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POLY (D,L-LACTIDE) BASED MICROSPHERES FOR PULMONARY DELIVERY OF PROTEINS

XIAOSONG SONG

Doctor of Philosophy

ASTON UNIVERSITY

August 2009

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Aston University

Poly (D,L-lactide) based microspheres for pulmonary delivery of proteins

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Within this thesis the potential of polymeric microspheres to deliver proteins via the pulmonary route was investigated. Initial work focused on the preparation, optimisation and characterisation of poly (D,L-lactide) (PLA) microspheres with the aim of optimising their formulation based on minimizing the particle size into the range suitable for pulmonary delivery to alveoli. In order to produce dry powders and to enhance their long-term physico-chemical stability, microspheres were prepared as the dry powder via freeze-drying. Subsequently, a multi-stage liquid impinger was used to evaluate the aerodynamic particle size and aerosolisation performance of the PLA microsphere powder delivered using a dry powder inhaler and nebulisers (air-jet and ultrasonic). Finally, cellular responses of a murine macrophage cell line to the optimised PLA microsphere formulations in cell proliferation, cytotoxicity, phagocytosis activity and cell activation. The immunomodulatory, trehalose dibehenate (TDB), was also added into the formulations to investigate its effect on the physico-chemical characters and cell responses in macrophages.

Optimisation studies showed that using appropriate concentrations of polymer 3% (w/v) in organic phase and emulsifier 10% (w/v) in external aqueous phase, the double solvent evaporation method produced high protein loading microspheres (72 ± 0.5%) with an appropriate particle size for pulmonary drug delivery. Combined use of trehalose and leucine as cryoprotectants (6% and 1% respectively, w/v) produced freeze-dried powders with the best aerosolisation profile among those tested. Although the freeze-dried PLA microspheres powders were not particularly respirable in dry powder inhalation, nebulisation of the rehydrated powders using ultrasonic nebuliser resulted in improved aerosolisation performance compared to the air-jet nebuliser. In terms of cellular responses, when tested in vitro using a macrophage cell line, the PLA microspheres system exhibited a low cytotoxicity and the microspheres induced phagocytic activity in macrophages. However, interestingly, the addition of an immunomodulator to the microsphere formulations (4%, w/w of polymer) reduced this phagocytic activity and macrophage activation compared to microspheres formulated using PLA alone. This suggested that the addition of TDB may not enhance the ability of these microspheres to be used as vaccine delivery systems.

**Keywords:** microsphere; PLA; pulmonary delivery; inhalation; cellular response.
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ACI  Andersen cascade impactor
ANOVA analysis of variance
APC antigen presenting cell
ARDS acute respiratory distress syndrome
BCG Bacille Calmette-Guérin
CFC chlorofluorocabons
COPD chronic obstructive pulmonary disease
DDA dimethylidioctadecyl ammonium
ddH2O double distilled water
DMEM Dulbecco’s Modified Eagle Medium
DNA deoxyribose nucleic acid
DPIs dry powder inhalers
ELISA enzyme-linked immunosorbent assay
FBS foetal bovine serum
FGF-2 fibroblast growth factor
FPF fine particle fraction
GSD geometric standard deviation
INT 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride
ISCOMs immunostimulating complexes
LDH lactate dehydrogenase
MMAD mass median aerodynamic diameter
MSCI multi-stage cascade impactor
MSLI multi-stage liquid impinger
MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADH nicotinamide adenine dinucleotide
NADPH nicotinamide adenine dinucleotide phosphate
NAG β-N-Acetylg glucosaminidase
NP-GlcNAc 4-Nitrophenyl N-acetyl-β-D-glucosaminide
PBS phosphate buffered saline
PDLLA poly (D,L-lactide)
PLA poly (lactide)
PLGA poly (lactide-co-glycolide)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PLLA</td>
<td>poly (L-lactide)</td>
</tr>
<tr>
<td>pMDIs</td>
<td>pressurized metered-dose inhalers</td>
</tr>
<tr>
<td>PMS</td>
<td>phenazine methosulfate</td>
</tr>
<tr>
<td>PSG</td>
<td>penicillin/streptomycin/l-glutamine</td>
</tr>
<tr>
<td>PVA</td>
<td>poly (vinyl alcohol)</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
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<tr>
<td>TB</td>
<td>tuberculosis</td>
</tr>
<tr>
<td>TDB</td>
<td>trehalose 6,6'-dibhenenate</td>
</tr>
<tr>
<td>TDM</td>
<td>trehalose dimycolate</td>
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<tr>
<td>Th1</td>
<td>Type 1 helper</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TSI</td>
<td>twin stage impinger</td>
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<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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<tr>
<td>W/O/W</td>
<td>Water-in-oil-in-water</td>
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Chapter 1

General introduction – pulmonary drug delivery
1.1. The pulmonary route

1.1.1. The respiratory track

The human respiratory track can be classified into two parts, the upper airway and lower airway. The upper airway includes the nasal and oral cavities, starting at mouth and nose and ending up at larynx (Figure 1.1.). The main functions of the upper airway are air conditioning and defending against airborne invaders (Lansley, 1993). The lower airway starts at larynx through the trachea, then divided into two bronchi and goes into the lungs where the bronchi divide into thousands of bronchioles and finally into alveoli. The airway region from larynx down to the terminal bronchioles is also called the conducting airway (Gerrity, 1990).

1.1.2. The lung

Figure 1.1. Human respiratory track (picture from Stocks & Hislop, 2002)
The lungs of a human are located inside chest, and they are made up of lobes, two in the heart side and three in the opposite side (Stocks & Hislop, 2002). They play the key role of respiration and motivating breath. As shown in Figure 1.1., the lung is full of fine bronchioli which end in millions of small air sacs called alveoli through which air exchanges and diffuses out and in of the blood vessels.

1.1.3. Lung diseases

There are many diseases that can limit or reduce the functionality of the lung, many of which are currently only treated symptomatically. Improved drug delivery to the lung may facilitate improved treatment of such diseases. However, to understand their treatment, the nature of the target and its pathology in the diseased state must be considered. Examples of diseases where the pulmonary drug delivery could improve/further improve treatment are discussed below.

1.1.3.1. Asthma

Asthma is a chronic airway disease as recurrent reversible bronchoconstriction or bronchospasm due to potentially permanent airway obstruction, airway hyper-responsiveness, and multicellular inflammation (Jeffrey & Linzer, 2007), affecting more than 300 million people all over the world causing more than 239 thousand deaths in 2002 (Msoli et al., 2004; World Health Organization, 2003). When asthma occurs, the muscles around airways tighten, causing obstruction in airway and less air passes through them into the lung (Figure 1.2.) (Jeffrey & Linzer, 2007).
Many drugs can be administered via the pulmonary route for asthma treatment with different mechanisms (Palmer, 2002): β-2 adrenergic agonists can induce bronchodilation and release bronchoconstriction; glucocorticosteroids were used to treat asthma for their anti-inflammatory action; theophylline and its derivative have actions against both of inflammatory action and bronchospasm; furthermore, leukotriene inhibitors antagonists work as bronchodilators, and also have anti-inflammatory action. The deficiency of vasoactive intestinal peptide (VIP) in airways was reported being involved in the pathogenesis of asthma (Said, 1989), indicating administration of VIP in airways may be an option for the therapy of asthma (Said, 1991). Ohmori et al. (2006) delivered VIP and its derivative into rat lung via dry powder inhalation, results suggested a large occupancy of lung VIP receptors.
1.1.3.2. **Acute respiratory distress syndrome (ARDS)**

ARDS was first mentioned by Ashbaugh et al. (1967), which is a severe lung disease of diffuse inflammation in lung parenchyma that following life-threatening systemic illnesses caused by direct or indirect lung injure due to inhaling smokes or toxic gas (Figure 1.3.) (Wong, 1998; Bellingan & Finney, 2006).

![Illustration removed for copyright restrictions](image)

The therapy of ARDS is usually based on pressure-controlled ventilation, positive end-expiratory press, permissive hypercapnia, body position changes, reduction of the pulmonary oedema, differential lung ventilation, extracorporeal respiratory support and inhalation of nitric oxide (Lewandowski & Falke, 1996).

1.1.3.3. **Chronic obstructive pulmonary disease (COPD)**

COPD is defined as a disease state characterised by slowly progressive development of a not fully reversible airflow limitation (Barnes, 1999), causing dyspnea, lung
failure and death (Sutherland & Cherniack, 2004). The disease may be caused by long-term smoking, but also related to genetic reason (Chappell et al., 2006). It caused 2.7 million deaths in 2002 (World Health Organization, 2003). Common symptoms of COPD include ongoing cough, shortness of breath, wheezing, chest tightness and expectoration (Celli et al., 2004). Inflammatory cells including T-cytotoxic lymphocytes, macrophages and neutrophils in airways and lung parenchyma are involved in COPD development (Figure 1.4.).

Inhaling bronchodilators including anti-cholinergics, β2-agonists and theophylline, with supplementary oxygen is the main therapy method against COPD (Barnes, 2003). In addition, according to the research of Onoue et al. (2004), the cytotoxicity of cigarette smoke extract on rat lung alveolar L2 cells was reduced in presence of VIP, indicating VIP could be beneficial in the treatment of COPD.
1.1.3.4. Lung cancer

Lung cancer is one of the most common cancers in the world leading more than one million deaths per year (Peto et al., 1996; Parkin et al., 2001) with a highest incidence in developed countries including Europe and North-America (Sasco, 2008). It develops over a long period from accumulation of gene alteration in lung cells (Figure 1.5.) (Kaye, 2001). Smoking is the main factor causing lung cancer (Boyle & Maisonneuve, 1995), about 85% in men and 47% in women are related to smoking in 2000 (Parkin et al., 2005), and other factors such as air pollution, second hand smoke exposure, heredity and COPD, also can cause lung cancer (Kaye, 2001).

Figure 1.5. X-ray picture of lung cancer. Accumulation of squamous cell carcinoma in left upper lobe (picture from Petty, 2001)

Lung cancer is usually treated with surgery, but cyclophosphamide (Jassem et al., 1994), adriamycin (Falk et al., 1993), vincristine (Morgan et al., 1987), carboplatin (Edelman, 2002) and mitomycin (Snee, 2001) are the commonly used in chemotherapy for lung cancer. Furthermore, new therapeutic methods were developed in recent years, including vaccines therapy and gene therapy (Hege & Carbone, 2003). Lung cancer vaccines are currently in development, including GM-CSF
gene-modified autologous tumor vaccine (GVAX®) (Salgia et al., 2003), carcinoma associated mucin (MUC1) (Palmer et al., 2001), GD3 anti-idiotypic antibody (BEC2) and melanoma associated gene (mage-3) (Gaugler et al., 1994); Gene therapy for lung cancer includes suicide-gene therapy, immunogene therapy (Hege & Carbone, 2003) and replacement of p53 gene which is the most commonly mutated gene in lung cancer (Swisher & Roth, 2002). Gendicine, the first commercial production of a gene therapy for its recombinant Ad-p53 gene treatment for head and neck squamous cell carcinoma, was approved by China in 2003 (Pearson et al., 2004).

1.1.3.5. Tuberculosis (TB)

TB is a major chronic communicable infectious disease caused by Mycobacterium tuberculosis which was firstly isolated in by Robert Koch in 1882 (Figure 1.6.) (Herzog, 1998), and it causes two million deaths every year with one-third of population infected all over the world (Kirby et al., 2008). Rifampin, isoniazid, pyrazinamide, streptomycin and fluoroquinolones are considered as effective drugs for TB therapy (Sacks et al., 2001), and combined chemotherapy of three or four drugs is a promising method to control TB based on bacillary load, drug resistance and bacillary sub populations (Singh, 2006).

Recent researches of TB therapy focus on delivering drugs directly into the lung: O’Hara and Hickey (2000) prepared and characterised rifampicin-loaded PLGA microspheres for pulmonary delivery, resulting physico-chemical properties suitable
for inhalation; Tsapis et al. (2003) used large porous particles entrapping para-aminosalicylic acid and administrated to male Sprague Dawley rats via pulmonary route, resulting a maximum plasma concentration of 11μg/ml in 15 minutes and 148 μg/ml in lung fluid at same time; Pandey and Khuller (2005) demonstrated that using inhalable solid lipid particles as carriers to delivery anti-tuberculosis drugs could improve drug bioavailability and reduce the dosing frequency; Hwang et al. (2008) evaluated the hyaluronan microspheres containing ofloxacin in vitro, indicating that delivering this formulation via pulmonary route would improve the therapeutic efficacy of ofloxacin against tuberculosis.

Figure 1.6. X-ray picture of human lungs infected by tuberculosis. Arrow indicates a calcified granuloma (picture from Lighter & Rigaud, 2009)

TB infection can be limited by vaccination. Mycobacterium bovis bacillus Calmette-Guérin (BCG) vaccine is the only current vaccines against TB, but the efficacy of it is very variable (Bramwell & Perrie, 2005; Wu et al., 2007). Recent research on tuberculosis vaccines based on DNA, sub-unit protein/peptide, and living attenuated vaccines were developed rapidly (Gupta et al., 2007). Findings from
Bivas-Benita et al. (2003) indicate pulmonary delivery of chitosan nanoparticles containing DNA vaccines against tuberculosis might improve immunization compared to intramuscular administration; Kirby et al. (2008) proved that PLGA (75:25) microspheres offered potential for delivery Ag85B-ESAT-6, a novel TB protein sub-unit vaccines against TB.

Therefore it appear that the lung remains a major challenge in terms of drug delivery for both small organic drugs and biopharmaceutics such as proteins and nucleotides.

1.2. Drug delivery to the lung

1.2.1. Brief history of inhalation

The first use of inhalation drug treatment can be tracked back thousands of years ago in smoking plant leaves containing atropine for the therapy of throat and chest diseases (Muthu, 1922; Gandevia, 1975; Labiris & Dolovich, 2003a). However the first application of modern medicine in pulmonary delivery can be dated back to 1802 when the Indian use of datura was introduced into England, and it was used for relief of asthma by smoking in a pipe or in a mixture with tobacco (Gandevia, 1975; Smith & Bernstein, 1996). Nebulisers have been used to produce liquid droplets in pulmonary therapy since 19th century, but they were driven by steam until the 1930s when compressed gas was used in nebulisation (Muers, 1997) and ultrasonic nebulisers were invented in the 1960s (Mercer, 1981).
Antiasthma therapy was developed with the invention of the metered-dose inhaler in the 1950s (O’Callaghan et al., 2002). The first pressurized metered-dose inhaler (pMDI) was in 1956, to delivery bronchodilators with multi doses (Newman, 2005). Due to inconvenient use caused by the poor mouth-hand coordination of patients, especially children and the elderly (Hickey & Dunbar, 1997; Podczeck, 1998), and the environmentally damaging propellants (Daniher & Zhu, 2008), dry powder inhalers were subsequently developed.

In addition to the topical effect of drug delivery to the lung the inhalation studies also focus on delivering drugs for the therapy of systemic diseases, such like diabetic (Huang & Wang, 2006) due to the high systemic absorption achievable via pulmonary drug delivery as most commonly exploited in nicotine delivery via smoking.

1.2.2. Advantages of pulmonary delivery

The lung is an ideal and attractive target for drug delivery and inhaled administration for the treatment of local disease, including asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (Daniher & Zhu, 2008). Drugs can get direct access to the disease site at a high concentration by using pulmonary drug delivery mechanisms and clinical responses can be immediate (Labiris & Dolovich, 2003; Daniher & Zhu, 2008). Pulmonary delivery also minimizes the required dose and systemic side effects (Sung et al., 2007) since no “first-pass” metabolism as occurs in the gastrointestinal track, and because of the low metabolizing enzyme environment in
the airways (El-Baseir & Kellaway, 1998). Furthermore, the slow mucociliary clearance mechanism in the lung periphery can prolong the residency of drug in the lung (Dolovich, 1997).

Pulmonary delivery is also a possible route for the therapy of systematic diseases and vaccine delivery. First of all, unlike invasive muscular or intravenous injection delivery system, it is needle free (Sung et al., 2007), and does not bring pain or anxiety to patients. It has the same therapeutic bioequivalence as an injection, which may be accounted by limited proteolytic activity, due to the existence of “antiproteases” contained in the lung fluid, which prevent proteins from enzymatic break down in the lung (Patton et al., 2004a). Most macromolecules and proteins are not suitable for oral delivery because they are digested in stomach and intestine. However, there is no such problem with pulmonary delivery (Wolff, 1998), and more than 10 times greater bioavailability was found compared to any of the other non-invasive delivery methods (Patton et al., 2004).

As a delivery site, the alveoli in adult human lung also provides a tremendous absorptive surface of about 70-140m² (Gronenberg et al., 2003). The lung has high solute permeability because alveoli have very thin epithelial cell walls of only 0.2 µm rather than about 50 µm in tracheal epithelium (Patton et al., 1996 & 2004), thus drugs that reach alveoli can be absorbed rapidly, Furthermore, therapeutic efficacy is not affected by diet which may affects the gastrointestinal absorption (Byron & Patton,
1994). Immunization by the pulmonary route can be induced by the airborne pathogens, thus delivering vaccines to the lung is a good way for getting systemic immune response.

1.2.3. Particulate pulmonary delivery systems

1.2.3.1. Liposomes

Liposomes are small vesicles similar in structure to cell membranes, with lipid bilayers surrounding an aqueous cavity formed by dispersing phospholipids, into aqueous liquid. The bilayer structure of liposomes is formed by combining between lipophilic hydrocarbon tails, repelled by water, of the two layers. Liposomes were firstly described by Bangham et al. (1965) and were used to study the biological membranes in 1970s (Kinsky & Nicolotti, 1977), and then the studies in liposomes were mainly focused on drug delivery. They are used widely to delivery peptides, proteins (Parmar et al., 1999) and DNA (Perrie et al., 2002 & 2003). It has been reported that liposomes offer immunological adjuvancy as the carrier of diphtheria toxoid (Allison & Gregoriadis, 1974).
Liposomes provide uniform deposition when delivered to the lung (Gilbert et al., 1991; Parthasarathy et al., 1999), prolonging the drug retention time and reducing the toxicity of drugs (Weinstein & Leserman, 1984). Drugs released sustainably from liposomes can be maintained at a therapeutic level in blood stream (Hung et al., 1995; Cabanes et al., 1998). The mechanism of sustained release from liposomes is not clear, but a popular hypothesis is that the structure of liposomes can be destabilised due to many factors, because the drug is absorbed in a free form (Yaoar & Bas, 2004). Another explanation is drugs release slowly because liposomes remain in the structure of tissue, forming a compressed depot (Kadir et al., 1992). The pulmonary delivery of liposomes is usually via dry powder inhalation and liquid nebulisation (Zaru et al., 2007), but nebulisation may cause structural damage of liposomes (Cook et al., 2005).
1.2.3.2. Niosomes

Niosomes are non-ionic surfactant based vesicles made up of lipids with bilayered structures (Carafa et al., 1998), and can be used for entrapping hydrophilic drugs either in an aqueous layer and serve as drug delivery carriers (Uchegbu & Vyas, 1998). Niosomes have all the advantages of liposomes but a particular class of amphiphile and aqueous media are needed to assemble the close bilayer structure (Uchegbu & Vyas, 1998; Kaur et al., 2004). It has been reported that niosomes were effective carriers to delivery vaccines (Bramwell & Perrie, 2005), DNA (Perrie et al. 2004) and sub-unit (Baillie et al., 2005). The records of niosomes for pulmonary delivery are limited, but Abd-Elbary et al. (2008) evaluated nebulised sucrose stearate-based proniosome-derived niosomes using a twin-stage impinger, and successfully got a fraction deposited in stage 2 over 60%, which indicated niosomes are potential carrier for pulmonary delivery.

Figure 1.8. Schematic illustration of a niosome
1.2.3.3. Immunostimulating complexes (ISCOMs)

ISCOMs are composed of Quil A, the subunit of Quillaja saponins which have adjuvant properties (Kensil, 1996; Bramwell & Perrie, 2005; Csaba et al., 2009), with a spherical cage-like structure about 40nm in size (Ozel et al., 1989; Bramwell & Perrie, 2005). ISCOMs used as carriers especially for vaccine delivery for their adjuvant properties, they can mediate an accelerated antibody response and increase the expression of major histocompatibility complex (MHC) class II in antigen presenting cells (APCs) that present antigen fragments to T-helper cells by binding to the CD4+ receptors on the T-helper cells (Watson et al., 1992). ISCOMs mainly used for mucosal immunity via nasal route (Csaba et al., 2009), and there is no literature record of direct pulmonary delivery of ISCOMs.

1.2.3.4. Emulsions and microemulsions

Emulsions are heterogeneous dispersed multiphase systems consisting of one liquid dispersed in another in the form of droplets (Schubert & Armbruster, 1992). The emulsion droplets are in a diameter range of 0.1 to 100μm depending on emulsification process, and emulsions are not a thermodynamically stable system (Van der Graaf et al., 2005). The high interfacial tension between emulsion droplets and continuous phase lead a trend that decreasing total interface area which resulting collapse on droplets. To enhance the stability of emulsions, emulsifiers are strongly recommended, including ionic, non-ionic and zwitterionic surfactants, proteins and amphiphilic polymers (Somasundaran et al., 2006).
Different from emulsion, microemulsions are optically isotropic and thermodynamically stable systems in the nanometer diameter range (Stray, 1994; Schwuger & Stickdorn, 1995; Lawrence & Rees, 2000). Microemulsions are widely used in drug delivery systems, not only via the transdermal (Delgado-Charro et al., 1997), ocular (Haße & Keipert, 1997) and intravenous (Von Corswant et al., 1998) routes, but also via pulmonary route - for example, Sommerville et al. (2000) used lecithin inverse microemulsions to deliver polar compounds using a metered-dose inhaler, resulting 36% fine fraction determined by a twin impinger.

1.2.3.5. Microspheres

Microspheres are defined as solid and spherical particles in a range from 1 to 1000μm in diameter (El-Baseir et al., 1997). It has been shown that poly(lactic-co-glycolic acid) (PLGA) microspheres had considerable potential application for the delivery of peptides (Newman et al., 1998), proteins (Ma et al., 1998) and DNA (Wang et al., 1999; Mohamed & van der Walle, 2006). Microspheres can protect proteins and antigens form from chemical and enzymatic degradation (Hanes et al., 1997).

