuvants such as organic solvents or tensides.

3.3 Preparation of Liposome by Dehydration-Rehydration Method (Kirby and Gregoriadis, 1993; Gregoriadis, 1999)

This method is a relatively mild procedure and has been used very often in the entrapment of peptide, protein, and DNA vaccine. The use of MLV as an initial vesicle preparation seems to result in lower encapsulation efficiency. In order to achieve high entrapment yields, drug-free SUV are typically prepared first in distilled water and then mixed with an aqueous solution containing the drug to be entrapped (Figure 10). The thus obtained vesicle suspension is then dried, either by freeze drying or by drying under vacuum without freezing or under a stream of nitrogen at slightly elevated temperatures. Dehydrating preformed vesicles followed by a controlled rehydration above T_m leads to the fusion of small preformed vesicles, resulting in multilamellar vesicles with high entrapment yields. After rehydration, the new vesicles formed—abbreviated as DRV—are considerably larger than the initially present SUV and multilamellar, and now contain to a large extend entrapped protein molecules, depending on the experimental conditions used, such as freezing procedure, initial vesicle properties, lipid composition and aqueous solution used.

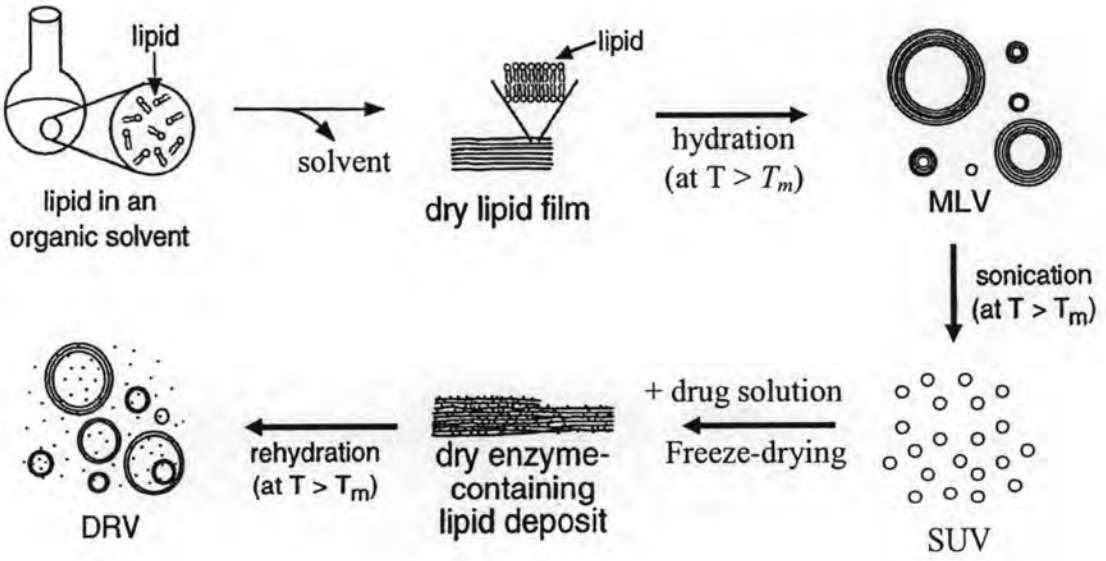

Figure 10 Schematic of dehydration-rehydration method (Adapted from Walde and Ichikawa, 2001)

3.4 Factors Affecting Drug Entrapment in Liposomes

To obtain optimum encapsulation of a drug into liposomes, parameters influencing both the liposome and the drug need to be carefully considered during the preformulation stage. Most of the factors studied so far are as follows:

3.4.1 Types of liposomes

MLVs are suitable for the encapsulation of bilayer-interacting hydrophobic drugs, and less appropriate for hydrophilic drugs because they have a low aqueous encapsulation volume. LUVs are more suitable for hydrophilic drugs due to their large entrapped aqueous volume, while SUVs have very low encapsulation efficiency for these drugs because of the low encapsulation volume (Stamp and Juliano, 1979; Szoka and Papahadjopoulos, 1980). However, entrapment of highly hydrophobic drugs is affected to a less extent by vesicle type and size since they remain entrapped within the phospholipids bilayers. Thus, for hydrophilic drugs, encapsulation appears to increase in the order of MLVs < SUVs < LUVs.