Previous studies showed microspheres prepared using different techniques e.g. solvent evaporation (Puapernpoonssiri et al, 2009) and spray drying (Seville et al, 2007) offer strong potential as pulmonary delivery systems and these will be discussed in more detail in section 1.3.
1.2.4. Mechanisms of particle deposition in respiratory tract.

The main mechanisms of deposition of particles in the respiratory track can be divided into inertial impaction, sedimentation and diffusion (Figure 1.7a.) (Gerrity, 1990; Thompson, 1998).

1.2.4.1. Inertial impaction

Inertial impaction usually occurs in the upper airway and conducting airway, trachea, bronchi and bronchioles (Gupta & Hickey, 1991; Timsina et al., 1994). The velocity and direction of the particles moving in the airway are changing by the airflow, so the particles with sufficient mass are likely to impact on the inner airway wall when they meet a bifurcation due to their inertia (Figure 1.9.) (Morén, 1987).

![Figure 1.9. Mechanism of inertial impaction deposition in the respiratory tract.](image)

1.2.4.2. Sedimentation

Sedimentation occurs in the lower airway including alveolar region with the particles moving through the airways falling on the inner airway wall due to the gravitational effect (Figure 1.10.) (Timsina et al., 1994).
1.2.4.3. Diffusion

Diffusion is based on Brownian motion, and influences particles in a ultrafine size $<0.5\ \mu m$ due to the collision with gas molecules in the air stream. Every collision changes the direction of motion and as a result the particles show an irregular and unoriented movement and cause contact with inner airway wall (Figure 1.11.) (Scheuch et al., 2006). Diffusion may happen anywhere in the whole respiratory track.

There are also some other mechanisms of particle deposition including interception and electrostatic interactions (Gerrity, 1990). Interception may happen anywhere in the respiratory tract when the particle size of the particles is large enough contact onto the surface of the inner walls of the airway during the movement (Balashazy et al., 1990). Electrostatic interactions, mostly happen in the upper airway, and are caused by
the attraction of charged particles to a surface of opposite charge (Gerrity, 1990).

1.2.5. Factors effect on particle deposition in the lung

The deposition and the distribution of particles in the lung are mostly effected by aerosol particle size, lung clearance, and lung disease (Labiris & Dolovich, 2003).

1.2.5.1. Particle size

Particle size is the one of the key factors affecting lung deposition (Dolovich & Newhouse, 2003). Particles in different size range are affected by different deposition mechanisms: inertial impaction occurs to the particles larger than 3 μm, while sedimentation may happen if particle size was in the range of 0.5 to 3 μm, particles smaller than 0.5 μm are influenced by Brownian motion and will diffuse in the airway (Scheuch et al., 2006). In general, most particles >10 μm are deposited in the oropharyngeal region and subsequently swallowed (Labiris & Dolovich, 2003), whilst particles with a mass median aerodynamic diameter of 5 to 10 μm are mainly deposited in at larynx and in conducting airways (Gerrity, 1990). Particles <5 μm can enter small airways and alveoli, and if the aerodynamic diameter smaller than 3 μm, a large amount particles (50-60% in many cases) can reach the alveoli (Laube et al., 1998).

1.2.5.2. Airway geometry

When moving through the respiratory track, particles have momentum due to their
mass and velocity (Morèn, 1987). They tend to retain their direction of movement due to the inertia. The chances of particles impacting onto the inner airway wall may be increased due to the complex branching structure of respiratory track, including bifurcations and small airway radius (Folkeson, 1990).

1.2.5.3. Humidity

The relative humidity of the air in the human respiratory tracks is about 99.5% (Ferron et al., 1988; Dua et al., 1995). Entering such humid condition from a low relative humid environment, the diameters of hygroscopic particles can increase dramatically (Li et al., 1992; Dua et al., 1995), which affects the particles deposition in the airways. Reponen et al. (1996) demonstrated that the aerodynamic diameter of cladosporium cladosporioids was increased from 1.8 to 2.3 µm as the relative humidity increased from 30 to 90%.

1.2.5.4. Lung clearance

The inner airway wall trachea and bronchi consist of airway ciliated epithelium with hair-like structures on the surface, over which is a mucus layer secreted by submucosal glands and epithelial goblet cells (Sleigh et al., 1988; Sleigh, 1990). Particles deposited in the conducting airways can be removed from the respiratory track by cough or trapped in the mucus and moved upward to the pharynx or if not removed may be absorbed through the epithelium and go into blood or lymphatic system, (Labiris & Dolovich, 2003). Particles deposited in the alveoli may be
phagocytosed by alveolar macrophages, subsequently removed by the lymphatic system or by the mucociliary clearance system (Folkesson et al., 1996).

1.2.5.5. Lung deseases

As already discussed there are a range of debilitating pathophysiological conditions which can affect the lung and particulate deposition. Distribution in the lung can be affected by pulmonary diseases that cause lung function abnormally, such as asthma, cystic fibrosis and bronchiectasis. These diseases can change the bend angle of bifurcations, narrow airways or block airways by accumulating mucus, modifying the deposition of aerosol deposition and distribution patterns (Labiris & Dolovich, 2003).

1.3. Microspheres for pulmonary delivery system

As noted previously in terms of the various drug delivery systems available, microspheres offer strong potential as pulmonary drug delivery carriers. Their deposition in the lung periphery may provide a prolonged drug release and they can be delivered to different part of the lung by control the particle size (Labiris & Dolovich, 2003). In terms of their size, a particle size of 0.5-3μm is appropriate for particles reaching alveoli, however various factors can influence their paricle size (El-Baseir et al., 1997).

1.3.1. Choice of materials used

Biodegradable microspheres can be produced from both natural such like chitosan,
dextran (Koten et al., 2003), albumin (Gallo et al., 1984) and starch (Fahlvik et al., 1990). However microspheres produced from synthetic polymers such like polymethyl methacrylate, polyanhydrides and polyesters, especially polyesters including polylactides, polyglycolides and their copolymers, are more attractive to researchers due to their reproducible and well characterised attributes. Biodegradability and biocompatibility are the two desirable characteristics of polymeric microspheres.

1.3.2. Methods of preparation of polymeric microspheres

Various methods and techniques are available for preparing microspheres, including emulsion-solvent evaporation, spray-drying and aerosol solvent extraction.

1.3.2.1. Solvent-evaporation method

Solvent-evaporation as a method for microsphere production has been widely used to formulate polymeric microspheres (Suzuki & Price, 1985; Jalil, 1990; El-Baseir et al., 1997; Kirby et al., 2008). In this method polymers are dissolved in a water-immiscible solvent, and then dispersed in an aqueous continuous phase to form discrete droplets, the microspheres then form after the evaporation of organic phase and solidification of the polymers droplets (O'Donnell & McGinity, 1997). The medical active ingredient can be either entrapped in the microspheres or loaded on the surface of microspheres. O/W solvent evaporation method is the most simple solvent evaporation method. The process involves emulsification of an organic solution containing polymer and medical active ingredient in an aqueous continuous phase, followed by an evaporation
process (Jeffery et al., 1991; Liu et al., 2007). This method is suitable for encapsulating drugs with poor water solubility (Suzuki & Price, 1985; Smith & Hunneyball, 1986), but is not suitable for water soluble drugs because the drug may transfer from organic phase into aqueous phase, subsequently decreasing entrapment efficiency or even inhibiting entrapment (O'Donnell & McGinity, 1997); this can be overcome using W/O/W form microspheres.

W/O/W double emulsion solvent evaporation method is the most popular technique to prepare polymeric microspheres (Bodmeier & McGinity, 1987; Ogawa et al., 1988). In this method the polymer is dissolved into an organic solution (e.g. dichloromethane or chloroform), then mixed with the solution containing drugs or active component and agitated or ultrasonic emulsified to form a primary w/o emulsion. This emulsion is then mixed into a second water phase with surfactant by high-speed homogenization to form a secondary w/o/w emulsion. Finally, the microspheres are solidified after organic solution evaporated by heating and agitating. Microspheres prepared using double emulsion solvent evaporation method have been widely used as carriers for proteins and peptides delivery because the high entrapment efficiency and generally less harsh processing conditions than faced in the preparation of the O/W type microspheres (Suzuki et al., 1985).

Microspheres prepared using O/O solvent evaporation method is an anhydrous system to load/entrapped water soluble drugs (Wada et al., 1990; Wang et al., 1991; Sturesson et
al., 1993). One organic phase containing active ingredient disperses into another immiscible phase, in absence of water the partition of drugs into continuous phase is inhibited (O’Donnell & McGinity, 1997). Other solvent evaporation methods include multiple emulsions solvent evaporation method can be used to form W/O/O or W/O/O/O/O types microspheres (Iwata & McGinity, 1992).

1.3.2.2. Spray-drying methods

Spray drying was defined as the transformation of a material from a fluid state into a dried particulated form by spraying a solution or suspension into a hot drying gas medium (Rattés & Oliveira, 2007). The process includes atomization of droplets through a spray orifice, spray-air contact, drying of the sprayed droplets, and collection of solid particles (Brodhead et al., 1992). Drug and polymer are dissolved/dispersed/emulsified, then the resulting solution/suspension/emulsion is sprayed into hot air where solvent evaporated rapidly. Microspheres can be formed with smooth appearance, high carrier ability and this technique has reproducibility and well-defined control of particle size and release.

During this process the quality of microspheres is affected by process parameters such as the concentration polymeric solution, the inlet air temperature and the spraying ratio (Conte wt al, 1994; Baras et al., 2000). Activity of bio-drugs can be maintained in the spray dried microspheres because of the reduced agitation and contact between drugs and organic solution during the process of preparation. However, exposure of
the entrapped agent can occur because of the high temperature, and for sensitive proteins, aggregation and denaturation are likely to be induced in such a conditions (Baras et al., 2000a).

1.3.2.3. Aerosol solvent extraction system (ASES)

In ASES, polymer and drug are dispersed into a solvent that is miscible with the supercritical gas (usually carbon dioxide) which the polymer/drug solution will be subsequently sprayed into, the solvent diffuses into the gas stream and being removed, and the polymer incorporates the drug and precipitates into microspheres (Müller & fisher, 1991; Engwitcht et al., 1999)

Finally there are also some other techniques involved in preparation of microspheres, such as salt-out (Allémann et al., 1993) and precipitation (Manca et al., 2008), both of them with various advantages and disadvantages.

1.4. Devices for inhalation

There are many options of devices for pulmonary delivery, but they can classified into three categories: pressurised metered-dose inhalers, dry powder inhalers and nebulisers.

1.4.1. Pressurised metered-dose inhalers (pMDIs)

The pMDI has multi-dose capability and was first introduced to deliver inhaled
bronchodilators for treatment of the symptoms of asthma and chronic obstructive pulmonary disease (COPD) in 1956 (Newman, 2005) It has been considered as the most convenient, versatile and cheapest inhalation device (Hickey & Dunbar, 1997). The pMDI consists of a container, actuator and metering valve (Figure 1.12.), the components of the drug formulation in the container are drug solution/suspension and propellants. The source of pressure driving the formulation out of the pMDI is from the propellants which used to be chlorofluorocarbons (CFCs). Although the CFCs are safe for use in humans when given in a recommended dosage using pMDI, an international agreement banned CFCs (the “Montreal protocol” in 1987) because the damage of CFCs to the ozone layer in the stratosphere (National asthma education and prevention program, 1998). Now CFCs have been replaced by other propellants, such as hydrofluoroalkanes (HFAs) which do not contain chlorine and so has no ozone-depleting potential (McDonald & Martin, 2000). However, HFAs are difficult to formulate due to their high vapour pressure compared to CFCs (Broadhead et al., 1996), thus the velocity of the emitted sprays is higher, resulting a higher oropharyngeal deposition (Adjei et al., 1996). It also has been reported that HFA-containing pMDIs can produce super fine droplets and improve the lung deposition (Leach, 1998).
The pMDI emits aerosols with large diameter and high velocity in 0.1-0.4 seconds (Hocharainer & Hökz, 2005), which causes 50-80% of the spray impact in the oropharyngeal region (Newman et al., 1981; Borgstrom & Newman, 1993). Only 10-20% of the dose emitted dose is deposited in the lung (Newman & Clarke, 1992). One important factor that affects the therapeutic efficiency using pMDI is the hand-mouth coordination, nearly half of the patients using the system have problems with it (Crompton, 1982). They usually fail to continuously inhale slowly after activation of the pMDI and exhale fully before the inhalation. Rather, they tend to inhale before the activation of pMDI or inhale after the end of it (Virchow et al., 2008), particularly children and elders (Hickey & Dunbar, 1997). Using a small volume spacer is recommended to help overcome such hand-mouth discoordination (Hardy et al., 1996).

In order to eliminate hand-mouth discoordination, new devices were developed,
including Autohaler (3M Pharmaceuticals, Minnesota, USA) and Easi-breathe (Baker Norton, Miami, USA), and both of them are breath-actuated pMDIs. The former was shown to improve the lung deposition up to 20.8% compared to 7.2% of conventional pMDI but the oropharyngeal deposition remains same (Newman et al., 1991).

1.4.2. Dry powder inhalers (DPIs)

The first commercial DPI was the Spinhaler (Fisons, UK), which was used to deliver disodium cromoglycate (Figure 1.13.) (Bell et al., 1971). Before inhaling, the needle inside the Spinhaler pierce the capsule on the side face by sliding the outer trigger of the inhaler. When inhaling, the air flows through the inhaler, the fan and capsule start revolving. Drug powders go through the pierced holes and are deagglomerated by the revolving fan, subsequently being inhaled out of the inhaler into the lung.

![Figure 1.13. Structure of Spinhaler.](image-url)
In general DPIs were developed due to inconvenient use caused by the poor mouth-hand coordination of patients and the environmentally damaging propellants in CFC-containing pMDIs (Hickey & Dunbar, 1997; Podczeck, 1998; Daniher & Zhu, 2008). There are many DPIs available in the market (Table 1.1.).

The DPI devices can be classified by dose type into single dose, multi-dose and multi-unit dose inhalers (Figure 1.14.) (Daniher & Zhu, 2008): in single dose DPIs, powders are pre-metered loaded in individual capsules, which are subsequently inserted into the inhaler for a single delivery, capsules are then removed and discarded after us. Multi-dose inhalers can be divided into two types: either containing multiple pre-metered loaded capsules or employing multi-dose reservoirs which stores powders in one chamber but dose is metered by the device.

The DPI devices can also be classified by actuation type i.e. passive and active inhalers (Daniher & Zhu, 2008). The first and second generations of DPIs are passive breath-actuated inhalers, and thus the drug deposition in the respiratory track is highly affected by the inspiration of patents. The drug powders have a fine particle size and are cohesive and likely agglomerate into large bulks due to interparticle force (Geldart, 1973). Sometimes these large bulks in passive DPIs cannot be deagglomerated by the inspiration of patents, subsequently decreasing the inhaled dose (Daniher & Zhu, 2008).
<table>
<thead>
<tr>
<th>DPI device</th>
<th>Type</th>
<th>Producer</th>
<th>Delivery method</th>
<th>Drugs</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>First generation: breath actuated single unit/dose</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinhaler</td>
<td>Single dose</td>
<td>Aventis</td>
<td>Capsule</td>
<td>SC</td>
<td>Asthma</td>
</tr>
<tr>
<td>Rotahaler</td>
<td>Single dose</td>
<td>GlaxoSmithKline</td>
<td>Capsule</td>
<td>SS, BDP, SS+BDP</td>
<td>Asthma</td>
</tr>
<tr>
<td>Inhalator</td>
<td>Single dose</td>
<td>Boehringer-Ingeheim</td>
<td>Capsule</td>
<td>Fenoterol</td>
<td>Asthma</td>
</tr>
<tr>
<td>Cyclohaler</td>
<td>Single dose</td>
<td>Pharmacemie</td>
<td>Capsule</td>
<td>SS, BDP, IB, BUD</td>
<td>Asthma</td>
</tr>
<tr>
<td>Handihaler</td>
<td>Single dose</td>
<td>Boehringer-Ingeheim</td>
<td>Capsule</td>
<td>Tiotropium</td>
<td>COPD</td>
</tr>
<tr>
<td>Aerolizer</td>
<td>Single dose</td>
<td>Novartis</td>
<td>Capsule</td>
<td>Formoterol</td>
<td>Asthma</td>
</tr>
<tr>
<td>FlowCaps</td>
<td>Single unit dose</td>
<td>Hovione</td>
<td>Capsule</td>
<td>-</td>
<td>Asthma</td>
</tr>
<tr>
<td>TwinCaps</td>
<td>Single dose</td>
<td>Hovione</td>
<td>Capsule</td>
<td>Neuraminidase inhibitors</td>
<td>Influenza</td>
</tr>
<tr>
<td><em>Second generation: breath actuated multi-unit/dose</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turbohaler</td>
<td>Multi-dose</td>
<td>Astra Zeneca</td>
<td>Reservoir</td>
<td>SS, TS, BUD</td>
<td>Asthma</td>
</tr>
<tr>
<td>Diskhaler</td>
<td>Multi-unit dose</td>
<td>GlaxoSmithKline</td>
<td>Blister package</td>
<td>SX, BDP, FP, Zanamivir</td>
<td>Asthma, Influenza</td>
</tr>
<tr>
<td>Diskus/Accuhaler</td>
<td>Multi-unit dose</td>
<td>GlaxoSmithKline</td>
<td>Strip pack</td>
<td>SS, SX, FP, SX+FP</td>
<td>Asthma</td>
</tr>
<tr>
<td>Aerohaler</td>
<td>Multi-unit dose</td>
<td>Boehringer-Ingeheim</td>
<td>-</td>
<td>IB</td>
<td>Asthma</td>
</tr>
<tr>
<td>Easyhaler</td>
<td>Multi-dose</td>
<td>Orion Pharma</td>
<td>Reservoir</td>
<td>SS, BDP</td>
<td>Asthma</td>
</tr>
<tr>
<td>Ultrahaler</td>
<td>Multi-dose</td>
<td>Aventis</td>
<td>Reservoir</td>
<td>-</td>
<td>Asthma</td>
</tr>
<tr>
<td>Pulvinal</td>
<td>Multi-dose</td>
<td>Chiesi</td>
<td>Reservoir</td>
<td>SS, BDP</td>
<td>Asthma</td>
</tr>
<tr>
<td>Novolizer</td>
<td>Multi-dose</td>
<td>ASTA</td>
<td>Reservoir Cartridge</td>
<td>BUD</td>
<td>Asthma, COPD</td>
</tr>
<tr>
<td>MAGhaler</td>
<td>Multi-dose</td>
<td>Boehringer-Ingeheim</td>
<td>Reservoir</td>
<td>SS</td>
<td>Asthma</td>
</tr>
<tr>
<td>Taifun</td>
<td>Multi-unit dose</td>
<td>LAB Pharma</td>
<td>Reservoir</td>
<td>SS</td>
<td>Asthma</td>
</tr>
<tr>
<td>Eclipse</td>
<td>Multi-unit dose</td>
<td>Aventis</td>
<td>Capsule</td>
<td>Sodium chromoglycate</td>
<td>Asthma</td>
</tr>
<tr>
<td>Clickhaler</td>
<td>Multi-dose</td>
<td>Innoveta Biomed</td>
<td>Reservoir</td>
<td>SS, BDP</td>
<td>Asthma</td>
</tr>
<tr>
<td>Asmanex</td>
<td>Multi-dose</td>
<td>Schering-Plough</td>
<td>Reservoir</td>
<td>MF</td>
<td>Asthma</td>
</tr>
<tr>
<td>Twistrhler Corporation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-----------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Third generation: active device</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exubera*</td>
<td>Single dose</td>
<td>Pfizer</td>
<td>Blistetr</td>
<td>Insulin</td>
<td>Diabetic</td>
</tr>
<tr>
<td>Airmax</td>
<td>Multi-dose</td>
<td>Norton Healthcare</td>
<td>Reservoir</td>
<td>Formoterol, BUD</td>
<td>Asthma, COPD</td>
</tr>
</tbody>
</table>

Figure 1.14. Illustration of DPIs classified by dose type (picture from Daniher & Zhu, 2008).
Active DPIs were developed in order to overcome the interparticle force, which have an integrated energy source and dispersion mechanism (Daniher & Zhu, 2008). They provide external energy based on vibration (Jaraiz et al., 1992), acoustic waves (Chirone & Massimilla, 1994; Chirone et al., 1993), stirring (Daniher & Zhu, 2008), or magnetic electrical field disturbance (Liu et al., 1991; Hristov, 2000) which supports thier deagglomerating in DPIs. Active DPIs are suitable for the patients who cannot provide inspiration flow rate from inspiration. However, the breath coordination is needed to use active DPIs (Daniher & Zhu, 2008).

Overall it has been summarised by Labiris and Dolovich (2003a) that about 12-40% of emitted dose can deposit in the lung using various DPIs, however still 20-25% of the dose is retained in the device and capsules.

1.4.3. Nebulisers

Nebulisers have been used for inhalations for more than one hundred years to atomize aqueous based drug formulations. Modern nebulisers are divided into two types based on their atomisation mechanism: jet and ultrasonic nebulisers (O’Callaghan & Barry, 1997).

1.4.3.1. Jet Nebulisers

The functional mechanism of a jet nebuliser has been described by O’Callaghan and Barry (1997), as shown in Figure 1.15: the airflow from a compressed gas source (e.g.
compressor or compressed gas cylinder) passes though a narrow orifice, called Venturi nozzle, and rapidly expands in the liquid reservoir, causing a negative pressure over the end of the feeding tube where the liquid is sucked up and drawn out into fine ligaments which subsequently collapse into droplets under the influence of surface tension. Small droplets are released following the airflow, while large droplets will impact on the baffle or the wall of the liquid reservoir and return for re-nebulisation.

Using jet nebulisers, the diameter of nebulised droplets, the output efficiency of jet nebulisers, nebulisation time and drug wastage can be influenced by following factors:

- increasing the driving gas flow rate through jet nebulisers can reduce the diameter of droplets,
• increase the output efficiency of nebulisers, and hence shortening nebulisation time (O’Doherty et al., 1990),

• increasing volume fill can increase the nebulised drug proportion, but prolong the nebulisation time (Clay et al., 1983; O’Doherty et al., 1990),

• increasing the baffle’s size can sufficiently decrease the droplets size and output, but increase residual volume and drug wastage, and prolong the nebulisation time (O’Callaghan & Barry, 1997),

• the drug concentration in droplets and solution reservoir increases during the nebulisation using jet nebuliser due to evaporation, and subsequently causes reduction in droplet size and increased in drug wastage (Phipps & Gonda, 1994),

• during the jet nebulisation the drug solution temperature falls, which increases solution viscosity and reduces output (Clay et al., 1983; Dennis et al., 1990).

1.4.3.2. Ultrasonic nebulisers

Ultrasonic nebulisers were invented in the 1960s (Mercer, 1981). They employ a piezoelectric crystal to generate high frequency vibrations which are transmitted on to the surface of the liquid making the surface unstable and forms a liquid fountain, subsequently droplets are formed in the nebuliser chamber and then nebulised out of the nebuliser (Figure 1.16.) (O’Callaghan & Barry, 1997; Taylor & McCallion, 1997; Labiris and Dolovich, 2003a).
Similar to jet nebulisers, many factors described above affect on nebulised droplets, the output efficiency of jet nebulisers, nebulisation time and drug wastage using ultrasonic nebulisers. Furthermore, the droplets are driven out based on the high frequency vibrations generated by piezoelectric crystal, and increasing the vibration frequency results reduction in droplets size and increase in output (O’Callaghan & Barry, 1997; Labiris and Dolovich, 2003a).

Compared to jet nebulisers, ultrasonic nebulisers have more output, shorter nebulisation time, and a smaller device size (Thomas et al., 1991), but jet nebulisers are more effective in atomizing viscous solutions (McCallion et al., 1995).

The disadvantages of nebulisers are that in general they are expensive, bulky, time consuming and inconvenient to handle, wash, and maintain (Byron, 1990; Newman, 1990; Timsina et al., 1994). However, compared to pMDIs and DPIs, nebulisers do
not require hand-mouth coordination, and they deliver large volume of drug solutions and suspensions, and the nebulised droplets can be inhaled during normal breathing through a mouth piece or face mask, which are suitable for infants, elders and patients who physically unable to use other devices (McCallion et al., 1996).

1.5. Protein conformation and stability.

As one of the most important class of biomacromolecules, proteins are polypeptide chains made up of 20 common amino acids, and the functional properties of them depend upon their three-dimensional structures which are refered to for distinct aspects: the primary structure is the sequence of the a protein's polypeptide chain held together by covalent or peptide bonds (Brändén & Tooze, 1999); secondary structure is the locally defined highly regular sub-structure, including α helix and β sheet (Janin & Chothia, 1985); tertiary structure is the structure of a single protein molecule formed by packing the secondary structure elements (Richardson, 1985); quaternary structure is a protein consisting of more than one peptide chains (Brändén & Tooze, 1999). The change of protein structure may cause the alter of its functional properties and bioactivities, thus a desirable protein delivery system should have the ability to maitain protein's structure.

1.6. Recent developments in pulmonary drug delivery.

Inhaled insulin became an attractive research area since Wigley et al. (1971) delivered insulin to rabbits via nebulisation which successfully resulted in a hypoglycemic
effect (Setter et al., 2007). Exubera (Pfizer) was the first inhaled insulin approved in Europe and the United States for the treatment of type 1 and 2 diabetes in adults in 2006 (Lenzer, 2006). It was based on a dry powder inhalation platform that powder particles had a diameter of 1-3 μm (Patton et al., 2004), and it has similar therapeutic efficacy but better patient preference profiles in comparison with conventional subcutaneous insulin (The Lancet, 2006; Arnolds & Heise, 2007; Skyler et al, 2008).

However, using Exubera was involved in an increased incidence cough, dyspnea and raised insulin antibody level (Ceglia et al, 2006; Skyler et al, 2008), and a small reversible decline in pulmonary function was also observed in long-term studies (Arnolds & Heise, 2007).

Furthermore, Arnolds and Heise (2007) have reviewed the factors that influence the absorption of inhaled insulin, and emphasized that the absorption of Exubera was reduced in the patients who have smoked in the last six months, or have lung diseases such like asthma or COPD.

Exubera was removed from market in 2007 (Mack, 2007) due to the poor acceptance of patients and lacking of long-term safety data in terms of lung damage (The Lancet, 2006; Mack, 2007).

Therefore it is clear there are a large range of options with regard to drug delivery
systems and inhaler devices available with both their characteristics in their own right and the effectiveness of the drug delivery system/inhaler device compatibility requiring consideration.

1.7. Aims and Objectives

Based on the need for a more effective non-invasive delivery of biopharmaceuticals including proteins, and the potential advantages the pulmonary route can offer, the aim of this research was to prepare and characterise biodegradable polymeric microspheres-based formulations for proteins, which were suitable for pulmonary delivery in pulmonary route.

Therefore the objectives of this thesis were as follows:

- Preparation and optimisation of PLA microspheres, and subsequent investigation of their stability and in vitro release profile.

- Formulation of PLA microspheres into dry powders to enhance their stability, and subsequent investigation of their physico-chemical characteristics.

- Evaluation of the aerosolisation performance of PLA microsphere formulations in both dry powders and dispersion.
• Investigation of the cellular responses of macrophages exposed to PLA microspheres and powders with/without addition of immunomodulators.
Chapter 2

Optimisation of PLA microsphere formulations for pulmonary delivery - physico-chemical studies
2.1. Introduction

Biodegradable polymer microspheres have been shown to offer considerable potential as drug delivery systems; various studies have demonstrated that moieties including peptides (Newman et al., 1998), proteins (Ma et al., 1998), DNA (Wang et al., 1999) and antigens (Tomasin et al., 1996) can be protected from chemical and/or enzymatic circumstances and maintained in their native forms by microsphere-based vehicles (Hanes et al., 1997).

Polylactide (PLA) appears to be a suitable candidate material for polymer microspheres, as it is a biocompatible and biodegradable macromolecule polymer which has been safely used in humans as material of vehicles for drug delivery for decades (Shive & Anderson 1997; Grube & Buellefeld, 2005). A number of products utilize PLA as excipient for sustained therapy can be found in the market and some examples are listed in Table 2.1.

<table>
<thead>
<tr>
<th>Product</th>
<th>Active Ingredient</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atridox</td>
<td>Doxycycline hyclate</td>
<td>Periodontitis</td>
</tr>
<tr>
<td>Decapeptyl</td>
<td>Triptorelin</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>Lupron Depot</td>
<td>Leuprolide acetate</td>
<td>Prostate cancer, Endometreosis</td>
</tr>
<tr>
<td>Sandostatin LAR</td>
<td>Octreotide</td>
<td>Acromegaly</td>
</tr>
<tr>
<td>Trelstar Depot</td>
<td>Triptorelin pamoate</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>Zoladex</td>
<td>Goserelin acetate</td>
<td>Prostate cancer, Endometreosis</td>
</tr>
</tbody>
</table>

Table 2.1. Examples of products available which use PLA as an excipient (Chaubal, 2002).

As an excipient, PLA has a good biodegradation profile - the metabolite of PLA is lactic acid, a natural metabolic by-product with human physiology, which can be
Further metabolized into carbon dioxide and water via the citric acid cycle and then be excreted from the body without accumulation (Maurus & Kaeding, 2004). PLA is also recognized as having low immunogenicity and safe for medical/pharmaceutical usage, within its structure it has no peptide chain which may be immunogenic (Kulkarni et al., 1996). Therefore, PLA can be used as a non-toxic, bio-degradable, controlled release matrix in various applications including pulmonary drug delivery (El-Baseir & Kellaway 1998).

As a polymer, PLA usually exists in two forms, Poly (L-lactide) (PLLA) and Poly (D,L-lactide) (PDLLA) (Figure 2.1.) (Maurus & Keating, 2004). Both forms are stereoirregular, but PLLA is more hydrophobic and semi-crystalline, and it may take more than two years for resorption in vivo (Middleton 2000). PDLLA is more hydrophilic and is amorphous in nature due to the random distribution of L- and D-lactide that are unable to organize into a crystalline structure (Middleton & Tipton, 1998), and therefore degrades faster (in 12 to 16 months) (Sinha & Trehan, 2003; Nair & Laurencin, 2007). In this study, PDLLA was selected as the material of microspheres for its faster degradation rate compared to PLLA.
2.1.1. Preparation of microspheres.

Many techniques are available for preparing PLA microspheres, such as emulsion solvent evaporation, spray-drying, aerosol solvent extraction, salting-out, emulsion-diffusion and non-solvent induced precipitation, and so on. However, emulsion solvent evaporation and spray-drying are the most common and widely used techniques due to their relatively simple procedures. Generally for preparing microspheres in small scale batches in the laboratory, the yield and efficiency of the emulsion solvent evaporation method is higher than that of spray-drying (Bitz & Doelker, 1996).
Briefly, in the double emulsion solvent evaporation method, the drug to be incorporated is dissolved in an aqueous solution. This solution is dispersed into an organic polymer solution to form primary water-in-oil (W/O) emulsion with e.g. ultrasonating, vortex mixing or agitating. This primary emulsion is then added into a secondary aqueous solution usually containing surfactant or emulsifier, followed by a high speed homogenization to form a secondary water-in-oil-in-water (W/O/W) emulsion. Evaporation of the organic solvents from the w/o/w results in the polymers solidifying to form microspheres (Ogawa et al., 1988; Whateley, 1993) (Figure 2.2.).

![Figure 2.2. Preparation of PLA microspheres using the double emulsion solvent evaporation method.](image)

Surfactant/emulsifier added in secondary aqueous solution stabilise the W/O/W emulsion by forming an interfacial film between the organic phase and aqueous phase and reducing the interfacial tension. Polyvinyl alcohol (PVA; Figure 2.3.) is a water-soluble polymer widely used as surfactant/emulsifier for emulsion and surfactant for polymer encapsulated vehicles because the harvested particles are relatively uniform and small (Panyan & Labhasetwar, 2003). Most commercial PVA has a large number of hydrophilic hydroxyl groups and also contains 4-12% acetate
groups (Figure 2.3.) because the polymer is prepared by partly hydrolysis of polyvinyl acetate. These hydrophobic acetate groups have an affinity with the oil phase and the hydrophobic polymer surface. PVA is therefore amphiphilic in nature (Tadros, 2009). During emulsifying, the acetate groups enter the organic phase and leave the hydroxyl groups in the aqueous phase, forming an interface between the two phases. Using PVA as emulsifier enhances the stability of the microdroplets and maintains them in a small size with a narrow size distribution (Zambaux et al., 1998). However, it is difficult to remove PVA from the hardened microparticle surface (Carrio et al., 1991). PVA was considered to have potential to cause cancer (Hueper, 1959), but has been used safely for medical treatment for many years now in products such as ophthalmic solutions for the treatment of keratoconjunctivitis (Norn, 1977).

![PVA and Commercial PVA](image)

Figure 2.3. Structure of polyvinyl alcohol and its commercial product.

The aim of the work outlined in this chapter was to investigate and optimise formulation parameters used in the preparation of PLA microspheres for the pulmonary delivery of proteins. Initial studies focused on particulate size as this was
deemed a key characteristic in pulmonary drug delivery. PLA microspheres were prepared and the following parameters investigated for their influence on mean particle size:

1. The method of preparing microspheres: investigated by preparing blank PLA microspheres either via double emulsion-solvent evaporation and single emulsion-solvent evaporation,

2. The molecular weight of polymer: investigated by preparing microspheres with PLA materials of 3 different molecular weights (MW: 50 kDa, 150 kDa, 300 kDa),

3. The concentration of PLA in the organic phase: investigated by preparing microspheres with varying amount of PLA (0.5, 1, 3, 5, 7 or 10 % w/v) in a fixed volume of organic phase (417 μl chloroform),

4. The concentration of PVA in the external aqueous phase: investigated by preparing microspheres with varying amount of PVA (0.3, 1.5, 3, 5 or 10% w/v) in a fixed volume of external aqueous phase (10 ml double distilled water).

From these data, optimised formulations were then tested for protein loading and short term-stability in terms of mean particle size, zeta potential and protein loading/retention.
2.2. Materials and methods

2.2.1. Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(D, L-lactide) (MW 50 kDa)</td>
<td>Polysciences, Inc. (Warrington, US)</td>
</tr>
<tr>
<td>Poly(D, L-lactide) (MW 150 kDa)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Poly(D, L-lactide) (MW 300 kDa)</td>
<td>Polysciences, Inc. (Warrington, US)</td>
</tr>
<tr>
<td>Poly(vinyl alcohol) (MW 13 - 23 kDa, 87 - 89% hydrolysed)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Chloroform, laboratory grade</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (fraction V, 98–99% albumin)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bicinchoninic Acid Solution</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Copper Sulphate Hydrate 98%</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Sodium Hydroxide Pellets</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
</tbody>
</table>

2.2.2. Methods

2.2.2.1. Preparation of PLA microspheres

Bovine serum albumin (BSA) was used as a model protein and BSA loaded PLA microspheres were prepared using a modified double emulsion solvent evaporation technique (Kirby et al., 2008). For these and all studies conducted, three separate independent batches of microspheres were prepared and tested. To prepare these formulations, 12.5 mg PLA was dissolved in 417 µl chloroform, an aqueous solution of BSA (1.25 mg in 10 µl) was dispersed into it by vortex mixing for 1.5 minutes to form the primary water-in-oil emulsion. This primary emulsion was then added to an
aqueous solution of PVA in double distilled water (10 ml, 10% (w/v)) by high speed homogenisation (Silverson SL2 homogeniser at 6000 rpm) for 3 minutes. This water-in-oil-in-water emulsion was then left stirring at room temperature for 18 hours to allow for the evaporation of chloroform. The solidified microspheres were separated and harvested from the continuous phase by centrifugation (20 minutes, 10000 x g), and then repeatedly washed by re-suspension in three 10 ml aliquots of double distilled water to remove the surfactant. BSA free microspheres were prepared both in w/o/w double emulsion and o/w single emulsion solvent evaporation methods: as described above, replacing the BSA solution used in the preparation of the primary emulsion with the same volume of double distilled water to prepare w/o/w microspheres; in o/w method, the preparation process was same as w/o/w except addition of internal aqueous phase.

2.2.2.2. Particle size distribution analysis of microspheres

Mean particle diameters and size distribution of microspheres were measured using laser diffraction technique (Washington, 1992) on a Malvern Mastersizer (Malvern Instruments, UK). This sizing method is based on the fact that particles moving in a monochromatic beam of light, of wavelength 633 nm produced by a helium-neon source, will scatter light at an angle correlated with their particle size (Mastersizer reference manual). Particles with large diameter will scatter the light at low angles, while as particles decreases in size, scattering angles will increase. Both scattered and unscattered light are received and formed into diffraction patterns by a receiving lens,
the resultant diffraction is collected by a series of concentric annular sectors.

PLA microspheres were added into a transparent cell with magnetic stirring (1200 rpm) until a 13-15% laser obscurity achieved. The mean particle size represents the volume mean diameter of PLA microspheres. The size distribution is profiled in percentile, D10, D50 and D90, which represent the volume of sample 10, 50, and 90% respectively with a size up to the value stated, which are determined by measuring the fraction of total laser power passing through the lens. Measurements of microsphere formulations were carried out for three independently prepared microsphere batches, to confirm reproducibility of sizing technique and an average percentile distribution was reported.

2.2.2.3. Zeta potential analysis of microspheres

Zeta potential was measured using ZetaPlus instrument (Brookhaven Instrument Corporation, NY) to indicate the surface charge on the microspheres. The zeta potential values were calculated from determined electrophoretic mobility data of the microspheres, measured from the direction and velocity of the microsphere movement in an electronic field, based on the Smoluchowsky mathematical equation (Patil & Panyam, 2009) by the Brookhaven software (Patil 2009). The samples were prepared by adding 75 µl of the microsphere dispersion into 2 ml double distilled water to achieve a count rate of 100 counts per second and determined by Zetaplus Analyzer at 25 °C. The results were reported as the mean zeta potential of three separate batches
each of which was the mean value of 10 measurements.

2.2.2.4. Freeze-drying of microspheres

After being washed and centrifuged in section 2.2.2.1., PLA microspheres were re-suspended in 1 ml of double distilled water, and then transferred to an appropriate container covered with paraffin film with punctures on it to let water remove out of the container. Freeze drying was carried out based on previous protocols developed (Mohammed et al., 2006) which involved freezing the samples at -80 °C for 30 min followed by primary drying at -50 °C for 24 h and secondary drying at -20 °C for 6 h under a vacuum.

2.2.2.5. Measurement of BSA loading efficiency using bicinchoninic acid

Quantification of BSA concentration was carried out by bicinchoninic acid (BCA) assay (Smith et al., 1985), a highly sensitive and selective colorimetric detection method. The assay works as follows: first, the cupric ions (Cu^{2+}) react with protein in an alkaline environment (biuret reaction) and then reduce to cuprous ions (Cu^{1+}), then two molecules of BCA chelated with one formed Cu^{1+} to produce a purple-coloured complex which shows a linear absorbance at 560 nm with increasing protein concentrations.

To calculate drug incorporation in freeze-dried microspheres 5 mg freeze-dried microspheres were resuspended in 1ml 0.1 M NaOH solution, and incubated for 48
hours at room temperature to degrade and release the protein. 10 mg BSA was also dissolved in 10 ml 0.1 M NaOH, and then diluted into 320, 240, 160, 120, 80, 40, 20, 10 μg/ml for the standards and treated in the same way as experimental. 0.1 M NaOH was used as background control. 75 μl each sample was plated out in triple into a 96 well microtitre plate and 75 μl of BCA reagent (prepared from 300 μl 4% v/v copper sulphate in 14.7 ml of bicinchoninic acid solution), was added to each well. The 96-well plate was then incubated at 37 °C for 30 minutes, the absorbance of purple-coloured BCA/ Cu¹⁺ chelate was measured using Microplate Reader (Bio Rad) at 560 nm. The entrapment efficiency of protein in the sample was calculated based on a calibration curve (Figure 2.9.).

\[
\text{Entrapment efficiency } \% \text{ (w/w)} = \frac{\text{Mass of the BSA in microspheres}}{\text{Mass of the BSA added}} \times 100\%
\]

2.2.2.6. Optimisation of formulation parameters for pulmonary delivery

For pulmonary delivery, particle size is one of the most important factors that influence deposition in the lung (Labiris & Dolovich, 2003), solid microspheres with aerodynamic mean size of 0.5-3 μm can reach the alveoli for effective delivery of pulmonary therapeutics (El-Baseir et al., 1997). Parameters mentioned in section 2.1 were therefore optimised to prepare suitable microspheres based on particle size.

2.2.2.7. PLA microsphere stability in aqueous environment

In order to evaluate the stability and the storage conditions of PLA microspheres, the
fresh made PLA microspheres were re-suspended in ddH₂O, sealed in container and stored at room temperature and 4 °C. The mean particle size, zeta potential and entrapment efficiency were measured as described in section 2.2.2.2 to 2.2.2.5 every 7 days over a period of 4 weeks.

2.2.2.8. Statistical Analysis

Results present the mean values of triplicate microsphere batches assays ± standard deviations (SD) of the mean values, differences between means were assessed by analysis of variance (ANOVA), with p values of <0.05 being considered significant and p value of <0.001 being considered extremely significant.

2.3. Results and discussion

2.3.1. Effects of formulation parameters on particle size distribution

2.3.1.1. Methods of preparation

Initially the double/single emulsion-solvent evaporation methods were invested for their effect on particle size and size distribution. In both methods, microspheres were prepared using PLA MW 50 kDa at a concentration of 3% (w/v) and with 10% (w/v) PVA as a starting formulation. In terms of the two methods tested, there was no significant difference observed between the particle size of microspheres made by the two methods (Figure 2.4.), and both of them showed a mono-modal distribution of size (Figure 2.5.), but the W/O/W microspheres prepared in double emulsion-solvent evaporation showed a trend of decreased microsphere size, particularly as shown in
the D90 distribution percentiles suggesting these systems may have a tighter size distribution. Both formulation types have been previously used to prepare microspheres e.g. O/W microspheres were successfully applied in encapsulating poor water soluble drugs (O’Donnell & McGinty, 1997), however the double emulsion-solvent evaporation tends to be more widely used due to the internal aqueous phase provides a stable environment for water soluble drugs and proteins. As consequence of these reasons, the double emulsion-solvent evaporation was employed in all subsequent studies.

![Graph showing particle size distribution](image)

Figure 2.4. Mean particle size and size distribution of empty microspheres prepared with molecular weight of 50 kDa PLGA, using water in oil in water double emulsion solvent evaporation and oil in water single emulsion solvent evaporation methods respectively (mean ± SD, n=3).
2.3.1.3. Concentration of polymer in the organic phase

The concentration of PLA phase was also investigated using 50 kDa PLA (from 0.5 to 7%, w/v) in the organic phase for their effect on particle size and size distribution. This concentration range was chosen based on the research of Kirby et al. (2008) that the particle size of PLGA microspheres was minimized in this range. Figure 2.7 shows that the particle size of the formulation made with 3% (w/v) PLA in organic phase is significant smaller than all other formulations (P<0.05) with a mean particle size of 1.13 μm and an overall decrease in particle size from 4.72 ± 1.01 μm to 1.13 ± 0.01 μm was seen as the concentration of PLA increased from 0.5 to 3% (w/v) respectively (Figure 2.7.). With increasing polymer concentration (0.5 - 3%) one might predict an increase in particle size due to the higher polymer content per emulsion globule. However the observed decreasing in particle size trend with increasing PLA
concentration noted may have been caused by the increase in the stability of the primary W/O emulsion, possibly due to the increased viscosity. On further increasing the PLA concentration, from 3% to 5% the particle size significantly (p<0.05) increased to $1.44 \pm 0.04 \, \mu m$; further increases in concentration to 7% resulted in no further significant changes in particle size (Figure 2.7.). These findings are in line with those previous reported (Jeffery et al., 1991; Benoit et al., 1999), where the authors concluded that higher concentrations of polymer in the organic phase might increase the frequency of collisions between the dispersed phases, leading to a fusion of semi-formed particles and an increase in particle size. In addition, higher viscosity due to a higher polymer concentration might reduce the efficiency of emulsification, resulting in an increase in globule size and ultimately increased particle size. Based on these results, a PLA concentration of 3% (w/v) was selected for the organic phase of the preparation.
Figure 2.7. Mean particle size and size distribution of empty PLA microspheres prepared with varying concentrations (% w/v) of PLA (mean ± SD, n=3). + denotes the value =19.74 ± 4.67 μm; * denotes significant difference between the two groups (p<0.05); ** denotes significant difference between the two groups (p<0.001).