3.4.2 Phospholipid selection

The choice of phospholipid is often limited to the family of the phosphatidylcholine (PC) and phosphatidylglyerol (PG) because of the availability and the cost. Increasing the alkyl chain length of phospholipid increases partitioning of hydrophobic drugs into the bilayers. The partitioning is also influenced by the fluidity of the membrane structures. An increase in temperature improves fluidity of the bilayers and thus improves drug encapsulation (Ma et al., 1991a, b). Encapsulation of a peptide drug is more entrapped in HPC liposomes than in egg PC liposomes due to the difference in fluidity state of the bilayer (Weiner, 1989).

3.4.3 Presence of charge

The presence of negatively charged lipids such as PS, phosphatidic acid and PG or positively charged lipids such as stearylamine tends to increase the interlamellar distance between successive bilayers due to charge repulsion in the MLV structure and thus lead to a greater overall entrapped volume. The effect of charge on entrapment efficiency depends on the characteristics of the drug being entrapped.

3.4.4 Effect of bilayer rigidity

The fluidity of a lipid bilayer, composed of a single species, depends on its lipid chain melting transition, T_m, relative to ambient or physiological temperature (Ulrich, 2002). The T_m depends on the length and saturation of the fatty acid chains and can vary from -20 °C to 90 °C. The lipids exist in different physical states above and below this temperature. Below the T_m, the lipids are in a rigid, well-ordered arrangement (gel phase), and above the T_m in a liquid-crystalline state (fluid phase). The presence of high T_m lipids (T_m > 37 °C) makes the liposome bilayer less fluid at the physiological temperature and thus, less leaky (Labiris and Dolovich, 2003b). Incorporation of cholesterol into the lipid bilayers has an influence on the onset position of the main transition temperature, rigidity and permeability of liposomes. Increasing cholesterol content at 50 mol% (1:1 ratio) is able to eliminate evidence of a phase transition and reduce the enthalpy of phase change to zero, and alter the fluidity of the membrane both below and above the T_c. Below this temperature, the phospholipids are pushed apart, the packing of the headgroups is weakened, and the fluidity of the ordered gel phase is increased. Above the transition temperature, the reduction of freedom of acyl chains causes the membranes to "condense", with a reduction in area, closer packing and a decrease in fluidity. These changes in fluidity are paralleled by changes in permeability of the membrane–decreased by high cholesterol at temperature higher than the T_c, but increased at lower temperatures.

3.4.5 Preparation methods

Method of preparation of liposomes can also affect drug location and overall entrapment efficiency (Weiner, Martin, and Mohammad, 1989). The cast film method is simple but the major drawback with MLV prepared is relatively low encapsulation in terms of aqueous space per mole of lipid. The reverse phase evaporation method gives high entrapment of water-soluble drugs but macromolecules may get denatured by organic solvent. The dehydration-rehydration method improves entrapment of macromolecules into liposomes (Gregoriadis et al., 1999; Walde and Ichikawa, 2001). Rehydration of a lipid film with the protein solution followed by freeze-thawing and extrusion is the most commonly used technique for preparation of protein-loaded liposomes (Colletier et al., 2002). However, this method may not be the best method for all proteins. Newer methods for encapsulation of proteins and peptides, involving injection techniques, allow pilot scale batches to be produced efficiently (Wagner et al., 2002). Care must be taken when using size reduction techniques (e.g. sonication, homogenization, extrusion), so that the stability of the protein/peptide is not compromised.

3.4.6 Physicochemical properties of drugs

Liposomes are capable of encapsulating a variety of drugs with a wide range of lipophilicities. Lipophilic drugs intercalate in the lipid bilayers (Figure 11), while others interact with the bilayer interface. Hydrophilic drugs partition in the aqueous spaces. This depends on the encapsulated aqueous volume. The internal or trapped volume and encapsulation efficiency greatly depends on liposomal composition, lipid concentration, method of preparation and drug used (Weiner et al., 1989). Drugs with intermediate solubility are poorly retained in liposomes. Efficient capture will depend on the use of drugs at concentrations which do not exceed the saturation limit of the drug in the aqueous compartment (for polar drugs) or the lipid bilayers (for non polar drugs) (Weiner et al., 1989). However, when the drugs are weak acids or weak bases, the pH and chemical composition of the internal aqueous

compartments can be manipulated so that the drug concentrates in the interior of the liposomes, resulting in a high degree of retention.

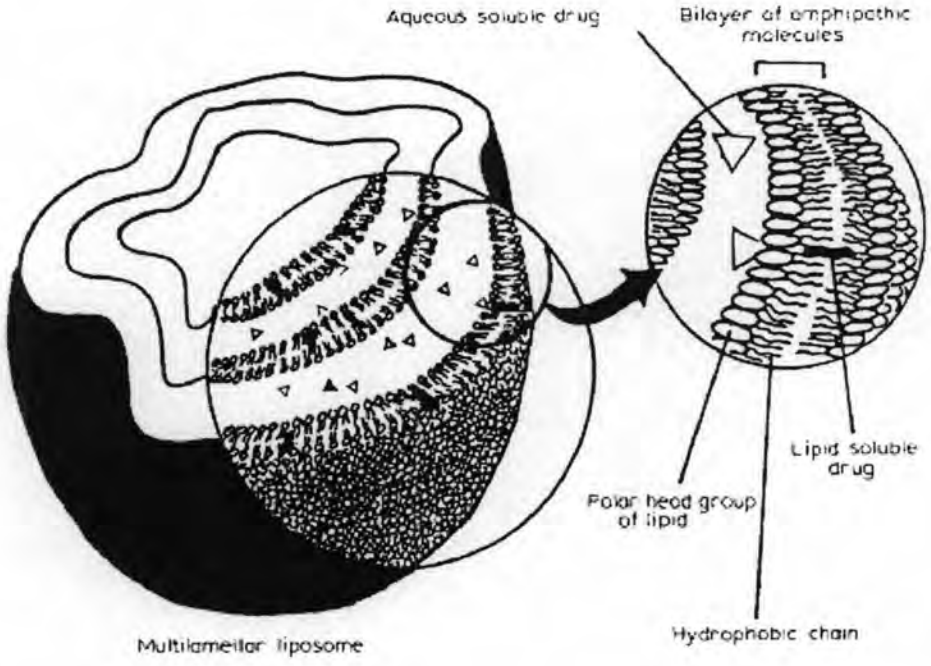

Figure 11 Schematic representation of a multilamellar vescilular structure in cut-way and magnified views (Weiner, 1989)

4. Liposomes for Pulmonary Drug Delivery

Phosphatidylcholine is the only excipient currently approved by the FDA for lung delivery. Liposomes have been studied in pulmonary delivery of drugs for years and used as a means of delivering phospholipids to the alveolar surface for treatment of neonatal respiratory distress syndrome (Labiris and Dolovich, 2003b). Since they are produced with natural and biodegradable compounds and also provide protection to the encapsulated material. The application of liposomes has been suggested for sustained release of several drugs and their interaction with the endogenous phospholipids was proposed as a contribution to the prolonged retention of peptides within the lung (Shahiwala and Misra, 2005). In addition, liposomes can enhance

absorption of proteins and reduce side effects (Liu et al., 1993; McAllister et al., 1996; Mitra et al., 2001).