2.3.1.4. Concentration of emulsion stabilizer in the external aqueous phase

Varying PVA concentrations (from 0.5 to 10%, w/v) in the external aqueous phase were investigated for their effect on particle size and size distribution. This was based on the research of Kirby et al. (2008), showing that optimisation of PLGA microsphere particle size, using PVA as emulsifier, was within this concentration range. Using a fixed PLA concentration of 3%, and a PLA molecular weight of 50 kDa, increasing PVA concentrations in the external aqueous phase decreased the mean particle size, with a linear correlation between PVA concentration and microsphere mean particle size as the concentration of PVA exceeded 3% w/v ($r^2=0.9987$) (Figure 2.8.). Given that the role of PVA is to stabilise the emulsion by reducing interfacial tension at the oil/water interface these finding are in line with PVA acting as an effective emulsifying agent. Indeed, a similar trend was reported in the observation of Mumper et al. (1992) and Benoit et al. (1999) with the later study preparing
microspheres from poly(ε-caprolactone) with PVA to stabilise the emulsion. They noted that the particle size reduced from $8.09 \pm 0.44 \mu m$ to $5.88 \pm 0.74 \mu m$ as the concentration of emulsifier increased from 0.5 to 5% w/v, respectively (Benoit et al., 1999). It has also been proposed that increasing the concentration of emulsifying agent in the formulation facilitates a smaller particle size by reducing droplet coalescence during emulsion preparation, again due to the reduced interfacial tension (El-Baseir & Kellaway, 1997). Among the six formulations tested, a PVA concentration of 10% was selected for the external aqueous phase for all subsequent studies, due to their significantly smaller and more uniformed particle size than the other formulations ($p<0.05$).

2.3.2. Effect of addition of BSA on particle size

Interestingly, protein loading was shown to have no significant effect on microsphere mean particle size when lower polymer molecular weights were used (50 kDa) (Table 2.2) however when 150 kDa or 300 kDa PLA was employed the presence of BSA within the formulation was shown to significantly ($p<0.05$) increase the mean particle size by approximately 41.32 and 152.07%. Other study showed that the haloperidol loading efficiency of PLG microspheres was increased from 13.5 to 46.1% as mean size increased from 0.8 to 8μm (Cheng et al., 1998).
Figure 2.8. Mean particle size and size distribution of empty PLA microspheres prepared with varying concentrations (%, w/v) of PVA in the external aqueous phase (mean ± SD, n=3). * denotes significant difference between the two groups (p<0.05); ** denotes significant difference between the two groups (p<0.001).

<table>
<thead>
<tr>
<th>Molecular weight of PLA (kDa)</th>
<th>Mean particle size (μm)</th>
<th>Span</th>
<th>Mean particle size (μm)</th>
<th>Span</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.14 ± 0.20</td>
<td>0.94 ± 0.082</td>
<td>1.21 ± 0.26</td>
<td>0.94 ± 0.07</td>
</tr>
<tr>
<td>150</td>
<td>1.50 ± 0.17</td>
<td>1.18 ± 0.01</td>
<td>1.71 ± 0.01</td>
<td>1.09 ± 0.01</td>
</tr>
<tr>
<td>300</td>
<td>2.51 ± 0.35</td>
<td>1.67 ± 0.11</td>
<td>3.05 ± 0.09</td>
<td>2.01 ± 0.10</td>
</tr>
</tbody>
</table>

Table 2.2. Particle size and span of BSA-free/loaded microspheres and BSA entrapment efficiency. The initial amount of BSA added was 10% (w/w) of polymer.

2.3.3. Effect of microsphere formulation parameters on protein loading.

10% BSA of the initial PLA amount was loaded, this ratio was based on the research of Feczko et al. (2008), where they successfully entrapped BSA into PLGA microspheres at entrapments more than 90% when the BSA was not higher than 10% (w/w) of the PLGA amount. The BSA loading efficiency was determined using a BCA assay, the measurement was based on a linear relationship of BSA concentration and absorbance read at 560nm (Figure 2.9.).
Figure 2.9. Calibration curve of BCA assay for BSA detection.

Similar to the effect of formulation on mean particle size, the effect of the polymer molecular weight was also investigated in terms of BSA loading within microspheres. Protein loading was investigated using a BCA assay as described above (section 2.2.2.5.). No significant difference in BSA loading was noted between each of the three microsphere formulations made with different molecular weight PLA, with entrapment efficiencies of ~72% being measured (Table 2.3.). These findings are consistent with previous summarisation by O’Donnell and McGinity (1997) that the loading efficiency was not affected by the molecular weight of polymers except when the molecular weight was very low (<30 kDa). Low molecular weight PLGA (<30 kDa) was also investigated for the entrapment of huperzine A in PLGA microspheres, the loading efficiency with respect to polymer molecular weights of 15, 20 and 30 kDa, as 62.75, 27.52 and 16.63% respectively (Fu et al., 2005).
Table 2.3. BSA loading efficiency of PLA microspheres with/without addition of 1% PVA (w/v) in inner aqueous phase (mean ± SD, n=3).

<table>
<thead>
<tr>
<th>Molecular weight of PLA (kDa)</th>
<th>Primary o/w emulsion in presence of PVA</th>
<th>Primary o/w emulsion without PVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>71.67 ± 0.59</td>
<td>71.71 ± 0.49</td>
</tr>
<tr>
<td>150</td>
<td>72.30 ± 0.75</td>
<td>71.87 ± 0.38</td>
</tr>
<tr>
<td>300</td>
<td>73.45 ± 3.14</td>
<td>72.41 ± 1.46</td>
</tr>
</tbody>
</table>

However the BSA entrapment efficiency in this study was not as high as reported in other reported studies. For example, Leo 2006 were able to achieve BSA loading of over 90% with PLA microspheres with similar initial BSA concentrations employed. The differences between these findings and those reported here could be related to the different type of emulsifier in external aqueous phase (Leo et al. used 0.5% pluronic F127, w/v, rather than PVA) and the preparation method in primary w/o emulsion process. In the studies conducted within this chapter vortex mixing was chosen instead of high-speed homogenisation in order to protect BSA from shear force and maintain the integrity of BSA, which could result in less efficient primary w/o emulsion formulation compared to homogenizing as was used by Leo et al. (8000 rpm homogenisation).

To investigate the effect of addition of emulsifier in o/w emulsion on BSA loading efficiency, 1% PVA (w/v) was added to the internal aqueous phase during in some preparations. Interestingly, the BSA loading efficiency was not significantly increased (p>0.05). Results from other researchers indicated the BSA loading efficiency of the formulations with emulsifier in primary o/w emulsion was even lower than of the
formulation without emulsifier. Blanco and Alonso (1998) prepared microspheres with 10 and 34 kDa PLGA and yielded a BSA entrapment efficiency of 91.53 ± 3.35 and 90.09 ± 1.57% respectively, compared to 68.24 ± 1.58 and 59.06 ± 4.18% for the formulations with 0.2% (w/v) poloxamer 188 added in primary w/o emulsion. A similar trend was found when using 20 kDa PLGA formulations entrapping lysozyme, but the decrease in entrapment efficiency on addition of emulsifier was not as pronounced as for BSA.

The high BSA loading efficiency with PLA microspheres could also be aided by the amphipathic nature of BSA, which led BSA formed an interfacial stabilizing film at the w/o interface working as surfactant in absence of a stabilizer (Schugens et al., 1994, Johanson et al., 1998). BSA was also used as internal emulsifier in PLA microspheres and improved the FIII9-10 protein entrapment efficiency up to 30% compared to 25% in which formulation PVA used as emulsifier (Bouissou et al., 2004). As the addition of PVA in primary w/o emulsion did not improved the BSA loading, the formulations without internal emulsifier was chosen for the further studies, which could avoid residual emulsifier/stabilizer inside the microspheres matrix.

2.3.4 Stability of PLA microspheres in aqueous environment

To measure the short term stability of the microsphere systems when stored at 4 °C or room temperature (distilled water and 0.2M PBS solution) their physico-chemical attributes were studied over a 1 month period. Measurement of the systems' zeta
potential indirectly indicates not only the surface charge related to the carboxylate end groups of PLA, but can also be used as an indicator for the stability of dispersed systems (Clarence 1985). Particles with high absolute values of zeta potential (e.g. above 40mV or below -40mV) will have a large surrounding electrical double layer and such particles will repel each other and prevent aggregation (Mu & Feng, 2001).

The zeta potential of all the microsphere formulations prepared from the three different molecular weight PLA were around neutral (Figures 2.9-12.) with only small fluctuations and no significant change in zeta potential of all formulations over the four weeks testing under all storage conditions. However the zeta potential measured in double distilled water were slightly positive in value (Figure 2.10 and 2.12.), while in PBS solution the results were slightly negative (Figure 2.11 and 2.13.), which indicated that the measurement was affected due to the ions in PBS solution. The carboxylate groups of PLA would be expected to produce at negative surface charge (Quellec et al., 1998). It could be predicted that PVA formed a layer between the carboxylate end groups of PLA and aqueous environment (Zambaux 1998) producing a more neutral surface.

In terms of mean particle size, no notable change was found for the formulations stored at 4 °C (Figure 2.14 and 2.15.). However, the mean particle size of the formulations stored in double distilled water at room temperature increased significantly (p<0.05) (Figure 2.16.). The microspheres made with 50 and 150 kDa
PLA stored in 0.2M PBS solution were also found increased in mean particle size (Figure 2.17.). However, at the same condition, no notable increase was found from 300 kDa PLA microspheres, which might caused by the relatively large standard deviation of the data. Although the zeta potential was not statistically changed, this might also due to the relatively large standard deviation of the data.

The results indicated that the microspheres show enhanced stability at 4 °C, with storage at room temperature resulting in aggregation of microspheres due to the neutral surface charge did not contribute to microspheres repelling each other. In the study of Kirby et al. (2008), the PLGA microspheres were stored in PBS solution at both 4 °C and room temperature, resulting stable in mean diameter and surface charge in a period of three month.

![Graph showing zeta potential over time of PLA microspheres](image)

Figure 2.10. Zeta potential over time of PLA microspheres (MW 50, 150 and 300 kDa) when stored at 4 °C in ddH₂O (mean ± SD, n=3).
Figure 2.13. Zeta potential over time of PLA microspheres (MW 50, 150 and 300 kDa) when stored at room temperature in 0.2M PBS solution (mean ± SD, n=3).

Figure 2.14. Mean particle size over time of PLA microspheres (MW 50, 150 and 300 kDa) when stored at 4 °C in ddH₂O (mean ± SD, n=3).
Figure 2.15. Mean particle size over time of PLA microspheres (MW 50, 150 and 300 kDa) when stored at 4 °C in 0.2M PBS solution (mean ± SD, n=3).

Figure 2.16. Mean particle size over time of PLA microspheres (MW 50, 150 and 300 kDa) when stored at room temperature in ddH2O (mean ± SD, n=3). * denotes significant increase in mean particle size (p<0.05) compared to the initial mean particle size at 0 day.
Figure 2.17. Mean particle size over time of PLA microspheres (MW 50, 150 and 300 kDa) when stored at room temperature in 0.2M PBS solution (mean ± SD, n=3).

Despite the retention of zeta potential at both storage medium, and the retention of particle size at 4 °C, storage of the microspheres under both temperature conditions resulted in loss of BSA loading over time (Figures 2.17-20). The BSA loading for the PLA microspheres store in double distilled water at 4 °C reduced by approximately 10% over the 4 weeks storage from an initial loading of around 72% (Fig 2.17). The rate of release was greater at room temperature, with the loading efficiency to 45.46 ± 5.32% (PLA Mw 50 kDa), 50.72 ± 2.83% (PLA Mw 150 kDa) and 57.34 ± 4.73% (PLA Mw 300 kDa) (Fig 2.19). At room temperature there was also a notable difference between the three molecular weight microsphere systems with the microspheres produced with the higher molecular weight PLA showing the least protein loss. This difference was not evident when the formulations were stored at 4 °C (Figure 2.18.). As similar trend was found in the presence of PBS, the leakage of BSA at 4 °C was minimal (Figure 2.19.), whereas the loading efficiency decreased to
44.32 ± 5.25% (PLA Mw 50 kDa), 46.36 ± 6.38% (PLA Mw 150 kDa) and 52.62 ± 4.78% (PLA Mw 300 kDa) at room temperature (Figure 2.21.).

Figure 2.18. BSA loading over time of PLA microspheres (MW 50, 150, and 300 kDa) when stored at 4 °C in ddH₂O (mean ± SD, n=3).

Figure 2.19. BSA loading over time of PLA microspheres (MW 50, 150, and 300 kDa) when stored at 4 °C in 0.2M PBS solution (mean ± SD, n=3).
Figure 2.20. BSA loading of PLA microspheres (MW 50, 150, and 300 kDa) when stored at room temperature in ddH₂O (mean ± SD, n=3). * denotes significant decrease in mean BSA loading efficiency (p<0.05) compared to the initial mean BSA loading efficiency at 0 day.

Figure 2.21. BSA loading of PLA microspheres (MW 50, 150, and 300 kDa) when stored at room temperature in 0.2 M PBS solution (mean ± SD, n=3). * denotes significant decrease in mean BSA loading efficiency (p<0.05) compared to the initial mean BSA loading efficiency at 0 day.
The three PLA microsphere formulations prepared with different molecular weight polymer exhibited poor stability at room temperature, based on the change in particle size and protein loss. All the formulations showed maintenance of zeta potential, but poor stability in particle size and BSA retention at room temperature. Although when stored at 4 °C, particle size and BSA leakage was slower, it could be predicted that extensive BSA loss occurs over prolonged times. These results suggested that the PLA microspheres were not suitable for storage in aqueous environments. Therefore, freeze-dried formulations were considered as an alternative means to providing long term stability.

2.4. Conclusion

The formulation parameters investigated in this study were the choice of PLA polymer molecular weight; PLA concentration and the PVA concentration. All three parameters were shown to influence mean particle size. To achieve microsphere formulations under 5 μm in aerodynamic diameter as needed for pulmonary delivery, microspheres were prepared using 3% w/w of PLA and 10% PVA. These parameters were selected based on smallest particle size and good uniformity, and subsequently being further studied. Increasing the molecular weight of PLA used in the formulation was shown to increase particle size, but it had no effect on protein loading. The BSA loading efficiency of all the formulations were around 70% for all three formulations both in presence or absence of PVA in the inner aqueous phase, which may be due to the stabilized interfacial film formed by protein-polymer interaction. Thus in the
following studies, PVA will not be added into the PLA microsphere formulations in order to avoid residual PVA inside the microspheres. Last but not least, the results of one month stability demonstrated that the PLA microsphere formulations are not stable for long term storage in an aqueous environment, and formatting these products in a dry dosage form may be a more suitable option.
Chapter 3

Freeze-drying of PLA microspheres
3.1. Introduction

Therapeutic protein and peptide drugs are increasingly being developed due to advances in the application of protein engineering and recombinant DNA technology (Hussain et al., 2004). However in terms of administration, proteins are difficult to deliver into the body due to their relatively fragile nature and large size. The lung is highly permeable to the proteins with the molecular weight less than 30 kDa, and the absorption of the proteins with large molecular weight (> 30 kDa) can be improved using absorption enhancer (Irngartinger et al., 2004). Given these characteristics, the pulmonary route has been considered as a potential route for protein delivery (Yu & Chien, 1997).

Proteins can be delivered into the lung either in the liquid or solid forms (Okumura et al., 1992; Irngartinger et al., 2004). However although the lung absorption of proteins can be improved in presence of absorption enhancer (Irngartinger et al., 2004), such formulations rarely provide sustained release, and lead to proteins being exposed to peroxidases, inflammatory and immunomodulatory mediators secreted by alveoli macrophages, causing protein degradation (Shen et al., 1999; Zhou et al., 1999). Nevertheless, in order to overcome the problems described above, particulate systems such as liposomes and micro/nanospheres have been developed as desirable carriers for pulmonary delivery of proteins, resulting in sustained release and improved drug efficacy (Kawashima et al., 1999; Huang & Wang, 2006; Sivadas et al., 2009).
It has been discussed in chapter 2 that the PLA microspheres have poor stability particularly in terms of protein retention when stored in aqueous solution, and formatting these products as a dry dosage form may be a more suitable option. Freeze-drying has been proved as a good technique to improve the long-term storage stability of microspheres (Chacón et al., 1999), and the resulted powders can be used in dry powder inhalation for pulmonary delivery (Puapermpoonsiri et al., 2009).

3.1.1. Freeze-drying

Freeze-drying (lyophilisation) is a dehydration process that is used in enhancing the shelf-life of various products within the pharmaceutical and food industries and has been used to improve the stability of a range of biopharmaceuticals including viruses, vaccines, proteins, peptides and colloidal carriers such as liposomes and microspheres (e.g. Abdelwashed et al., 2006a; Duncan et al., 2005; Mohammed et al., 2006).

Lyophilisation (freeze-drying) allows for the removal water from the system and produces a solid product with low molecular mobility. During freeze-drying frozen water is transformed directly from the solid phase to gas phase due to sublimation (Figure 3.1.) This results in the production of a dry product of which the stability is improved (Franks, 1998).
Figure 3.1. Schematic illustration of water phase during freeze-drying process. The blue line a-b represents freezing step; b-c represents decreasing of pressure by vacuum; c-d represents sublimation and drying step.

Freeze-drying basically has three steps: freezing, primary drying and secondary drying. In the first step of freeze-drying, the suspension samples are solidified by freezing. With ice crystals separating out of the liquid, the liquid suspension becomes more and more concentrated and subsequently yielding amorphous, crystalline or a combined amorphous-crystalline phase (Abdelwashed et al., 2006a). The choice of temperature used during freezing will dictate the size of ice crystals formed with lower temperatures increasing the rate of freezing and producing smaller ice crystals which may be advantageous since large ice crystals may cause more mechanical damage to samples. After the sample is frozen a vacuum is applied such that sublimation can occur. After primary drying residual moisture can remain (Pikal et al., 1990a) either bounded as hydrate water on the crystalline product surface or adsorbed in the solute, and/or it may also be dissolved in an amorphous solid (Pikal et al., 1990b). To remove
the adsorbed water, temperature needs to be raised higher in secondary drying step than in the primary drying step to break the hydrogen bonds or van der waals forces formed between the frozen material and the water molecules (Aulton, 2002).

3.1.2. Cryoprotectants

The formation of ice crystals during the freezing process can result in damage to lyophilised products in terms of destabilisation and aggregation due to the dehydration of the samples (Abdelwahed et al., 2006a). To address these issues and protect the samples against drying stress, cryoprotectants are often added to the sample suspension or solution before freezing (Abdelwahed et al., 2006b). Saccharides such as sucrose and trehalose (Figure 3.2.) have been proved efficient as cryoprotectants in microparticle freeze-drying (Miyajima et al., 1986).

There are several hypotheses that have been proposed to explain the role of saccharides in protecting microparticles during freeze-drying:

- The hydroxyls of sugars may form hydrogen bonds with the polar groups on the surface of particles forming a “hydrate membrane” in replacement for the water molecules lost during the freeze-drying and thereby prevent particles from exposure to the surroundings (Crowe & Crowe, 1988a; Zhang et al., 2006).

- Saccharides can also form a glassy layer which associates with the vitrification on the surface of particles, which makes the system more ‘sticky’ and reduces
the mobility of the particles. The high viscosity and low mobility of the particulate system reduces their aggregation and interaction (Levine & Slade, 1992; Crowe et al., 1994)

- Saccharides can also combine with the water molecules on the surface of solutes by hydrogen bonds, causing a lower stabiliser concentration than in the solution and a higher surface tension, blocking the interaction between solutes, known as preferential exclusion (Arakawa & Timasheff, 1984). Furthermore, the increase of solute concentration in the unfrozen fraction, due to the solutes separation out from water as it freezes, leads to particle isolation and can also prevent particles aggregation or diffusion (Crowe & Crowe, 1988b; Allison et al., 2000).
In addition to sugars, amino acids such as proline, histidine, arginine, lysine and histidine have been successfully used as an alternative to traditional sugar-based cryoprotectants (Crowe et al., 1994; Sterberg & Wadsten, 1999; Mohammed et al., 2007). In the case of amino acids, the functional group on the side chain of the amino acids may be involved in forming hydrogen bonds with the phosphate of the lipid head group and forming a stable amino layer to block the interaction between particulates such as liposomes (Mohammed et al., 2007), which functions similar to trehalose (Mohammed et al., 2006).
It has been reported that leucine has surfactant-like properties and a hydrophobic nature (Gliński et al., 2000), which may be involved in changing the surface morphology of spray-dried particles improving their dispersion behaviour and aerosolisation properties (Seville et al., 2007; Learoyd et al., 2009). However, the effects of leucine on the properties of microspheres prepared using W/O/W double emulsion solvent evaporation method have not been reported.

In this chapter, PLA microspheres were prepared in a freeze-dried format. The addition of sucrose or trehalose was investigated as cryoprotectant; L-leucine was also added into formulations both individually and combined with trehalose to see its effect on the physico-chemical microspheres.
3.2. Materials and methods

3.2.1. Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(D, L-lactide acid) (MW 50 kDa)</td>
<td>Polysciences, Inc. (Warrington, US)</td>
</tr>
<tr>
<td>Poly(vinyl alcohol) (MW 13 – 23 kDa, 87 - 89% hydrolysed)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Chloroform, laboratory grade</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (fraction V, 98–99% albumin)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bicinchorinic Acid Solution</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Copper Sulphate</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Sodium Hydroxide pellets</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>D(+)Trehalose dehydrate</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS) tablets</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
</tbody>
</table>

3.2.2. Methods

3.2.2.1. Preparation of freeze-dried PLA microspheres

PLA microspheres were prepared using double emulsion solvent evaporation method as described in section 2.2.2.1. The harvested PLA microspheres were then suspended in an aqueous solution containing cryoprotectant, and subsequently freeze-dried (Figure 3.3.).
Figure 3.3. Process of preparing PLA microspheres by using double emulsion solvent evaporation method.

3.2.2.2. Freeze drying

PLA microspheres were suspended in 1ml of aqueous solution containing cryoprotectant, and then freeze-dried as described in session 2.2.2.4. An investigation into the effects of addition and concentration of cryoprotectants on particle size after rehydration, zeta potential and BSA entrapment efficiency were determined. A range of cryoprotectants were investigated:

- **Sucrose**: The effect of addition of sucrose was investigated by resuspending the microspheres in 1ml sucrose solution at varying concentrations (3, 7 and 10%, w/v) before freeze-drying.