4.1 Factors Affecting Pulmonary Delivery of Liposomes

The drug carrying capacity, release rate, deposition and in vivo behavior of liposomes in the lungs are dependent on the lipid composition, size, charge, drug/lipid ratio, and method of delivery (Zeng et al., 1995; Labiris and Dolovich, 2003b; Cryan, 2005). Liposome composition can be altered to enhance transport across the epithelium for systemic delivery, to improve drug retention within the lung, or to delay release. Three major factors must be taken into consideration: (1) the interaction of the protein with the lipids, (2) the interaction of the formulation with the lungs, and (3) the efficiency of delivery from a given device. The vesicle size and number of bilayers are critical parameters in determining the circulating half-life. The preferred size range for clinical applications has been suggested to be 50-200 nm in diameter. Bilayer fluidity also affects the interaction of liposomes with macrophages, with high T_m lipids having a lower uptake (Sharma and Sharma, 1997). The type and density of charge on the liposome surface are also important parameters (Labiris and Dolovich, 2003b). A negative charge increases liposome-cell interactions and charged liposomes may be cleared faster than neutral liposomes. Cationic liposomes deliver their contents to cells by fusion with the cell membrane.

Table 3 summarizes anatomic/functional deposition and clearance patterns of inhaled liposome aerosols (Schreier et al., 1993). One of the major problems concerning the pulmonary administration of particulate systems is the rapid capture by the alveolar macrophages, a process known to be affected by several factors such as particle size, surface properties, composition and local concentration (Rudt and Muller, 1992; Ahsan et al., 2002; Makino et al., 2003). However, this problem of liposomes may be taken advantages for targeting specific cells to treat respiratory intracellular infections or immunization purposes by their ability to deliver entrapped antigens into selected immune cells and to stimulate an immune response (Frezard, 1999; Gregoriadis, 2003). Liposomes offer a number of advantages as carriers of vaccines in that they are biodegradable and nontoxic, can elicit both human immunity

and cell-mediated immunity (Kersten and Crommelin, 1995), and can be prepared entirely synthetically. Furthermore, they are highly versatile in their structural characteristics, which allow for the precise manipulation of their immunoadjuvant properties. Figure 12 illustrates the current presumed mode of action of liposomes as immunological adjuvants (Frezard, 1999).

Table 3 Anatomic/functional deposition and clearance patterns of inhaled liposome aerosols (Schreier et al., 1993)

Anatomy	Deposition mechanism (s)	Droplet size (μm)	Affecting factor (s)	Clearance mechanism (s)
Naso/oro-pharynx	Inertial impaction	< 100	Anatomic obstruction Secretions Edema	Swallowing Expectoration Mucociliary
Tracheo-bronchial	Inertial impaction Sedimentation Diffusion	< 40	Bronchospasm Anatomic obstruction Mucus hypersecretion Respiratory pattern (depth/rate)	Cough Mucociliary Bronchial lymphatics via macrophages
Pulmonary (alveolar)	Sedimentation Diffusion	< 5	Respiratory pattern Fibrosis Atelectasis	Endocytosis Surfactant incorporation Lymphatics via macrophages

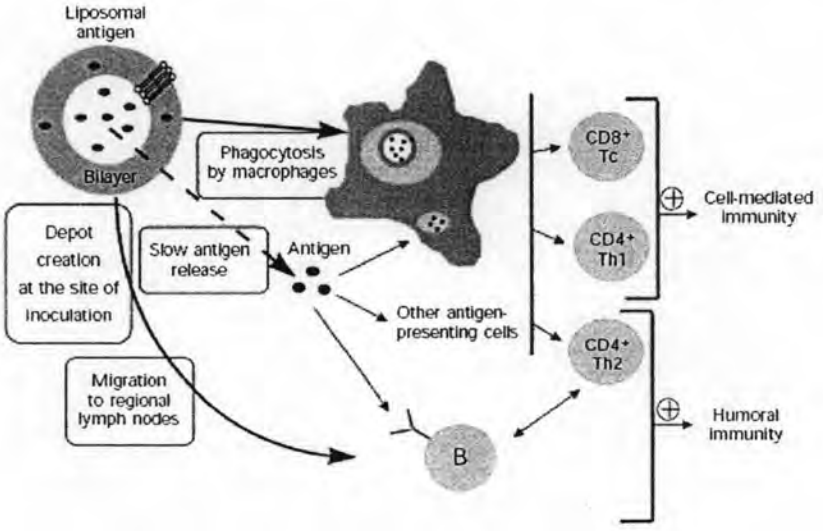

Figure 12 Schematic representation of the mechanisms proposed for the immunoadjuvant action of liposomes (Frezard, 1999)