- **Trehalose**: The effect of addition of trehalose was investigated by resuspending
the microspheres in 1ml trehalose solution at varying concentrations (3, 7 and 10%, w/v) before freeze-drying.

- **L-leucine:** The effect of addition of L-leucine was investigated by resuspending the microspheres in 1ml L-leucine solution at varying concentrations (0.1, 0.3, 0.5, 0.7 and 1%, w/v) before freeze-drying.

### 3.2.2.3. Powder Characterisation

#### 3.2.2.3.1. Decreasing powder size by manual grinding

Initially, the freeze-dried products were directly rehydrated in double distilled water for the measurement of particle size and zeta potential to investigate the effects of cryoprotectants. Then the particle size of freeze-dried powders prior to hydration was reduced by manual grinding using a pestle and a mortar for 1, 5 and 10 minutes, in order to study the effect of grinding time on particulate diameter. The particle size of the freeze-dried powder products after freeze-drying but before manual particle size reduction could not be measured due to they were in the form of a freeze-dried cake.

#### 3.2.2.3.2. Mean size and size distribution analysis

The particle size and size distribution of the PLA microspheres contained within the freeze-dried powders was measured using laser diffraction (HELOS particle sizer plus CUVETTE dispersion unit, Sympatec, Germany) following dispersing the dry powder in double distilled water. In addition, the diameters of freeze-dried powders were
measured using dry powder laser diffraction (HELOS particle sizer plus VIBRO/RODOS dispersion unit, Sympatec, Germany).

3.2.2.3.3 Zeta potential analysis

Zeta potential was measured using ZetaPlus instrument (Brookhaven Instrument Corporation, NY) double distilled water at 25 °C as mentioned in section 2.2.2.3.

3.2.2.3.4 BSA loading efficiency

Measurement of BSA loading efficiency was carried out using BCA assay (section 2.2.2.5.)

3.2.2.3.5 Water content

The water content in the freeze-dried microsphere formulations was investigated by measuring the mass loss of the freeze-dried powders which was determined using a thermogravimetric analyzer (PerkinElmer Instruments) with a dry nitrogen purge and Pyris software. An open pan and a heating rate of 10 °C /min were used to determine the mass loss from 40 °C to 150 °C.

\[
\text{Water content (\%) } = \frac{\text{Weight loss}}{\text{Initial mass}} \times 100\%
\]

3.2.2.4 Stability of freeze-dried powders

Freeze-dried powders were sealed in a 4ml glass vial and stored in a condition
avoiding light at room temperature and 4°C. The mean particle size of freeze-dried powders. The mean particle size of rehydrated freeze-dried powders, zeta potential and BSA retention were measured every 7 days over a period of 4 weeks.

3.2.2.5. *In vitro* release

15 mg of freeze-dried microspheres or the weight of powders equivalent to 15 mg microspheres were suspended in 15 ml 0.2 M PBS solution, pH 7.4, with sodium Azide (0.02%, w/v) and incubated in a 37 °C water bath with horizontal shaking. Samples (1 ml / 15 ml) of the suspension were removed at fixed time intervals (0, 1, 2, 4, 8, 24hr, 2days, 4 days, 7 days, 14 days, 21 days and 28 days) and replaced with equivalent amounts of fresh PBS solution to maintain constant volume/sink conditions. The samples were centrifuged (10000 g) for 10 min and the supernatants collected. The amount of BSA in the supernatant was determined using a BCA assay. The *in vitro* release profiles were generated in terms of cumulative protein release versus time.

3.3. Results and discussion

3.3.1 Effects of addition and concentration of cryoprotectants on particle size and zeta potential

3.3.1.1. Sucrose

The diameter of PLA microspheres in the absence of cryoprotectant was significantly (p<0.001) increased from 1.13 ± 0.01 to 5.5 ± 0.17 µm after being freeze-dried (Figure 3.4.), with a multi-modal distribution being seen (Figure 3.5.) which indicated microsphere aggregation and/or interparticulate cohesion occurred during the freeze-drying process caused by freezing and drying stresses. The addition of sucrose
was effective in maintaining mono-modal size distribution (Figure 3.3.) and circumventing the particle size growth, and its efficiency was observed to be a concentration-dependent effect (Figure 3.4). Overall a decrease in mean particle size of the microspheres from $2.53 \pm 0.28$ to $1.07 \pm 0.03 \mu m$ was seen as the concentration of sucrose increased from 3 to 7% (w/v). Further increasing the sucrose concentration from 7 to 10% produced microspheres not significantly different in size compared to those noted prior to freeze-drying (Figure 3.4).

![Graph showing particle size distribution](image)

Figure 3.4. Mean particle size and size distribution of PLA (Mw 50 kDa) microspheres before and after freeze-drying in the presence of varying concentrations of sucrose (w/v) solution or double distilled water (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.05); ** denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.001).
In terms of zeta potential, no significant change was found across the various concentrations of sucrose, indicating the surface charge of the microspheres was not affected by freeze-drying and addition of sucrose solution at all the concentrations studied (Figure 3.6.). These results also suggested the function of sucrose as a cryoprotectant is more likely to be vitrification, the concentration of sucrose solution was increased due to the ice formation and vacuum sublimation, and this concentrated solution will transform into a viscoelastic rubber and then finally vitrify to a stable glass as water removing (Sun et al., 1996). Furthermore, The multi-hydroxyl of sucrose may involved in maintaining the physical characters of PLA microspheres,
hydroxyl can interact with H₂O molecules on the surface of microspheres that lead to an increasing of the concentration and the viscosity of the microsphere suspension, which may reduce the growth of ice crystals and preserve the microparticles from mechanical damage (Zhang et al., 2008). According to the review of Hancock et al. (1997), amorphous or imperfect ice crystalloids will be produced due to the eutectic formed from multi-hydroxy compounds in the presence of water and the microparticles will therefore be protected during the process of the ice crystallisation.

![Graph showing Zeta potential of PLA microspheres](image)

Figure 3.6. Zeta potential of PLA microspheres (Mw 50 kDa) before and after freeze-drying in the presence increasing concentrations of sucrose solution, measured in double distilled water (mean ± SD, n=3).

### 3.3.1.2. Trehalose

Trehalose was also shown to influence the particle size of microspheres when present during the freeze-drying process. However, in contrast to sucrose, microspheres lyophilised in the presence of trehalose showed an increase in mean particle size from 1.25 ± 0.02 to 3.56 ± 0.03 μm as the trehalose concentration increased from 3 to 10 %
(w/v; Figure 3.7.). Compared with sucrose, trehalose is more hydrophobic, and it has fewer internal hydrogen bonds which allows it to create more flexible bonds with microparticles during the freeze-drying process (Abdelwahed et al., 2006a), which may be the reason for the difference between the concentration-dependent effect of the two cryoprotectants. However, other studies (Taylor & Zografi, 1998) have proposed the reason for this to be due to sucrose forming an amorphous matrix with a lower glass transition temperature. Nevertheless, similar to sucrose, no significant change in zeta potential occurred with the addition of trehalose solution at all the concentrations studied (Figure 3.8.)

![Graph showing mean particle size and size distribution of PLA microspheres before and after freeze-drying](image)

**Figure 3.7.** Mean particle size and size distribution of PLA (Mw 50 kDa) microspheres before and after freeze-drying in the presence of varying concentrations of trehalose (w/v) solution or double distilled water (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.05); ** denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.001).

Previous studies mention that trehalose forms hydrogen bonds through its hydroxyl groups bonding with the polar region of the lipid head group. This results in the
replacement of the water around the bilayer of liposomes and circumvents freezing and drying stress (Crowe & Crowe, 1988a). Further, trehalose can form hydrogen bonds with nanoparticles during lyophilisation due to the absence of internal hydrogen bounds (Abdelwahed et al., 2006a). These two views suggest that trehalose may also substitute for water replacement role if the surface of the PLA microspheres have polar groups. According to the studies of Guo et al (2007), the PVA molecules adsorbed on the surface of microspheres offer polar groups. However, the surface charge of the PLA microspheres is nearly neutral, which means the surface of these microspheres have relatively few polar groups, thus the water replacement may not be the main cryoprotective function of trehalose in this case.

It seems vitrification is one of the most reasonable cryoprotective functions of trehalose: trehalose can form a more stable glass layer around particles due to its higher glass transition temperature than sucrose (Crown et al., 1996). Cryoprotection may also be caused by the higher viscosity of trehalose compared with than other disaccharides at the same concentration (Sola-Penna & Meyer-Fernandes, 1998). Furthermore, the occupied hydration volume of trehalose is 2.5-fold larger than sucrose in bulk water, which may improve the blocking effect on the interaction between the solute (Sola-Penna & Meyer-Fernandes, 1998).
Figure 3.8. Zeta potential of PLA (Mw 50 kDa) microspheres before and after freeze-drying in the presence increasing concentrations of trehalose solution, measured in double distilled water (mean ± SD, n=3).

Whilst in these studies trehalose was slightly less effective than sucrose at maintaining the particle size of the microspheres after freeze-drying compared to sucrose, trehalose is generally considered as a preferable cryoprotectant due to its advantages in comparison with other sugars, such as less hygroscopicity, flexible formation of hydrogen bonds, low chemical reactivity and higher glass transition temperature (Crowe et al., 1992; Crowe et al., 1996; Abdelwahed et al., 2006). Thus trehalose was chosen as the sugar-based cryoprotectant for the following studies.

3.3.1.3. L-leucine

L-leucine also offers a concentration-based cryoprotective effect on particle size of PLA microspheres: inclusion of increasing amounts of L-leucine from 0.1 to 1 % (w/v) as cryo-protectant resulted in a decrease in the mean size of microspheres from 5.20 ± 0.17 to 1.37 ± 0.01 μm with a leucine concentration of 0.7 and 1% (w/v) producing
microspheres not significantly different in size compared to the sizes noted prior to freeze-drying (Figure 3.9.). It is notable that the mean particle size is mostly affected in the D50 and D90 factions with no significant difference in D10 for the formulations in presence of leucine at concentrations in the range of 0 – 0.5% (w/v), and this was seen to some extent with sucrose and trehalose but most notable with L-leucine.

![Bar chart showing particle size distribution](image)

**Figure 3.9.** Particle size and size distribution of PLA (Mw 50 kDa) microspheres before and after freeze-drying in the presence of varying concentrations of L-leucine solution (w/v) or double distilled water (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.05); ** denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.001)

The zeta potential results (Figure 3.10.) showed no notable change was found before and after freeze-drying or in the presence of L-leucine solution at all the concentration studied, similar to the previous results with sucrose and trehalose. However as previously noted, as zeta potential is measured in an aqueous media, these results only verify that L-leucine does not change the zeta potential of the particles. L-leucine is one of the most hydrophobic amino acids structurally similar to isobutanol (Dixit et al., 1997), and the poor solubility of L-leucine may enhance the particle isolation thus
improve the cryoprotection. Furthermore, L-leucine has been shown to have very weak surface activity (Gliński et al., 2000), which may also contribute to its cryoprotective effect.

![Graph showing zeta potential of PLA microspheres before and after freeze-drying in the presence of increasing concentrations of L-leucine solution](image)

Figure 3.10. Zeta potential of PLA microspheres before and after freeze-drying in the presence of increasing concentrations of L-leucine solution, measured in double distilled water (mean ± SD, n=3).

### 3.3.2. Mixture of trehalose and L-leucine as cryoprotectant

As both trehalose and L-leucine had the ability to maintaining the physico-chemical properties of the PLA microspheres, and the L-leucine has potential of to improve aerosolisation performance of powders (Seville et al, 2007; Learoyd et al, 2009). The combination of trehalose and L-leucine together was investigated to improve cryoprotection and dispersion. Initially 7% trehalose + 1% L-leucine (w/v) was used but due to the reduction in particle size being proportional to the concentration of trehalose, the subsequent study decreased the concentration of trehalose to 6% (w/v).
Prior to freeze-drying, the microspheres had a mean particle size of 5.50 ± 0.17 μm. Freeze drying in the presence of either 7% trehalose or 1% L-leucine resulted in significant (p<0.001) decreases in mean particle size to 1.48 ± 0.08 μm and 1.37 ± 0.01 μm respectively. Trehalose and L-leucine together resulted in a smaller mean particle size than when used separately (Figure 3.11.), with microspheres of mean particle size of 1.09 ± 0.03 μm and 1.33 ± 0.01 μm being produced when freeze-dried in the presence of 1% L-leucine with either 6% or 7% trehalose respectively. This suggested that the combination of trehalose and L-leucine offered enhanced protection to the microsphere formulations and can maintain particle size more efficiently during the drying process. Of the combinations, 6% w/v trehalose and 1% w/v L-leuince as cryoprotectants was found to give the smallest particle size (1.09 ± 0.03 μm) following freeze-drying and subsequent rehydration of the dry powder, and this combination was subsequently used for cryoprotection in further studies. The synergistic effect of this combination is unknown and could be due to surfactant action of leucine enhancing the ability of trehalose to interact with the microspheres and replace the presence of water at the particle surface.
Figure 3.11. Mean particle size and size distribution of PLA (Mw 500,000 Da) microspheres before and after freeze-drying in the presence of trehalose and/or L-leucine solution (w/v) or double distilled water (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.05); ** denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.001).

Figure 3.12. Zeta potential of PLA microspheres before and after freeze-drying in the presence increasing concentrations of L-leucine solution, measured in double distilled water (mean ± SD, n=3).
There was no notable change found in zeta potential for any of the preparations with near neutral zeta potentials being noted (Figure 3.12.) as would be predicted based on previous results; neither the trehalose or L-leucine showing an interaction with the microsphere particle surface when suspended in an aqueous media.

Due to their desirable diameters, the following formulations were chosen for the further studies: a. 7% trehalose; b. 1% L-leucine; c. 7% trehalose and 1% L-leucine; d. 6% trehalose and 1% leucine. A formulation without cryoprotectant was used as a control.

3.3.3. Decreasing powder size by manual grinding

The resulting freeze-dried products were initially “cake-like” due to the existence of the cryoprotectants, and obviously not suitable for inhalation. In order to make them inhalable, the freeze-dried products were grinded into powders using a pestle and a mortar.

In order to investigate the effect of grinding time on particulate diameter, the freeze-dried formulations containing 6% trehalose and 1% leucine (w/v) were sized either in aqueous media or as the dry powders after being ground for 1, 5 and 10 minutes.
Figure 3.13. Mean particle size and size distribution of PLA (Mw 500,000 Da) microspheres freeze-dried in the presence of trehalose (6%, w/v) and L-leucine (1%, w/v) using dry powder laser diffraction following manual grinding for 1, 5 or 10 minutes (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison between the two groups (p<0.05); ** denotes significant difference in distribution parameter in comparison between the two groups (p<0.001).

Figure 3.13 shows the effect of manual size reduction on dry powder particle size; after 5 minutes manual grinding, the mean particle size of the freeze-dried powders was reduced to 47.14 ± 9.87μm from a cake like bulk. Grinding for a longer time was shown a further decrease in the diameter of the freeze-dried powders, and the particle size could reach 12.76 ± 3.14μm and 5.10 ± 0.54μm after grinding for 5 and 10 minutes respectively. Although the particle size was reduced significantly (p<0.001), it was still far from the size required for lung deposition (<3μm in aerodynamic diameter, indicating the physical size should be even smaller). However, grinding may be a potential method to decrease particle size.

Although the particle size of the freeze-dried powders was significantly reduced...
during the 10 minutes grinding ($P < 0.001$), the diameter of the PLA microspheres contained in the freeze-dried powders was not affected at all, with the microspheres maintaining their particle size and mono-modal size distribution after grinding for 1, 5 and 10 minutes (Figure 3.14 & 3.15). After rehydration, trehalose and L-leucine as cryoprotectants were dissolved in double distilled water, while the PLA microspheres remained, which indicated that the grinding action was able to break down the cryoprotectant component of the freeze-dried bulk, but did not influence the microspheres.

![Graph showing particle size distribution](image)

Figure 3.14. Mean particle size and size distribution of re-hydrated PLA (Mw 500,000 Da) microspheres freeze-dried in the presence of trehalose (6%, w/v) and L-leucine (1%, w/v) after being manual grinding for 1, 5 or/and 10 minutes (mean ± SD, n=3).
3.3.4. Dry powders sizing of other formulations

The formulations in absence of L-leucine were also ground for 10 minutes, followed by sizing in the powder format. Results were in comparison with the formulations containing L-leucine to investigate the effect of L-leucine on dry powder size.

As shown in Figure 3.16., the assessment of the dry powder particle size after freeze-drying revealed mean particle sizes in excess of 20 microns. These sizes were at least 4 fold larger than those reported for the microspheres measured after resuspension in aqueous media, suggesting significant aggregation of the microspheres with the cryoprotectant in the dried state. These larger sizes would prohibit the delivery of microspheres to the lung as a dry powder format and therefore further particles size was undertaken by manual particle size reduction.

Assessment of the particle size distribution of the freeze-dried powder following
manual particle size reduction for 10 minutes indicated that use of 6% w/v trehalose and 1% w/v L-leucine as cryoprotectants during freeze-drying also resulted in the smallest dry powder particles (5.10 ± 0.54 μm) (Figure 3.16).

![Particle size distribution chart](image)

Figure 3.16. Mean particle size and size distribution of PLA (Mw 50 kDa) microspheres powders after freeze-drying measured using dry powder laser diffraction following manual particle size reduction (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.05); ** denotes significant difference in distribution parameter in comparison to before freeze drying (p<0.001)

### 3.3.5. Effect of addition of cryoprotectants on BSA loading efficiency

The BSA loading efficiency of the freeze-dried formulations was also determined using a BCA assay. As shown in Figure 3.17, using 7% trehalose or 1% L-leucine (w/v) alone as cryo-protectant did not affect the BSA loading efficiency in comparison with the formulation without any cryo-protectant. Furthermore, the combined use of 6 or 7% trehalose with 1% leucine (w/v) also resulted a maintained loading efficiency as initial about 72% (w/w). Results indicated the freeze-drying did not affect the BSA loading efficiency.
3.3.6. Water content

The residual moisture in all the freeze-dried PLA microspheres powders in this case have been showed in Table 3.1 In all cases the moisture content was around 4% with no significant difference between the various preparations. These results are consistent with the previous studies by Konan et al. (2003) investigating the water content in meso-tetral (4-hydroxylphenyl) porphyrin-loaded PLA/PLGA nanoparticles with residual moisture of 4.2 to 5.1 % (w/w) being reported. The moisture content is primarily dictated by the freeze-drying protocol employed. Some water may not separate out as ice during the freezing step, so would not sublime during the first drying step, but instead may absorb to the surface of the crystalline products, or exist in the solute phase either as hydrate water or dissolved in an amorphous solid to form a solid solution (Pikal & Shah, 1990b). Secondary drying is the main process that
removes this unfrozen water (Abdelwahed et al., 2006a). Furthermore, the presence of cryoprotectants can also influence residual moisture content due to the possible presence of residual moisture caused by unfrozen water trapped in the trehalose matrix (Abdelwahed et al., 2006a).

<table>
<thead>
<tr>
<th>Cryoprotectants</th>
<th>Initial mass (mg)</th>
<th>Weight loss (mg)</th>
<th>Water content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no cryo</td>
<td>3.197 ± 0.340</td>
<td>0.124 ± 0.008</td>
<td>3.90 ± 0.16</td>
</tr>
<tr>
<td>7% trehalose</td>
<td>3.323 ± 0.239</td>
<td>0.140 ± 0.005</td>
<td>4.23 ± 0.20</td>
</tr>
<tr>
<td>1% L-leucine</td>
<td>3.252 ± 0.162</td>
<td>0.136 ± 0.003</td>
<td>4.18 ± 0.17</td>
</tr>
<tr>
<td>7% trehalose + 1% L-leucine</td>
<td>3.071 ± 0.075</td>
<td>0.124 ± 0.007</td>
<td>4.05 ± 0.15</td>
</tr>
<tr>
<td>6% trehalose + 1% L-leucine</td>
<td>3.141 ± 0.083</td>
<td>0.132 ± 0.003</td>
<td>4.19 ± 0.11</td>
</tr>
</tbody>
</table>

Table 3.1. Moisture content of BSA-loaded PLA (MW 50 kDa) in presence of different cryoprotectants (Mean±SD, n=3).

3.3.7. Stability of freeze-dried powders

To investigate the stability of these various freeze-dried microsphere formulations their stability in terms of retention of physico-chemical attributes (mean particle size, zeta potential and protein loading) was measured over four weeks storage under an anhydrous environment at either room temperature and 4 °C.

The particle size of the dry powders of the various formulations when stored at room temperature and 4 °C are shown in Fig 3.16 and 3.17 respectively. Storage under these conditions resulted in no significant change in particle size for all powder formulations tested. The particle size and zeta potential of the microspheres after rehydration was also measured: under both storage conditions the mean particle size
of the microspheres (Figure 3.20 & 3.21) or their zeta potential (Figure 3.22 & 3.23) did not significantly change over the time course of the study. These results confirmed that the ~4% residual moisture did not induce an aggregation and was not a problem in terms of short term storage of these formulations and that the freeze-dried powders with addition of cryoprotectants could rapidly redisperse into water to produce microspheres with mean diameters similar to those initially formulated (Figure 3.20 & 3.21).

Protein (BSA) retention was also measured over the 4 weeks under the above conditions (Figure 3.24 & 3.25). In both conditions no significant change in protein loading was observed with BSA retention remaining consistent (~72% w/w) with or without addition of cryoprotectant. This suggests that any aggregation occurring (e.g. in the absence of cryoprotectant) does not influence protein-loaded systems.
Figure 3.18. Stability of mean particle size over time of PLA (MW 50 kDa) microsphere powders after freeze-drying in the presence of various cryoprotectants when stored at room temperature (mean ± SD, n=3).

Figure 3.19. Stability of mean particle size over time of PLA (MW 50 kDa) microsphere powders after freeze-drying in the presence of various cryoprotectants when stored at 4 °C (mean ± SD, n=3).
Figure 3.20. Stability of mean particle size over time of PLA (MW 50 kDa) microsphere powders after freeze-drying in the presence of various cryoprotectants when stored at room temperature (mean ± SD, n=3).