4.2 Formulation of Liposomal Dry Powders

Liposomes may be prepared for inhalation in liquid or dry powder form. Drug release can occur during nebulization, but manipulation of lipid composition, size, and operating conditions can minimize this loss (Niven and Schreier, 1990; Desai, Hancock, and Finlay, 2002). However, dry powders have been considered because of the stability of drugs and formulations (Prime et al., 1997).

In liposomal DPI formulations, drug encapsulated liposomes are homogenized, dispersed into carrier and converted into DPI by spray and / or freeze drying (Shahiwala and Misra, 2005; Chougule et al., 2007). On inhalation, drug encapsulated liposomes get rehydrated in lung and release drug over a period of time. Proliposome powders for inhalation have been produced by freeze-drying using tertiary butanol as a solvent (Bystrom and Nilsson, 1996). They compose of a single phase discrete particles of a biologically active component together with a lipid or mixture of lipids having a liquid phase transition temperature of less than or equal to 37 °C on hydration and that of greater than 57 °C in dry form. On inhalation, the drug spontaneously encapsulates into lipid inside lungs. The disclosed formulation is useful in treatment of anthrax infection on inhalation (Weers et al., 2005).

A disclosure of phospholipid based powders for rapid absorption of the delivered active agent has been made. Emitted dose and lung deposition of the DPI formulation are claimed to be independent of device resistance and inspiratory flow rates (Mezel and Hung, 2004; Tarara et al., 2004). A novel lipid particle formulation for delivery of steroids into deep lung claims prolonged release of the drug, improved therapeutic ratio, lower toxicity, reduced systemic side effects, and stability for several months. The formulation is in particular suitable for treatment of interstitial lung diseases (Radhakrishnan, 1991).

Dry powder liposomes have been produced by lyophilization followed by milling (Joshi and Misra, 2001) or by spray-drying (Skalko-Basnet et al., 2000; Kim and Kim, 2001). Because of liposomal powders as drug carriers for inhalation therapies, the lyophilized liposomal powders should be micronized to particles of ~1-

6 μm in diameter for efficient delivery to the lung. This micronization has normally been achieved by jet-milling. Jet-milling leads to high amounts of leakage of the encapsulated drug because of high-energy collision during milling, leading to perturbation of the liposome structure (Mobley, 1998; Desai, Wong, et al., 2002). Among recent developments techniques of DPIs, the spray drying technique offers a number of potential advantages over freeze drying technique (Bosquillon et al., 2004; Lo et al., 2004). Spray drying technique is utilized for development of homogeneous particle size powder for inhalation to overcome problems associated with lyophylization technique such as formation of hard cake, need of micronization and heterogeneous size distribution. In addition, spray-freeze-drying technique has been utilized to manufacture a liposomal powder formulation which forms liposomally encapsulated ciprofloxacin when wetted (Sweeney et al., 2005).

5. Spray Drying Technique

Spray drying is the method the most widely used for drying of bio-molecules, such as proteins, peptides and enzymes for inhalation (Okamoto et al., 2002). Spray drying also offers some advantages to other drying methods; particle size of the powder is controlled in a single step, and the morphology and the density of the particles can be controlled (Broadhead, Rouan, and Rhodes, 1992; Louey, Oort, and Hickey, 2004; Elversson and Millqvist-Fureby, 2005).

Spray drying converts a liquid into a powder in a one-step process. The spray-drying process involved four stages: atomization of the product into a spray nozzle, spray-air contact, drying of the spray droplets and separation of the dried product from the drying air (Figure 13) (Masters, 1991; Broadhead et al., 1992). For two-fluid nozzles, the feed fluid and atomizing air are passed separated to the nozzle where they mix and the air causes the feed to break up into a spray. Droplet drying (water evaporation) starts instantaneously after atomization.