Figure 3.21. Stability of mean particle size over time of PLA (MW 50 kDa) microsphere powders after freeze-drying in the presence of various cryoprotectants when stored at 4 °C (mean ± SD, n=3).
Figure 3.22. Stability of zeta potential over time of PLA (MW 50 kDa) microsphere powders after freeze-drying in the presence of various cryoprotectants when stored at room temperature (mean ± SD, n=3).

Figure 3.23. Stability of zeta potential over time of PLA (MW 50 kDa) microspheres powders after freeze-drying in the presence of various cryoprotectants when stored at 4 °C (mean ± SD, n=3).
Figure 3.24. BSA loading over time of PLA (MW 50 kDa) microsphere powders after freeze-drying in the presence of various cryoprotectants when stored at room temperature (mean ± SD, n=3).

Figure 3.25. BSA loading over time of PLA (MW 50 kDa) microsphere powders after freeze-drying in the presence of various cryoprotectants when stored at 4 °C (mean ± SD, n=3).
3.3.8 *In vitro* release

It has been well known that the drug release profile of a polymeric microparticle systems always start with a rapid burst release (Kwong et al., 1986), this may due to the fast release of the drug loaded/absorbed on the surface of the microparticles.

In the case of the PLA microspheres formulated in these studies, an initial burst release occurred for all the formulations, with over 50% of loaded BSA being released from PLA microspheres in the first 8 hours, and then another 10% over the next 16 hours at a relative slow rate (Figure 3.26.). The rest of the loaded BSA would be released gradually over a period of 4 weeks (Figure 3.27.). The release profiles for all formulations (without cryoprotection or with the various cryoprotectants) were not significantly different, demonstrating that aggregation resulting from freeze-drying did not influence protein release nor did the presence of any of the cryoprotectants employed. This again confirmed previous results suggesting that the cryoprotectants used did not influence the integrity of the systems compared to unprotected systems and that upon rehydration the microspheres were resuspended with no associated interactions with the cryoprotectants.

In general, drug release from polymeric microspheres involves a diffusive process through the polymer matrix and polymer degradation (Johnson et al., 1996; Sinha & Trehan, 2003). The dispersion of the drug on the microsphere surface leads to an initial burst release. It has been argued that maintaining a small microsphere particle
size (e.g. by employing cryoprotectants) could promote a fast release rate, with the small volume diameter leading to a large surface area contact with the dissolution media as was previously proposed by Ei-Baseir & Kellaway (1998). However the results shown in our studies do not support this given that microspheres freeze-dried in the absence of any cryoprotection were around 20 fold larger in size than those cryoprotected with the trehalose/leucine combinations.

![Graph showing cumulative BSA release](image)

Figure 3.26. Cumulative BSA release (%, w/w) vs time in the first 24 hours (mean ± SD, n=3).
Figure 3.27. Cumulative BSA release (%, w/w) vs time in a period of 4 weeks (mean ± SD, n=3).

3.4. Conclusion

Addition of cryoprotectants is definitely necessary to preserve the PLA microsphere formulations studied from the freezing and drying stress during lyophilization, which results in particle aggregation. Both sugar-based cryoprotectants and amino acids showed good cryoprotective ability on PLA microspheres, with sucrose offering the most cryoprotection. In all cases the zeta potential was unchanged from its initial value, and particle diameter can be regulated by varying the type or concentration of cryoprotectant. Because of the neutral surface of the microspheres in this study, the stabilisation mechanism of sucrose and trehalose, was more likely to be vitrification, forming a glassy layer around the microspheres, rather than interaction with the surface forming hydrogen bonds. In contrast, L-leucine functions by increasing in concentration in solution and then performing particle isolation. Although the mechanism of combined use of trehalose and L-leucine as cryoprotectant is uncertain, it did result in a smaller particle size than the formulations cryoprotected with these
agents separately suggesting a synergistic action.

All freeze-dried microsphere formulations were stable in terms of the characteristics measured when sealed in an anhydrous environment and stored for up to four weeks, with no changes in particle size, surface charge, or BSA loading efficiency. Furthermore, the freeze-dried powders showed an instantly dispersion in double distilled water and maintain the physico-chemical characteristics before being freeze-dried. Release profiles for all the formulations were similar with the microspheres demonstrated an initial burst release followed by a gradual release of the residual BSA load, offering the potential for sustained drug release. This initial burst release supported the need for these systems to be stored in a dried format.
Chapter 4

Aerosolisation profiles of PLA microsphere freeze-dried powders by dry powder inhalation and nebulisation
4.1. Introduction

In terms of pharmaceutical devices, drug delivery to the lung mainly employs either: dry powder inhalers (DPI), pressured metered-dose inhalers (pMDI) or nebulisers (Daniher & Zhu, 2008). However, it has been discussed in section 2.3.3. that the PLA microspheres previously developed in these studies are not suitable for long term storage in aqueous environment, thus such microspheres are not suitable for pMDI because of its moisture condition. However, the stability of these systems in a freeze-dried formulation showed promise (section 3.3.7) suggesting the possibility of delivering microspheres to the lung in this format. In this study PLA microspheres were prepared into a dry powder form with the aim to deliver them using a DPI. These powders were compared to the delivery of rehydrated microspheres using a nebuliser.

4.1.1. Dry powder inhalers (DPIs)

DPIs were developed to be an alternative of pressurised metered dose inhalers (pMDIs) to avoid the request for propellants such as chlorofluorocabons (CFC) (Daniher & Zhu, 2008). DPIs are breath-actuated, thus no breath-hand co-ordination is required, but the lung deposition dose delivered using DPIs is affected by the patient’s inhalation abilities (Hickey et al, 1994; Chavan & Dalby, 2002). Within this chapter the potential of using DPI to deliver microsphere powders was investigated using an Eclipse dry powder inhaler (Aventis). The Eclipse DPI is a multi-unit dose inhaler and can be loaded with four size 2 capsules; the powders are then released after the capsules are cut open by the knife in the device when screwing the trigger (Figure 4.1.)
4.1.2. Nebulisers

Nebulisers have been used for the treatment for respiratory diseases such as asthma since late 19th century (Labiris & Dolovich, 2003). It has been reported that nebulisers can be used in atomizing most aqueous based drug solutions (Pettis et al, 2000 dipeptide), suspensions (Massinde & Hickey, 1993), liposomes (Taylor & Farri, 1993; Zaru et al, 2007), biodegradable microspheres (Massinde & Hickey, 1993) and nano particles (Kawashima et al, 1999; Dailay et al, 2003). Particularly in the research of Kawashima et al (1999), insulin was delivered via nebulised PLGA nanospheres to the guinea pig, resulting in a prolonged hypoglycaemia (48 hours) compared to nebulisation of insulin solution (6 hours). Pettis et al (2000) delivered muramyl dipeptide to the lung of guinea pig, and successfully activated the alveolar macrophages. Nebulisers deliver large doses over multiple breaths, especially suitable for infants, elderly and critically ill patients who are disable to operate the inhalation devices, and no breath-hand co-ordination required. There are two main type of nebulisers, air-jet nebulisers and ultrasonic nebulisers.
4.1.2.1. Air-jet nebulisers

Jet nebulisers are able to propel liquid droplets via the action of compressed air from a compressor, passing though a narrow orifice. This produces a low pressure area at the outlet of the liquid feed chamber, which leads the liquid being drawn up to collapse into droplets which are subsequently sprayed out of jet nebuliser. Large droplets impact on the baffle and then return to the liquid feed chamber for renebulisation (O’Callaghan & Barry, 1997; Labiris & Dolovich, 2003). A Pari LC air-jet nebuliser (Pari, Germany) was used to deliver the suspension of PLA microsphere powders in the current studies (Figure 4.2).

![Image of Air-jet nebuliser (Pari LC)](image)

Figure 4.2. Air-jet nebuliser (Pari LC)

4.1.2.2. Ultrasonic nebulisers

In contrast to air jet nebulisers, ultrasonic nebulisers employ a piezoelectric crystal to generate high frequency vibrations which are transmitted on to the surface of the liquid making the surface unstable. Subsequently, droplets are formed in the nebuliser chamber which are then nebulised out of the nebuliser (O’Callaghan & Barry, 1997;
Labiris and Dolovich, 2003). A Pari eFlow nebuliser (Pari, Germany) (Figure 4.3) was also used to deliver the suspension of PLA microsphere powders in these studies and compared to the air-jet system.

![Ultrasonic nebuliser (Pari eFlow)](image)

Figure 4.3. Ultrasonic nebuliser (Pari eFlow)

### 4.1.3. Aerodynamic diameter sizing and distribution by *in vitro* impaction

Aerodynamic diameter, defined as “the diameter of a sphere of unit density (1g/ml) that has the same settling velocity as the particle in question” (Mohr, 1991), is one of the most important factors which effects on the deposition of aerosols in the lung (Holzner & Müller, 1995).

#### 4.1.3.1. Methods to determine aerodynamic diameter

There are various methods that can be used to measure particle size including light scattering, microscopy, sieving and cascade impaction. Sizing of the particulates using light scattering technologies and microscopy results an estimated physical particle size being noted whilst cascade impaction has been applied widely for evaluating the
aerodynamic behaviour of particles which can be correlated to their deposition in the respiratory air way (Timsina et al, 1994). The mechanism of cascade impaction is based on the theory of inertial separation wherein particles with different aerodynamic diameters have different velocities and directions in the air flow, which affects their deposition site of the particles (Figure 4.4.; Hickey, 1990). Particles with larger diameters (and sufficient inertia) will impact on to the retention surface of the upper stages, which have a bigger effective cut-off diameter. In contrast, particles with small diameters are more likely to enter the lower stages due to their improved flow properties.

![Figure 4.4. Schematic illustration of cascade impaction](image-url)

4.1.3.2. Mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD)

Mass median aerodynamic diameter (MMAD) is a size parameter used to reveal the inertial properties and size distribution of particles, which measured using cascade
impaction method in order to present aerodynamic diameter (Hickey & Martonen, 1993). It is the diameter of a sphere of unit density that has the same aerodynamic properties as a particle of median mass from aerosol (O'Callaghan & Barry, 1997). Furthermore, MMAD can be also defined as the diameter at the 50% mark of a plot of cumulative fraction versus effective cut-off diameter (Seville et al, 2007).

The geometric standard deviation (GSD) indicates the variability of the particle size and the width of the size distribution in the impactor or impinger (Hickey et al, 1990; Labiris & Dolovich, 2003). It can be calculated from the ratio of the MMAD to the effective cut-off diameter at 16% point on the cumulative distribution curve (Dunbar et al, 2005).

4.1.3.3. Fine particle fraction (FPF)

The fine particle fraction indicates the respirable particle portion of the total dose. Usually, the dose of sub-5μm particles in aerodynamic diameter is considered as the fine particle fraction (Borgström & Newman, 1993; Smith et al, 1998). In some research, the FPF has been limited to 1-3 μm, to ensure the particles can reach the alveolar region (Zanen et al, 1992).

4.1.3.4. Devices for cascade impaction

There are three popular types of impactors: twin stage impinger (TSI), multi-stage cascade impactor (MSCI) and multi-stage liquid impinger (MSLI).
1. **Twin stage impinger (TSI):** This separates the aerosol dose into two parts by the cut-off between of the two stages (Figure 4.5.); the cut-off size is 6.4 μm at an air flow rate of 60 L/min (Miller et al, 1992). Thus all the particles larger than 6.4 μm will remain in the upper stage while particles smaller than 6.4 μm can reach the lower stage (Smyth et al, 2005), the aerosols entering the lower stage are considered as the respirable dose, the others are considered unrespirable (Hallworth & Westmoreland, 1987) (Fig 4.4). Although the results from the twin stage impinger was correlated with the clinical performance of aerosols (Hallworth & Westmoreland, 1987), it only separates samples into two size categories (due to its dichotomous sampler) and is unable to reveal the size distribution of the aerosols, which is critical in lung deposition (Zeng et al, 2001).

![Figure 4.5. Twin stage impinger (schematic illustration from Smyth et al, 2005)](image-url)
2. **Multi-stage cascade impactors (MSCI):** Examples of these include the Andersen cascade impactor (ACI) and the next generation impactor (NGI).

ACI is an eight-stage impactor with the effective cut-off diameters of each stages calibrated at the flow rate of 60 L/min are 6.18, 3.98, 3.23, 2.27, 1.44, 0.76, 0.48 and 0.27 μm (Figure 4.6.;Pharmacopeial Forum, 1996). ACI also divides aerosols by their diameters, and the small particles follow the air flow and enter the next stage while large particles with sufficient inertia will impact on to the collection plate (Swanson et al, 1996; Dunbar et al, 2005). ACI is the most widely used impactor for characterising pharmaceutical aerosols (Stein, 2008), and yield more detail in terms of aerodynamic size distribution due to the multiple stages (Zeng et al, 2001). However, particles may bounce from the impaction surface of the collection plate when the impulse of the impacting particles is greater than adhesion force (Dunbar et al, 2005). The rebounded particles can deposit on the subsequent stages following the air flow, causing a bias towards a smaller mass median aerodynamic diameter (MMAD) (Tzou, 1999). Although particle bouncing to subsequent stages is a serious problem with inertial impactors, Dunbar et al (2005) minimized these bounce effects by placing glass fiber filters with a pore size of 20 μm, saturated in water, on inverted impaction plates.
The next generation impactor (NGI) is high performance cascade impactor developed specifically for pharmaceutical aerosol testing with appropriate effective cut-off size over a wide range of air flow rates (30-100 L/min) (Marple et al, 2003). In comparison with ACI, NGI is easy to be operated and can be get ready to for the next test quickly, providing better sample recover and less internal particle losses (Mitchell et al, 2003).

3. **Multi-stage liquid impinger**: This consists of a throat with a rubber mouthpiece, four impaction stages and an integral filter stage, the aerosol particles in an air flow through MSLI were separated based on their aerodynamic particle size (British Pharmacopeia, 2001) (Figure 4.7.). At an air flow rate of 60 L/min, the effective cut-off diameters of impaction stages 1 to 4 are 13, 6.8, 3.1 and 1.7 µm
respectively (Zeng et al, 2001). A piece of filter paper is put in the filter stage to capture the remaining fraction of particles less than 1.7 μm. Particle bounce and reentrainment can be minimised because of the higher moisture environment in the stages 1 to 4 of MSLI and there is minimal inter-stage losses because all the inner surfaces of the impinger are easily rinsed (Asking & Olsson, 1997; Zeng et al, 2001;). However, MSLI may not suitable for evaluating the aerosolisation performance of the aerosols that are unstable in aqueous environments.

![Illustration removed for copyright reasons](image)

Figure 4.7. Multi-stage liquid impinger (schematic illustration of Clark & Borgström, 2002).

The aim of the work in this chapter was to investigate the aerosolisation profile of the freeze-dried microsphere powders using the Eclipse DPI, or using ultrasonic (Pari eFlow) and air-jet nebulisers (Pari LC) after resuspension in ddH₂O. The two key factors measured were:

1. Delivery of the PLA microsphere powder formulations in the presence of the
cryoprotectants (7% trehalose and/or 1% leucine, or 6% trehalose and 1% leucine, w/v, respectively) using the Eclipse DPI, in order to investigate their *in vitro* deposition in MSLI.

2. Delivery of the suspension of PLA microsphere powders using the ultrasonic and air-jet nebulisers to investigate their *in vitro* deposition of nebulisation in MSLI and also be in comparison with DPI.

4.2. Materials and methods

4.2.1. Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(D, L-lactide acid) (MW 50 kDa)</td>
<td>Polysciences, Inc. (Warrington, US)</td>
</tr>
<tr>
<td>Poly(vinyl alcohol) (MW 13 - 23 kDa, 87 - 89% hydrolysed)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Chloroform (laboratory reagent grade)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (fraction V, 98-99% albumin)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bicinchoninic Acid Solution</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Copper Sulphate</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Sodium Hydroxide pellets</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>D(+)Trehalose dehydrate</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS) tablets</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
</tbody>
</table>
4.2.2. Methods

4.2.2.1. Preparation of BSA-loaded microspheres

BSA-loaded PLA (Mw 50 kDa) microspheres, prepared by the double emulsion solvent evaporation method, were suspended in an aqueous solution containing trehalose and/or L-leucine, and subsequently freeze-dried, as described in sections 3.2.2.1 and 3.2.2.2.

4.2.2.2. Aerosolisations

4.2.2.2.1. Dry powder inhalation

To investigate the effect of trehalose and/or L-leucine on *in vitro* deposition of the freeze-dried powders, 150 mg aliquots of the freeze-dried powders cryoprotected by varying concentrations of cryo-protectant (7% trehalose, 1% L-leucine, 7% trehalose and 1% L-leucine or 6% trehalose and 1% L-leucine, w/v) were filled in 8 gelatin capsules (size 2) for dry powder inhalation using an Eclipse DPI.

4.2.2.2.2. Nebulisation

To investigate the effect of different types of nebulisers on nebulisation efficiency, 150 mg aliquots of the freeze-dried microsphere powders containing 6% trehalose and 1% L-leucine (w/v) were resuspended in double distilled water and filled in both ultrasonic nebuliser (Pari eFlow) and air-jet nebuliser (Pari LC), using the same fill volumes of 4 ml (the maximum volume of the Pari eFlow ultrasonic nebuliser).
To investigate the effect of different fill volumes in air-jet nebuliser, 150 mg aliquots of the freeze-dried powders cryoprotected in the presence of 6% trehalose and 1% L-leucine (w/v) were resuspended in double distilled water and filled in the Pari LC nebuliser, using fill volumes of 4 or 8 ml (the maximum volume of Pari LC).

4.2.2.3. Scanning electron microscopy

The morphology of BSA-loaded microspheres and freeze-dried powders was determined by scanning electron microscopy (SEM). Freshly made or rehydrated microspheres were loaded onto carbon adhesive disk mounted onto aluminium stubs and left in the air until dry; freeze-dried powders were also sprinkled onto carbon coated aluminium stubs. Samples were then coated with a thin layer of gold and images were obtained using a Philips XL-30 scanning electron microscope at 20kV.

4.2.2.4. In vitro deposition

The in vitro deposition pattern of the freeze-dried powder was determined using a multi-stage liquid impinger (Copley Scientific Instruments, UK). The impaction stages 1 to 4 of the MSLI were filled with 20 ml double distilled water. A vacuum pump was connected to the outlet of the MSLI to supply air flow.

The inhalation devices were connected to the MSLI to be ready for operation. The Eclipse inhaler was operated at an air flow rate of $60 \pm 0.5$ L/min for 5 seconds twice; the nebulisers were operated at an air flow rate of $60 \pm 0.5$ L/min for 10 minutes. The
deposition of the microspheres or freeze-dried powders in the device, the throat, all
the impaction stages and the filter paper and the amount of BSA present at each stage
was determined by BCA assay.

4.2.2.5. Calculation of mass median aerodynamic diameter (MMAD) and
general standard deviation (GSD)

MMAD was read directly as the diameter at the 50% point of a plot of cumulative
fraction versus aerodynamic diameter indicated by effective cut-off diameter of each
impaction stages of the MSLI (Figure 4.8.; Seville et al, 2007).

![Diagram](image)

*Figure 4.8. Schematic illustration of calculation of mass median aerodynamic diameter.*
The GSD ($\sigma_g$) was calculated as following equation (Dunbar, 2005):

$$\sigma_g = \frac{\text{MMAD}}{d_{0.16}}$$

where $d_{0.16}$ is the diameter at 16th percentile of the cumulative distribution under size.

**4.2.2.6. Fine particle fraction (FPF)**

The total dose of the particles with aerodynamic diameters smaller than 5μm was considered as fine particle fraction (FPF), obtained by interpolation from the cumulative fraction undersize of 5μm from the plot of the cumulative fraction against aerodynamic diameter indicated by effective cut-off diameter of each stages (Figure 4.9.) (Pilcer et al, 2008; Learoyd et al, 2009).

![Figure 4.9. Schematic illustration of calculation of fine particle fraction.](image-url)
4.3. Results and discussion

4.3.1. Morphology of microspheres and freeze-dried microsphere powders

The morphology of the microsphere formulations in presence/absence of 6% trehalose and 1% leucine (w/v) was investigated using Scanning Electron Microscopy (SEM) to see the effect of cryoprotectants. Under SEM inspection, the freeze-dried microspheres without addition of cryoprotectant showed a large aggregation even after being ground for 10 minutes (Figure 4.10.a.), while much smaller particles were found in the formulations of freeze-dry powders with cryoprotectants applied (6% trehalose + 1% leucine, w/v) (Figure 4.10.d.). The particle size was seen to decrease as the grinding time increasing (Figure 4.10.b-d). Within Figure 4.10.a aggregates of microspheres can be seen.

Visualising freshly prepared microspheres which were not freeze-dried show these microspheres to have a spherical shape without pores or cavities, and also demonstrated the particle distribution and uniformity (Figure 4.11.a.). Microspheres which were freeze-dried in presence of 6% trehalose and 1% leucine (w/v), subjected to particle size reduction for 1, 5 or 10 min and then resuspended in distilled water are shown in Figure 4.11.b, c and d respectively. These micrographs show that the microspheres retain their size and morphology independent of the time they were subjected to particle size reduction. These studies support the previous particle size studies shown in section 3.3.2 and again demonstrate that the co-use of trehalose and L-leucine protected the microspheres during freeze-drying against the freezing and
drying stress possibly by cohering on the surface of the particles and then cause small particles to aggragat into large fragments as previously suggested by Mohammed et al, (2007). SEM is widely used in visualising particulate drug delivery system in particle size, surface morphology and structural, it has been reported that the aerosolisation performance and *in vitro* deposition of spray-dried spherical lactose powders were enhanced with the incorporation of leucine and other amino acids, because the surface morphology of the powders was changed by the amino acids, which was observed using SEM (Seville et al, 2007).