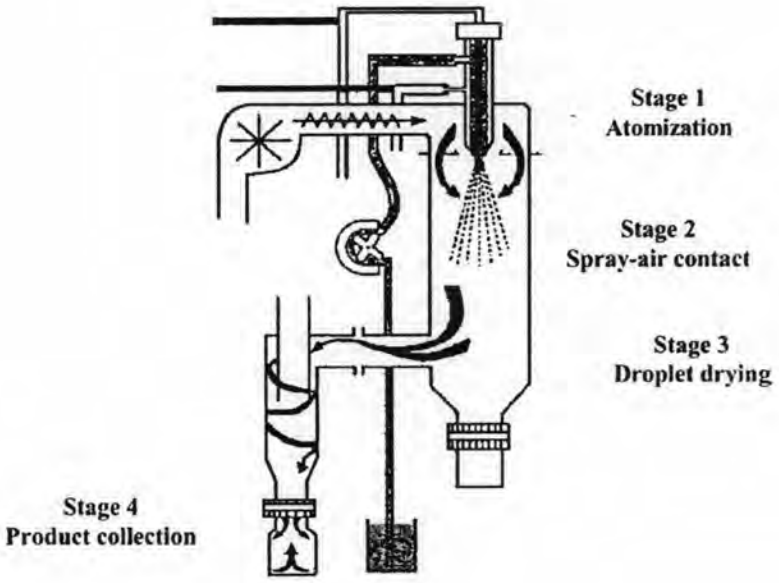

Figure 13 The main stages in spray-drying processing (Alves et al., 2004)

The independent variables of spray drying processes are liquid feed rate, atomizing air flow rate, drying air flow rate, and inlet air temperature. Outlet temperature linearly depends on each of these variables, suggesting that it can be estimated if the regression lines between outlet temperature and the independent variables are available for a spray drier. It is likely that proteins, such as insulin and β-galactosidase, are susceptible to degradation upon spray drying due to the relatively high temperatures (Broadhead et al., 1994; Costantino et al., 1998; Stahl et al., 2002). As the feed droplets come into contact with the hot air, the liquid begins to evaporate. A skin forms at the surface of the droplets and as it thickens, it restricts further moisture evaporation. At this time (the falling drying rate period) particles form, then their temperature begins to rise and thus they become more heat sensitive (Broadhead et al., 1992). Stabilizer (e.g. sugars and amino acids) is required as a good water-replacing agent for the protein formulation. Surface denaturation at the air-liquid interface of sprayed droplets may play a significant role in protein degradation because atomization generates fine droplets with an extremely high specific surface area (Mumenthaler, Hsu, and Pearlman, 1994; Maa and Prestrelski, 2000). These proteins are such as recombinant human growth hormone (rhGH), bovine serum albumin and lactate dehydrogenase. The structure of most proteins is more or less

amphiphilic. These protein molecules tend to be adsorbed to the air-water interface where the unusual surface energy might cause the protein molecule to unfold and to expose its hydrophobic regions. The unfolded molecules then undergo aggregation by the interaction of their hydrophobic region until precipitation. Adding polysorbate-20 into the liquid feed significantly reduces the formation of insoluble rhGH aggregates, and adding divalent metal zinc ions effectively suppresses the formation of soluble rhGH aggregates.

Spray drying process parameters play a vital role on properties of powders obtained such as yield, particle size, shape, topographical features, density, moisture content and drug retention (Elversson, Millqvist-Fureby, and Alderborn, 2003). Regarding the production of particles in the inhalable size range, the aerodynamic diameter of the particles must be less than 5 μm which makes the droplet formation important as well as the choice of the proper feed concentration, feed flow rate, feed temperature and atomization pressure (Masters, 1991).