![Figure 4.10](image)

*Figure 4.10. Morphological analysis of the freeze-dried powders by SEM: a. Non cryoprotected freeze-dried powders after grinding for 10 minutes; b. Cryoprotected freeze-dried powders after grinding for 1 minute; c. Cryoprotected freeze-dried powders after grinding for 5 minutes; d. Cryoprotected freeze-dried powders after grinding for 10 minutes.*
Figure 4.11. Morphological analysis of the rehydrated freeze-dried powders by SEM: a. Fresh prepared microspheres; b. Cryoprotected freeze-dried powders after grinding for 1 minute; c. Cryoprotected freeze-dried powders after grinding for 5 minutes; d. Cryoprotected freeze-dried powders after grinding for 10 minutes

4.3.2. Aerosolisation studies of freeze-dried microsphere powders

4.3.2.1. Dry powder inhalation

The *in vitro* aerosolisation deposition of the freeze-dried powders was profiled using a MSLI. As expected the powder obtained from the formulations using 7% w/v trehalose as cryo-protectant, with grinding to reduce the particle size, showed a poor aerodynamic distribution (Figure 4.12.): ~24% of the dose remain in the inhaler and
capsules and more than 54% deposited on the inner wall of the MSLI throat and mouthpiece. When sized, these powder particles had a mean particle size of over 10 microns and a D10 of 1.66 ± 0.28 μm so would be expected to produce poor deposition. Similarly, microspheres freeze-dried with 1% L-leucine under the same studies caused 28% of the dose to remain in the inhaler and capsules and 51% of the dose to deposit in the inner wall of the MSLI. This formulation would be predicted to exhibit a high oropharyngeal deposition following inhalation. Less than 14% of the dose of both the formulations reached the impaction stage 1 and 2, with negligible deposition in the lower stages. Therefore whilst freeze-drying was able to produce microspheres of around 1 micron after resuspension (Figure 4.11.) as a freeze-dried powder the dry particle size was still well outside the size ranges required for lower stage deposition <3.1 μm.

Employing the combination of trehalose and L-leucine as cryoprotectants interesting did produce a different deposition profile, with significantly more (p<0.05) microspheres reaching stages 1,2 and 3 (with effective cut-off diameters of 13, 6.8 and 3.1μm respectively), compared to those freeze-dried in the presence of trehalose of leucine alone (Figure 4.12.). This is in line with the previous results presented in section 3.3.3, where the choice of cryo-protection was shown to influence not only the size of the resuspended microspheres but also of the freeze-dried powder particle size. However, despite these reduced dry powder particle sizes with the trehalose/leucine combinations none of the powders obtained from either formulation could enter
impaction stage 4 (or filter stage) of which the cut-off size is 1.7 \( \mu m \) and below. This indicated that the aerodynamic diameter of the powders was bigger than 3.1\( \mu m \) (cut-off size of stage 3). In previous studies (section 3.3.3.), manual grinding was conducted for 10 minutes, resulted a reduction in particle size of freeze-dried powders to \( \sim 5 \) microns with a D50 of 2.86 \( \pm \) 1.60 \( \mu m \). This would predict a large dose passing through stages 2 and 3 of the MSLI, but even in this case, the depositions were quite limited even in stage 2.

![Diagram of MSLI deposition profile](image)

Figure 4.12. MSLI deposition profile of freeze-dried BSA-loaded PLA microspheres powders administrated by Eclipse inhaler at an air flow rate of 60L/min (mean \( \pm \) SD, n=3). * denotes significant difference in distribution parameter in comparison between the two groups (p<0.05); ** denotes significant difference in distribution parameter in comparison between the two groups (p<0.01)

The poor aerosolisation performance of the freeze-dried various powders might be caused by the poor efficiency of the manual grinding which could not reduce the particle size as expected. Whilst manual grinding was able to significantly reduce the particle size of the powder (section 3.3.3.) the powder particles were still larger than
the cut off for stage 4 (1.7 microns). Also the manual grinding produced in irregular powder shape as noted in Figure 4.10 which could also contribute to these deposition profiles. Sizing by laser diffraction is based on assuming the particles being spherical, so the angle of the laser scattered is highly dependent on which orientation the particles were in when the laser was and defracted by them (Figure 4.13)

![Diagram of laser diffraction setup]

Figure 4.13. Schematic illustration of sizing irregular particles by laser diffraction.
Use of both trehalose and leucine resulted in the freeze-dried powders with a lower residual fraction in the inhaler and capsules and lower deposition in the MSLI throat and the mouthpiece, and greater deposition on stages 1, 2 and 3 of the MSLI (with a higher FPF of $-1.1\%$) (Figure 4.14.). In chapter 3, it was shown that using trehalose and leucine together resulted in a smaller particle diameter. Thus the better aerosolisation profile may due to the smaller particle size of the freeze-dried powders. However a second factor which may also contribute is the powder flow property. It has been reported that the disodium cromoglycate (DSCG) powders containing leucine were less cohesive and so better dispersed. The mechanism may have been partly due to the interaction between leucine and the DSCG molecules (Najafabadi et al, 2004; Chew et al, 2005). It is unclear whether a similar interaction exists between the leucine and the PLA/PVA molecules.
4.3.2.2. Nebulisation

In this section, the studies focused on comparison between the ultrasonic nebuliser and the air-jet nebuliser and the aerosolisation profiles of the nebulised mist. Only one formulation, PLA microsphere freeze-dried powders in presence of cryoprotectant consisting of 6% trehalose and 1% leucine (w/v, respectively), was used to evaluate the parameters described above, and also compared to the DPI.

4.3.2.2.1. Characterisation of the nebulised microspheres

The nebulised microspheres were collected to investigate the effect of nebulisation on particle size and zeta potential (Table 4.1.). The mean diameter of the microspheres was not significantly changed by nebulisation by either the ultrasonic nebulizer or the air-jet nebuliser (Table 4.1). Similarly the different fill volumes (4 ml or 8 ml) employed in air-jet nebulizer made no significant difference to the microsphere mean diameter measured after nebulisation (Table 4.1). The zeta potential of the microspheres was also unaffected by nebulisation using both systems and both volumes with zeta potentials measured after nebulisation showing no significant difference to those of microspheres not subjected to nebulisation (Table 4.1). Beck-Briochsitter et al. (2009) also recently studied the effect of nebulisation on the mean particle size and the zeta potential of the propylcarbamateco-vinyl acetate-co-vinyl alcohol]-graft-poly (d,l-lactide-coglycolide) nanoparticles nebulised using a vibrating mesh nebuliser, with the aim to use these systems to deliver 5(6)-carboxyfluorescein. The nanospheres prepared in this study were 195 ± 7.1 nm in
size and had a zeta potential of $-28.3 \pm 0.3$ mV, which was not altered after nebulisation (Beck-Broichsitter et al, 2009).

<table>
<thead>
<tr>
<th></th>
<th>Before nebulisation</th>
<th>After ultrasonic nebulisation</th>
<th>after air-jet nebulisation 4 ml</th>
<th>after air-jet nebulisation 8 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle size (µm)</td>
<td>1.13 ± 0.01</td>
<td>1.14 ± 0.02</td>
<td>1.12 ± 0.02</td>
<td>1.11 ± 0.03</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>6.98 ± 3.61</td>
<td>9.21 ± 3.33</td>
<td>4.94 ± 2.56</td>
<td>6.26 ± 3.57</td>
</tr>
</tbody>
</table>

Table 4.1. Mean diameter and surface charge of rehydrated microsphere powders before/after nebulisation (mean ± SD, n=3).

4.3.2.2. Aerosolisation studies using different type of nebulisers

The aerodynamic size distribution of the nebulised microspheres from the formulation using 6% w/v trehalose and 1% w/v L-leucine together as cryoprotectant was also tested and is shown in Figure 4.15.

![Figure 4.15. MSLI deposition profile of PLA microsphere nebulized by ultrasonic nebuliser and air-jet nebuliser (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison between the two groups (p<0.05); ** denotes significant difference in distribution parameter in comparison between the two groups (p<0.01); *** denotes significant difference in distribution parameter in comparison between the two groups (p<0.001)
Overall the total output rate and the respirable dose of the ultrasonic nebuliser was higher than of the air-jet nebuliser (Figure 4.15.). The decreased output rate of the air-jet systems has been proposed to be a result of the air-drive mechanism of the air-jet nebuliser leading to a faster evaporation of solvent in the fluid cell, resulting in an increasing concentration of the reservoir fluid which may physically block the orifice of the nebuliser and cause a higher residual in the fluid cell (Cockcroft et al, 1987; McCallion et al, 1996a). In contrast, the concentration in the reservoir of the ultrasonic nebuliser does not increase during the nebulisation process (Rau, 2002). This supports the results presented in Figure 4.15 where it can be seen that significantly more (p< 0.01) microsphere dose remained in the reservoir after air-jet nebuliser compared with the ultrasonic nebuliser. Studies by Kawashima et al., (1999) also reported that ultrasonic nebulisation was more efficient in delivering insulin-loaded PLGA nanospheres to a cascade impactor, compared with an air-jet nebuliser, according to the higher output efficiency and respirable fraction.

The aerodynamic distribution of the microspheres was improved by nebulisation (using either system; Figure 4.15) in comparison with delivery of the dry powders by DPI (Figure 4.12.). The deposition dose onto the throat region was reduced from 54.3 ± 6.3% to 13.1 ± 0.3% when delivered using the ultrasonic nebuliser, and the reservoir dose of the microspheres in the nebuliser was 9.7 ± 2.2% which was also much lower than the dose (17.0 ± 2.7%; Figure 4.15.) remained in the Eclipse inhaler and capsules (Figure 4.12.). The nebulised microspheres were able to not only reach the impaction
stage 1 and 2, but also entered into the lower stages with 34.9±2.6% being deposited in these stages (Figure 4.15.).

Furthermore, the ultrasonic nebulisation resulted a greater deposition performance using the MSLI, an increase in mean FPF <5 μm, based on three individual reading from the cumulative distribution curve at the cut-off size 5 μm (Figure 4.16.; Table 4.2.), was also found; using the Pari LC nebuliser, 56.6% of the dose entered the impaction stages of the MSLI, the respirable output was also improved (21.2% in mean FPF <5 μm; Figure 4.16; Table 4.2.). Thus the mean FPF <5 μm was increased by both nebulisation system in comparison with DPI (Table 4.2.). The microspheres nebulised using air-jet nebuliser could also enter impaction stage 3, 4 and filter stage, although not as much as ultrasonic nebulisation (Figure 4.15).

![Graph showing cumulative fraction distribution](image)

**Figure 4.16.** Cumulative fraction distribution of freeze-dried BSA-loaded PLA microsphere powders administrated using ultrasonic nebuliser and air-jet nebuliser (mean ± SD, n=3).
<table>
<thead>
<tr>
<th></th>
<th>Ultrasonic nebuliser</th>
<th>Air-jet nebuliser 4ml</th>
<th>Air-jet nebuliser 8ml</th>
<th>DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPF &lt;5 μm (%)</td>
<td>32.50 ± 2.12</td>
<td>21.15 ± 2.26</td>
<td>26.14 ± 4.21</td>
<td>1.07 ± 1.22</td>
</tr>
</tbody>
</table>

Table 4.2. Fine particle fraction of nebulisations and DPI

4.3.2.2.3. Aerosolisation studies using air-jet nebuliser with different fill volumes

The effect of the fill volume of the air-jet nebulizer on *in vitro* deposition was also tested. As before, 150 mg aliquots of freeze-dried powders were resuspended in 4 or 8ml ddH₂O and filled in the Pari LC air-jet nebuliser, and the *in vitro* deposition of the microspheres was tested by MSLI (Figure 4.17.). These studies showed that the residual dose in reservoir and the MSLI throat deposition were significantly decreased (P<0.01) from 23.4 ± 2.9% to 13.3 ± 0.9% when the fill volume was increased from 4 to 8 ml respectively (Figure 4.17.)

The respirable dose (FPF <5 μm) increased from 21.2±2.3% to 26.1±4.2% but no significant statistic difference found (Figure 4.18.; Table 4.2.). The improved aerosolisation profile can be explained by that the increase volume fill improving the output efficacy due to the lower residual volume (Figure 4.17.). Despite the nebulisation time, larger fill volumes led to lower concentrations in the reservoir and subsequently results in lower concentrations in the residual volume, which finally resulting a lower proportion of nebulised drug being left in the residual volume. Also more concentrated suspensions may cause higher viscosity and higher surface tension. It has been reported that highly viscous solutions can nebulise more slowly and less
effectively (Newman et al., 1985). Furthermore these results could also be attributed to the effect of solvent evaporation during nebulisation: solvent evaporation could produce a less marked effect on the concentration of the 8 ml solution, thereby less aggregation would occur at the orifice, allowing more droplets atomised out of the nebuliser.

![Bar chart](image)

Figure 4.17. MSLI deposition profile of PLA microsphere nebulized by air-jet nebuliser filled with 4 and 8ml microsphere resuspension respectively (mean ± SD, n=3). * denotes significant difference in distribution parameter in comparison between the two groups (p<0.05); ** denotes significant difference in distribution parameter in comparison between the two groups (p<0.01).
Figure 4.18. Cumulative fraction distribution of freeze-dried BSA-loaded PLA microsphere powders administered using air-jet nebuliser with the fill volumes of 4 and 8ml respectively (mean ± SD, n=3).

4.3.2.2.4. MMAD and GSD of nebulised PLA microspheres

Table 4.3 shows the aerodynamic parameters of PLA microsphere as freeze-dried powders or in suspension. The chosen formulation included 6% trehalose and 1% leucine (w/v), since this showed the smallest particle diameter sized using laser diffraction and the best in vitro deposition profile.

The PLA microsphere suspension nebulised using the ultrasonic nebuliser showed a MMAD of 8.2 μm. This was significantly smaller than the MMAD of the mist nebulised by air-jet nebuliser (12.5 μm), when filled with same volume of microsphere suspension (p<0.01; Table 4.3.). As the fill volume in the reservoir of air-jet nebuliser increased from 4 to 8ml, a significant reduction in MMAD of the nebulised microspheres mist was observed, from 12.5 to 8.8 μm (p<0.05; Table 4.3.), which was almost the same value obtained using ultrasonic nebuliser (P>0.05).
<table>
<thead>
<tr>
<th></th>
<th>MMAD (μm)</th>
<th>GSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasonic nebuliser</td>
<td>8.23 ± 0.27a</td>
<td>3.54 ± 0.15</td>
</tr>
<tr>
<td>Air-jet nebuliser 4 ml</td>
<td>12.50 ± 1.36</td>
<td>3.01 ± 0.47</td>
</tr>
<tr>
<td>Air-jet nebuliser 8 ml</td>
<td>8.76 ± 0.79b</td>
<td>2.28 ±0.25</td>
</tr>
</tbody>
</table>

Table 4.3. MMAD and GSD of PLA microspheres/powders aerosolised by ultrasonic nebuliser and air-jet nebuliser (Mean ± SD, n=3). The MMAD and GSD of dry powders delivered by DPI were unable to measured due to the cumulative fraction in the MSLI was less than 50%. a denotes a significant decrease in MMAD in comparison to air-jet nebuliser filled with 4ml PLA microsphere suspension (p<0.01); b denotes a significant decrease in MMAD in comparison to air-jet nebuliser filled with 4ml PLA microsphere suspension (p<0.05).

It has been widely accepted that most particles with MMAD larger than 10 μm will deposit in the oroparyngeal region with a large dose, and subsequently being swallowed (Labiris & Dolovich, 2003). According to this theory, the mist containing PLA microspheres nebulised by the Pari LC air-jet nebuliser with a fill volume of 4 ml would be expected not to pass through the larynx efficiently in order to reach the alveoli. However, as shown in Figure 4.16 and Table 4.2, more than 20% of the total dose had a diameter less than 5 μm (which are mainly deposited in the alveolar region or the small airways close to it (Gerrity, 1990)), and about 7% of the total dose had a cut-off under 3.1 μm, which are mainly deposited into the alveoli (Patton, 1996; El-Baseir et al, 1997). It is also notable that the GSD of these nebulised mist was >1.2, indicating a heterodisperse aerosol that made up of particles in many different sizes (O’Callaghan & Barry, 1997), although the microspheres themselves showed a good uniformity.

Increasing the fill volume enhanced the nebulising efficiency of the air-jet nebuliser, since the MMAD decreased in the range of 5 – 10 μm, indicating that the nebulised
mist would mainly enter and deposit in the conducting airways (Gerrity, 1990). Also, about 26.1% of the total dose had a particle size <5 µm and would be expected to reach the alveoli (Table 4.2). The mist nebulised using the ultrasonic nebuliser had a similar MMAD and GSD and so may have the same aerodynamic properties (Table 4.3).

4.4. Conclusion

From the results outlined above, manual grinding can reduce the freeze-dried microsphere powders prepared using double emulsion solvent evaporation method, the grinding efficiency is depended on the length of grinding duration. In the current study, manual grinding was conducted for 10 minutes, but from the data in Figure 4.10 it can be seen that the size was not reduced sufficiently to facilitate pulmonary delivery; a further size reduction would be needed. Although the powders were not particularly respirable via DPI, some other methods may work better on decreasing the particles, such like ball milling. However, combined use of trehalose and L-leucine as cryoprotectant offers a better aerosolisation performance than using either individually.

Nebulisation is an alternative method for pulmonary drug delivery of the formulated microspheres because of the fine particle size of the PLA microspheres contained within the freeze-dried powders. The FPF of the nebulised microspheres indicates a promising deposition to the lung periphery. Nebulisation of the rehydrated powder
results in statistically improved aerosolisation performance, with the ultrasonic nebuliser (Pari eFlow) being more efficient than the air-jet device (Pari LC). However, this \textit{in vitro} model has only an inhaling process while breathing of course includes exhaling too. It has been reported that two thirds of the atomized dose may be breathed out of respiratory track into the air during expiration (Kradjan & Lakshminarayan, 1985), thus the \textit{in vivo} aerosolisation deposition may be lower.

The MMAD is a key size parameter correlated to the aerodynamic properties of aerosols. It can be used alone to reveal the lung deposition of aerosols with high uniformity in size, but it is better to consider GSD together with MMAD due to a heterodisperse aerosol.
Chapter 5

Cellular responses of macrophages to PLA microspheres
and freeze-dried powders
5.1. Introduction

It has been reported that microparticle-based delivery systems made of PLA or PLGA works not only as drug carriers, but also as immunostimulators which induce and promote immune response activity (O’Hagan & Singh, 2003; Jiang et al., 2005; Kirby et al., 2008). As vaccine delivery systems microparticles are able to deliver a range of antigens and offer the ability to enhance immune responses to sub-unit antigens such as Ag85B-ESAT6, an antigen used in the development of possible tuberculosis vaccines (Kirby et al., 2008). Alone, sub-unit antigens offer a good safety profile with a generally low immunogenicity. By loading such antigens onto particulates such as microspheres, the antigens are not only protected from rapid degradation, but are also more effectively targeted to phagocytic cells such as macrophages and dendritic cells. This is due to the large particulate morphology of microspheres which the immune system recognises as ‘non-self’ which results in the particulates being taken up. The loaded antigens are then processed for appropriate presentation such that immune responses to the loaded antigens are generated (Bramwell and Perrie, 2005).

For particles delivered via the pulmonary route, alveolar macrophages in the lung are the first line of defence against inhaled particles (Hocking & Glode, 1979). When small particles are inhaled through the airway and finally reach the alveolar region in the lung, they will be phagocytosed by antigen-presenting cells, such as dendritic cells and alveolar macrophages because of their non-specific phagocytic nature (Pettis et al., 2000; Elamanchili et al., 2007). An inflammatory response will be induced,
leading to the released of cytokines following phagocytosis of particles (Driscoll & Maurer, 1991). Quantifying the released cytokines is required to understand the conditions of macrophages such like proliferation, death ratio, phagocytic activity and response to external stimulations. These cellular responses can be affected by some factors of the polymeric spheres, such as particle size (Cleland et al., 1998), surface charge, surface hydrophobicity (Rafati et al., 1997), and adjuvants used in the formulation (Singh & O’Hagan, 1999).

To investigate cellular responses in vitro a range of markers may be measured such as cell proliferation, using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Figure 5.1.). The tetrazolium compound, MTS, in the presence of phenazine methosulfate (PMS), is converted to its formazan product by the dehydrogenase coenzymes, nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH), which only exist in the metabolically active cells. The formazan product is water soluble and can be quantitatively measured by the absorbance at 490-500nm (Cory et al., 1991).
Figure 5.1. Structure of MTS tetrazolium and its formazan product

In addition to cell proliferation, cellular damage can also be measured via lactate dehydrogenase (LDH), a cytoplasmic enzyme which is released upon cell lysis or damage of the cell membrane, as a marker of cytotoxicity (Wang et al., 2009). The released LDH is proportional to the number of lysed cells and can be considered as a quantitative marker for cell death (Koh & Choi, 1987). Lactate is converted to pyruvate with the reduction of NAD\(^+\) to NADH, converting the tetrazolium salt, 2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride (INT), into a red, optically-detectable formazan product which is proportional to the LDH release (Figure 5.2.).
Figure 5.2. Reactions of LDH assay and structure of relative compounds.

β-N-Acetylglucosaminidase (NAG, also know as N-acetyl-β-D-glucosaminidase) is a lysosomal enzyme secreted by mammalian cells when phagocytosis takes place, and therefore can be used as a marker indicating phagocytic activity levels (Pettis et al., 2000). Assay of NAG content can be undertaken using a colorimetric method based on the hydrolysis of 4-Nitrophenyl N-acetyl-β-D-glucosaminide (NP-GlcNAc) by NAG (Figure 5.3.), the consequent product o-Nitrophenol can be measured at 405nm (Product introduction of β-D-glucosaminidase Assay kit).
Tumour necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine secreted by macrophages after recognition of pathogen following phagocytosis (Thomas, 2001), which may induce innate immunity through increasing toll like receptor induced production of additional pro-inflammatory cytokines (Beutler, 1999; Avni et al., 2009). TNF-α also induces type 1 helper T cell (Th1) immune response which enhances the anti-pathogen ability of macrophages and promotes the immunity (Wang, 2009). TNF-α secretion can be measured using assay is based on an enzyme-linked immunosorbent assay (ELISA) (Meager et al., 1987; Hirai et al., 1991). Cell supernatant is added to a plate pre-coated with a capture antibody; antigen, if any, will bind the capture antibody, enzyme-linked secondary antibody is then added, washed and followed by substrate, which is converted into a detectable form by enzyme.
5.1.1 Enhancing the immunogenicity of microspheres.

Examples of immunomodulators include derivatives of myolic acid, a cell wall component of mycobacteria, and trehalose-6,6'-dimycolate (TDM, cord factor), an adjuvant which promotes T helper type 1 (Th1) migration to the intracellular infection, particularly, anti-bacterial infection (Oiso et al., 2005). Trehalose 6,6'-dibhenenate (TDB) is a synthetic analog of TDM because of its shorter fatty acids chains (Toubiana et al., 1977; Plmm et al., 1979; Olds et al., 1980). TDB is considered as a promising immunomodulator which may induce a substantial cell-mediated immune response and high levels of immunoglobulins when combined with dimethyldioctadecylammonium (DDA) in a cationic liposome formulation (Davidsen et al., 2005).