6. Preparation of Liposomal Powders by Spray Drying Technique

In general, the dehydration of the phospholipid bilayer is known to result in bilayer fusion (Strauss, Schurtenberger, and Hauser, 1986). The fusion process influences the integrity and the permeability barrier of the lipid bilayer. Then bilayer becomes leaky and as a result, the aqueous contents in liposomes exchange and equilibrate with the aqueous medium surrounding the vesicles. Therefore, preparation of liposomal powders from preformed liposomes was initially concerned about stabilization of phospholipid vesicles during spray drying process using sugars as an additive. Hauser and Strauss (1987) and Hauser (1993) reported that sucrose is the best stabilizer for dehydration of SUV liposomes by spray drying technique. As the effectiveness of different sugars, disaccharides are more potent than monosaccharides and polyalcohols (Crowe et al., 1988). Mechanism of stabilization by sugars was proposed by Crowe et al. (1988) in term "water replacement" hypothesis. Water of hydration around the polar group of phospholipids is replaced by sugar molecules,

which are assumed to be hydrogen bonding. Koster et al. (1994) suggested that sugars depress the gel-to-liquid crystalline transition temperature (T_m) in dry phospholipids to a temperature close to that of the hydrated phospholipid. This might be ascribed to vitrification (glass formation) of the stabilizing solute rather that the direct interaction between saccharide and PC. However, Crowe et al. (1996) and Crowe et al. (1998) suggested that the vitrification is not in itself sufficient to depress the T_m. The sugar-phospholipid polar group interactions apparently expand the intermolecular spacing of the phospholipids because sugar molecules are intercalated between lipid polar groups. As a result, the hydrocarbon chains become more mobile.

The spray drying of liposomes has been reported to good chemical stability of phospholipids after spray drying using lactose as a bulking agent (Goldbach et al., 1993a). However, the leakage of a hydrophilic drug (atropine sulfate) was observed after reconstitution the spray-dried liposomal powders with deionized water although size of the reconstituted liposomes did not change when compared to the initial liposomes (Goldbach et al., 1993b).

Spray-dried liposomal powders were studied for pulmonary delivery of lipophilic drugs such as amiloride hydrochloride (Chougule et al., 2006) and dapsone (Chougule et al., 2008) for controlled and prolonged drug retention at the site of action. These lipophilic drugs could be efficiently retained into liposomes after reconstitution of the spray-dried powders. Most additives used for producing liposomal powders by spray drying are carbohydrates such as sucrose, lactose, trehalose and mannitol (Lo et al., 2004; Lu and Hickey, 2005; Chougule et al., 2006; Grenha et al., 2008). Chougule et al. (2006) reported that the spray-dried liposomal powders with mannitol exhibited the best aerosol powder performance compared to those with lactose and sucrose. Liposomal powder formulation containing DPPC and sucrose can preserve the activity of superoxide dismutase during spray drying process due to surface activity of DPPC and its tendency to adsorb on the droplet surface (Lo et al., 2004). Grenha et al. (2008) proposed the preparation of lipid/chitosan nanoparticles complexes as well as microspheres containing this complexes using mannitol as an excipient by spray drying for pulmonary delivery of insulin. The presence of phospholipids is

determinant in controlling the release of the encapsulated insulin. In addition, phospholipid dry powders for pulmonary application was produced by spray drying process using ethanol as solvent and mannitol as an additive (Alves et al., 2004).

Dispersing agent such as amino acids and water soluble polypeptides may be required in the preparation of spray-dried liposomal powders to improve dispersibility of dry powders (Eljamal et al., 1998). Of all the amino acids tested, leucine appears to show the most promising dispersion enhancing effect for a number of spray-dried pulmonary formulations such as disodium cromoglycate (Najafabadi et al., 2004). Weers et al. (2002) reported that addition of polyvalent cation such as calcium chloride in the spray-dried phospholipid-based powders increases the T_m of particles which improves thermal stability and dispersibility of the powders.

In addition, the presence of povidone (polyvinylpyrrolidone, PVP) in liposomal powders with mannitol gives the highest retained protein activity for lyophilization and storage stability (Lu and Hickey, 2005). Crystallization of saccharides may be inhibited in the presence of povidone due to its ability to increase the T_g of the saccharides, and the larger molecular weight PVP, the stronger prevention of crystallization (Zeng, Martin, and Marriot, 2001).