In this chapter the cellular interactions of microspheres with macrophages will be investigated. To achieve this, a murine macrophage J774 cell line was used as the model cell line, which has been widely used in in vitro because of its similar response to stimulation as naïve macrophages (Chen et al., 1999; Stumpo et al., 2003). The objective of this work was to investigate the cellular responses of macrophages exposed to PLA microspheres and freeze-dried powders. Serial formulations containing TDB were also prepared to evaluate their effect on cellular responses and to investigate the potential of such systems as vaccine delivery systems.
5.2. Materials and methods

5.2.1. Materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(D, L-lactide acid) (MW 50kDa)</td>
<td>Polysciences, Inc. (Warrington, US)</td>
</tr>
<tr>
<td>Poly(vinyl alcohol) (MW 13 – 23 kDa, 87 - 89% hydrolysed)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Chloroform, laboratory grade</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bovine Serum Albumin (fraction V, 98–99% albumin)</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Bicinchoninic Acid Solution</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Copper Sulphate</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Phosphate Buffered Saline (PBS) tablets</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Sodium Hydroxide pellets</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>D(+)Trehalose dehydrate</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>L-Lecine</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>CellTiter 96® AQuous One Solution</td>
<td>Promega UK Ltd. (Southampton, UK)</td>
</tr>
<tr>
<td>CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit</td>
<td>Promega UK Ltd.</td>
</tr>
<tr>
<td>mouse TNF-α/TNFSF1A DuoSet ELISA Development Kit</td>
<td>R&amp;D Systems Europe, Ltd (Abingdon, UK)</td>
</tr>
<tr>
<td>Reagent Diluent</td>
<td>R&amp;D Systems Europe, Ltd (Abingdon, UK)</td>
</tr>
<tr>
<td>Substrate Solution</td>
<td>R&amp;D Systems Europe, Ltd (Abingdon, UK)</td>
</tr>
<tr>
<td>Stop Solution (2NH₂SO₄)</td>
<td>R&amp;D Systems Europe, Ltd (Abingdon, UK)</td>
</tr>
<tr>
<td>β-N-Acetylglycosaminidase Assay Kit</td>
<td>Sigma-Aldrich Co. Ltd. (Dorset, UK)</td>
</tr>
<tr>
<td>Dulbeccoo's modified eagle medium (DMEM)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Foetal bovine serum (Heat-Inactivated)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
<tr>
<td>Penicillin-Streptomycin-glutamine (100X)</td>
<td>Invitrogen, Paisley, UK</td>
</tr>
</tbody>
</table>
5.2.2. Methods

5.2.2.1. Preparation of PLA microsphere formulations

Formulations were divided into two series: non-freeze-dried microspheres and freeze-dried powders, to determine the effect of cryoprotectant on cellular response. Non-freeze-dried microspheres were prepared using double emulsion solvent evaporation method (see chapter 3), while freeze-dried powders were lyophilised microspheres with cryoprotectants, 6% (w/v) trehalose and 1% (w/v) leucine. Some formulations included 4% TDB (w/w of polymer) added into the organic phase as immunomodulator, and were prepared to investigate the effect of TDB on cellular response.

5.2.2.2. Cell culture

Macrophages, J774 Balb/C cell line, were grown in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) penicillin/streptomycin/l-glutamine (PSG), and incubated at 37°C in a humid 5% CO₂, under a sterile condition.

5.2.2.3. Optimisation of cell number

The concentration of macrophages was optimized using MTS assay by CellTiter 96® AQ™ One Solution reagent, which is widely used for determining the number of viable cells in proliferation: Suzuki et al. (2003) evaluated the effect of oxytocin on proliferation of human endometrial endometrioid adenocarcinoma cell lines; O’Hare et
al. (2005) measured the cell viability of Ba/F3 cells transfected with Bcr-Abl kinase; Garcia-pedrero et al. (2007) measured the cell proliferation of BT549 breast cancer cells; the CellTiter 96® AQ<sub>acous</sub> One Solution also can be used to access cytotoxic effects (Case et al., 2008).

Macrophages were plated at serial concentrations of 0.2, 0.4, 0.8, 2, 4 and 8×10<sup>4</sup> cells/well into a 96-well plate in 100μl medium in triplicate, and 100μl medium was also added into the plate in triplicate as blank. The plate was then incubated for 24 hours at 37°C in a humid 5% CO₂ under sterile conditions to let macrophages attach the bottom of wells. 20μl CellTiter 96® AQ<sub>acous</sub> One Solution reagent was added into each well followed by further 4 hours incubation. The absorbance was read at 490nm hourly to optimise the incubation time. The initial number of cells that produces an assay signal near the low end of the linear range of the assay (Product introduction of CellTiter 96® AQ<sub>acous</sub> One Solution reagent) was selected.

To confirm cell number optimization, macrophages were also plated at serial concentrations of 0.2, 0.4, 0.8, 2, 4 and 8×10<sup>4</sup> cells/well into a 96-well plate in 100 μl medium in triplicate, 100μl medium was also added into the plate in triplicate as blank. 50 μl of the supernatant in each well was transferred into a new 96-well plate followed by addition of 50 μl of the substrate mix to each well. The plate was then covered with foil to protect from light and incubated at room temperature for 30 minutes, then read at 490 nm every 15 minutes after the addition of stop solution. The
absorbance of optimised cell number should be at least two times of the absorbance of the blank.

5.2.2.4. Optimisation of microsphere concentration

The optimization of microspheres concentration was carried out using a LDH assay with the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit. This is a colorimetric method of assaying cellular cytotoxicity and is an alternative to 51Cr release cytotoxicity assays and widely used in cytotoxicity study (Korzeniewski & Callewaert, 1983) and including:

- Hornick et al. (1997) who studied antibody dependent cell-mediated cytotoxicity against ARH-77 human plasma cells,
- Meghji et al. (1997) who monitored the cytotoxicity of the human MG63 osteoblast-like cells to peptides derived from mycobacterium tuberculosis chapeonin 10 protein,
- van der woude et al. (1997) use this assay to assess the cytotoxicity of COS-7 SV40-transformed monkey kidney cells treated by lipid-based transfection reagents,
- Hertel et al. (1997) and Kim et al. (2000) both determined the toxicity of PC12 cells with regard to β-amyloid peptide and cell cytotoxicity;
- Sheehan et al. (1997) also used the CytoTox 96® Non-Radioactive Cytotoxicity assay used to quantify cell number to evaluate the cell proliferation.
Macrophages were also plated at serial concentrations of 0.2, 0.4, 0.8, 2, 4 and $8 \times 10^4$ cells/well into a 96-well plate in 100 µl medium in triplicate, and then incubated under cell culture condition for 24 hours to let cells attach the bottom of the wells. Fresh made PLA microspheres containing 4% TDB (w/w polymer) were re-suspended in medium at different concentration of 1, 10, 100, and 1000 µg/ml. The supernatant was replaced by 100 µl microspheres suspension (suspended in culture medium) after the plate was centrifuged at 250 x g for 4 minutes, the wells containing untreated cells as control groups were aspirated and fill with 100 µl fresh culture medium. The plate was then incubated for 24 hours incubation under the same condition of cell culture.

50 µl of supernatant in each well was transferred into a new 96-well plate as experimental for sample wells or experimental spontaneous for control groups respectively, the residual supernatant in the wells of control groups was replaced by 100 µl fresh medium with addition of 10 µl lysis solution (Triton X100) followed by 45 minutes incubation at 37°C in a humid 5% CO2 to ensure the cells were thoroughly lysed. The plate was then centrifuged at 250 x g for 4 minutes, and 50 µl of supernatant in the wells of control group was transferred into the new 96-well plate as target maximum. 10 µl of the lysis solution was added in triplicate into the new 96-well plate as volume correction, 50 µl of reconstituted substrate mix solution was added to each well, and then covered the plate with foil to protected from light and incubated at room temperature for 30 minutes, followed by the addition of 50 µl of the stop solution to each well. The absorbance was read at 490nm in a period which
optimised in section 5.2.2.3. Culture medium was set as negative control and target maximum was set as positive control (100% LDH release). The cytotoxicity was calculated using the following equation:

\[
\text{Cytotoxicity (\%) } = \frac{\text{Experimental - Experimental spontaneous}}{\text{Target maximum - Volume correction}} \times 100
\]

5.2.2.5. Preparation for Assays

Macrophages were plated into 96-well plates at the concentration optimised in section 5.2.2.3., 100 µl per well and subsequently incubated at 37°C in a humid 5% CO₂ under sterile conditions for 24 hours. Samples in different formulations were re-suspended in culture medium at the concentration optimised in section 5.2.2.4., and added into the wells to replaced the supernatant in each well; the supernatant of the untreated macrophage wells was replaced by fresh culture medium as control; 100 µl of culture medium in triplicate was as background or blank. The plate was ready for assays after 24 hour incubation under the same condition described above.

5.2.2.6. Cell proliferation

The cell proliferation was measured using the MTS assay with CellTiter 96® AQ₅₆₀₆₈ aqueous One Solution. Macrophages were plated and treated with PLA microsphere samples as described in section 5.2.2.5., 20µl CellTiter 96® AQ₅₆₀₆₈ One Solution reagent was added into each well followed by further incubation in a period which also optimised in section 5.2.2.2., the absorbance was measured at 490 nm. The reading of
background well was as negative control; the reading of untreated cell well was as positive control.

5.2.2.7. Cytotoxicity study

The cytotoxicity of microsphere formulations was determined using the LDH assay with CytoTox 96® Non-Radioactive Cytotoxicity Assay kit. Macrophages were plated and treated with PLA microsphere samples as described in section 5.2.2.5., and the assay process was same as described in section 5.2.2.4.. Culture medium was set as negative control and target maximum was set as positive control (100% LDH release).

5.2.2.8. Phagocytic activities

Phagocytic activities were determined using a NAG assay with β-N-Acetylglucosaminidase Assay kit following the product bulletin. Macrophages were plated and treated with PLA microsphere samples as described in section 5.2.2.5., 10 μl of supernatant in each well was mixed with 90 μl of substrate solution as test sample; 2 μl of NAG control enzyme and 98μl of substrate solution were mixed as positive control; 100 μl of substrate solution was the blank; 300 μl of standard solution was the standard. The reaction components above were added into a 96-well plate in triplicate. After incubation at 37°C for 10 minutes, the absorbance was measured at 405 nm. The concentration of NAG was calculated using the following equation:
Units/ml = \frac{(A_{405\text{sample}} - A_{405\text{blank}}) \times 0.05 \times 0.3 \times DF}{A_{405\text{standard}} \times \text{time} \times V_{\text{enz}}}

- Units denotes 1 unit will hydrolyze 1.0 μmole of 4-Nitrophenyl N-acetyl-β-D-glucosaminide to p-nitrophenol and N-acetyl-β-D-glucosaminide per 1 minute at pH 4.7 at 37°C.
- $A_{405\text{sample}}$ denotes the absorbance of the sample at 405 nm.
- $A_{405\text{blank}}$ denotes the absorbance of the blank at 405 nm.
- 0.05 denotes the concentration (μmole/ml) of 4-nitrophenol in the standard solution.
- 0.3 denotes the final volume (ml) of the reaction components after the addition of the stop solution.
- DF denotes enzyme dilution factor, the value of it in this case is 100.
- $A_{405\text{standard}}$ denotes the absorbance of the standard solution at 405 nm.
- Time denotes the incubation time (minute).
- $V_{\text{enz}}$ denotes the volume (ml) of the sample.

5.2.2.9. Macrophage activation

The TNF-α assay was run with Mouse TNF-α/TNFSF1A DuoSet ELISA Development Kit following the product bulletin. A 96-well microplate was coated with the diluted capture antibody at the working concentration of 0.8 μg/ml in 0.2 μm filtered PBS solution, and then the microplate was sealed. After being incubated at room temperature for 18 hours, each well of the microplate was aspirated and washed with 400 μl washing buffer (0.05% Tween 20 in PBS, pH 7.2-7.4, 0.2 μm filtered).
After a total of three times aspiration and wash, each well was filled with 200μl reagent diluent and then incubated at room temperature for 1 hour. The microplate was ready for sample addition after three times aspiration and wash with 400μl washing buffer each time.

Macrophages were plated and treated with PLA microsphere samples as described in section 5.2.2.5., 100 μl of supernatant in each well were added into the coated 96-well plate in triplicate. Same volume of standard in serial dilutions (31.25, 62.5, 125, 250, 500, 1000 and 2000 pg/ml) were also added into the plate in triplicate. The plate was then sealed and incubated at room temperature for 2 hours. After three times aspiration and wash with 400 μl washing buffer each time, the plate was filled with 100 μl of streptavidin-HRP into each well, sealed and incubated at room temperature, and protected from light for 20 minutes. After another 3 times aspiration and wash with 400 μl washing buffer, 100 μl of substrate solution was added into each well, sealed and incubated at room temperature, and protected from light for 20 minutes. Then 50 μl of stop solution was added into each well, the plate was tapped gently to let thorough mixing. The optical density was read at 450nm.

A calibration curve was constructed by plotting the mean absorbance for each standard on the y-axis against the TNF-α concentration on the x-axis. Alternatively, by plotting the log value of the mean absorbance and log value of the concentration of TNF-α, and drawing a best fit line. The TNF-α concentration of the samples could be
gained by regressed calculation from the linear one of the two the calibration curve.

5.3. Results and discussion

5.3.1. Effects of addition of TDB

In order to compare the effect of addition of an immunomodulator to the PLA microspheres, microspheres were prepared with and without the addition of 4% TDB (w/w of polymer). Previous studies (Kirby et al., 2008) have shown that the addition of 4% TDB was able to enhance the immune response to incorporated sub-unit antigen, compared to microspheres formulated from PLA alone. Therefore, to further within these studies 4% w/w TDB was also adopted.

Incorporation of 4% TDB within the PLA microspheres led to a 2-fold increase in the mean particle size in both lyophilised and non-lyophilised BSA-loaded formulations (2.34 ± 0.21 and 2.49 ± 0.36 µm respectively), compared to the both formulations without TDB (1.13 ± 0.08 and 1.09 ± 0.04 µm, respectively) (Figure 5.4.). The increase in size of microspheres with addition of TDB was also found in microspheres formulated in the absence of BSA.

In terms of surface charge, there was no significant difference between microspheres prepared with and without TDB (p>0.05) (Figure 5.5). Previous studies have shown that the higher positive surface charges of microparticles leads to the higher cytotoxicity (Kuo et al., 2005; Liang & Chou, 2009). However within these studies all
microspheres formulated were similarly cationic, therefore any difference noted due to the incorporation of TDB could be related to a toxicological nature of TDB rather than surface charge.

Figure 5.4. Mean particle size of the PLA microspheres and the freeze-dried powders with/out addition of TDB. Measured using laser diffraction (HELOS particle sizer plus CUVEETTE dispersion unit, Sympatec, Germany) following dispersing the microspheres or dry powder in double distilled water. (Mean ± SD, n=3).* denotes significant difference in mean particle size in comparison to the formulation without TDB (p<0.05).

Figure 5.5. Surface charge of the PLA microspheres and the the microspheres in freeze-dried powders with/out addition of TDB. measured using ZetaPlus instrument (Brookhaven Instrument Corporation, NY) following dispersing the microspheres or dry powder in double distilled water. (Mean ± SD, n=3).
In terms of protein loading, the addition of TDB caused a significant reduction in BSA loading efficiency from $71.68 \pm 1.71$ to $63.85 \pm 2.76\%$ for non-lyophilised microspheres and from $70.86 \pm 2.39$ to $62.34 \pm 3.45\%$ (Figure 5.6.)

![BSA Loading Efficiency](image)

Figure 5.6. BSA loading efficiency of the PLA microspheres and the freeze-dried powders with/out addition of TDB. Measured using BCA assay. (Mean ± SD, n=3). * denotes significant difference in BSA loading efficiency in comparison to the formulation without TDB (p<0.05).

4% TDB (w/w of polymer) was used in this study, based on previous work in our laboratories (Kirby et al. 2008). The particle size of Ag85B-ESTA-6-loaded microspheres was dramatically decreased to 3.16 μm upon addition addition of TDB (from 20.26 μm compared to PLGA/DDA microspheres), which indicated TDB that may be functional in maintaining the emulsion stability. Kirby et al. mentioned that in presence of TDB, the interfacial tension of the primary w/o emulsion droplets might be affected by the TDB molecules with their large trehalose head-group, which contributed to a homogeneous coalescence of emulsion droplets forming and subsequently decrease the particle size compared with PLGA/DDA. However,
according to their results, the size of PLGA/TDB microspheres was significantly bigger than the diameter of PLGA microspheres (1.50 μm), which was similar to the result in this study and the increase in size could be due to the loading of TDB in the PLA microsphere matrix.

5.3.2. Optimisation of cell number

Varying concentrations of macrophages were investigated using the CellTiter 96® AQ~w~ One Solution Cell Proliferation Assay in order to optimise the cell number for the assays, the initial number of cells per well that produced an assay signal near the low end of the linear range of the assay would be selected as the optimised cell number. The incubation time after the addition of the CellTiter 96® AQ~w~ One Solution reagent was also investigated in order to optimise the assay condition.

The assay signals were determined hourly (up to 4 hours) (Figures 5.7a & b), and a general trend was found that increasing cell numbers resulting in increased absorbance values, and comparison between the results the incubation times showed that the longer incubation leads a higher absorbance levels which remained in the reading range of the micro-plate reader. However, the results of the 2 and 3 hours incubation after the addition of the CellTiter 96® AQ~w~ One Solution reagent showed larger and better signal range than that of 1 and 4 hours incubation which resulted loss of linearity at higher cell concentrations (>4 × 10⁴ cells/well) and would not support differentiation of cell concentrations at these level. Finally, 2 hours
incubation was chosen as the incubation time for all subsequent experiments, for its more linear curve ($r^2 = 0.9954$) than that of 3 hours ($r^2 = 0.9926$). Although the linear range of the curve all started from the cell number of 8000 cells/well, the concentration of macrophages of $2 \times 10^4$ cells/well was chosen as the optimised cell number in order to make sure the potential negative proliferation was still in the linear range (Figure 5.7b).
Figure 5.7a. Effect of cell number of macrophages on absorbance at 490nm measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (mean ± SD, n=3). Cells were incubated for 1 to 4 hours after the addition of the CellTiter 96® AQueous One Solution reagent.
Figure 5.7b. Effect of cell number of macrophages on absorbance at 490nm measured using the CellTiter 96® AQuantiOne Solution Cell Proliferation Assay (mean ± SD, n=3). Cells were incubated for 1 to 4 hours after the addition of the CellTiter 96® AQuantiOne Solution reagent.
Figure 5.8 shows the CytoTox 96® Non-Radioactive Cytotoxicity Assay results for different concentrations of macrophages after incubation for 15, 30, 45 or 60 minutes (Figure 5.8 a to d respectively). The incubation time after the addition of stop solution was one of the parameters which influenced the absorbance record, and was investigated in order to optimise the assay conditions. Similar to the proliferation colorimetric assay, increasing both cell concentration and incubation time increased the reaction conditions and the subsequent absorbance measurements (Fig 5.8). Indeed, with higher cell concentrations, above $4 \times 10^4$ cells/well, incubation times of over 45 minutes resulted in absorbance readings out with the range of the micro-plate reader (Figure 5.8 e and d). The absorbance values of incubation times of 15 and 30 minutes after adding the stop solution showed that the assay signal in the range of cell number between 8000 to 40000 cells/well was linear and the absorbance value was more than two times the background. However, similar to the CellTiter 96® AQuous One Solution Cell Proliferation Assay, to make sure the potential negative proliferation was still in the linear range, the concentration of macrophages of $2 \times 10^4$ cells/well was chosen as the optimised cell number.

In line with our interpretations, Scheel et al. (2009) evaluated the effect of hydroxyapatite on the RAW 264.7 macrophage cell line in cytotoxicity and activation using an sodium-30-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate assay, and Kim and Ha (2009) determined the protective effect of paoniflorin from on murine macrophages in
LPS-induced cytotoxicity using the MTT assay - the results of both studies showed that the cytotoxicity to murine macrophages was best measured at a cell number of $2 \times 10^4$ cells/well.

Figure 5.8a. Effect of cell number on absorbance at 490nm measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (mean ± SD, n=3). Cells were incubated for 15 minutes after the addition of the stop solution.

Figure 5.8b. Effect of cell number on absorbance at 490nm measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (mean ± SD, n=3). Cells were incubated for 30 minutes after the addition of the stop solution.
Figure 5.8c. Effect of cell number on absorbance at 490nm measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (mean ± SD, n=3). Cells were incubated for 45 minutes after the addition of the stop solution. The readings of the absorbance of the cell number over $4 \times 10^4$ cells/well were beyond the reading range of the micro-plate reader.

Figure 5.8d. Effect of cell number on absorbance at 490nm measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (mean ± SD, n=3). Cells were incubated for 60 minutes after the addition of the stop solution. The readings of the absorbance of the cell number over $4 \times 10^4$ cells/well were beyond the reading range of the micro-plate reader.
5.3.3. Optimisation of sample concentration

To optimise the microsphere concentration relative to cell number, preliminary studies using PLA/TDB microspheres were used. Figures 5.9-15 show the cytotoxicity to macrophages exposed to the PLA microspheres containing 4% TDB (w/w), at varying cell numbers of 2,000 to 80,000 cells/well. As demonstrated within these figures, at high microsphere to cell concentrations, higher toxicity levels were measured, especially at a low cell number: more than half of the macrophages were killed at a cell number of 2000 cells/well when exposed to the PLA microspheres at a concentration of 10 µg/well. As the concentrations of microspheres was reduced, the cytotoxicity also decreased to 13.34 ± 11.62 and 13.18 ± 9.95% when exposed to the 1 and 0.1 µg/well microspheres, respectively. Furthermore, the cytotoxicity decreased as the cell number increased, only 8.89 ± 1.90 and 7.93 ± 3.31% macrophages lysed when exposed to 100 and 10 µg/well microspheres at a cell number of $4 \times 10^4$ per well. A very low cytotoxicity (4.45 ± 0.86%) was measured when $2 \times 10^4$ cells/well macrophages were exposed to 10 µg/well PLA microspheres. No statistic difference was found for macrophage lysis at the same cell number when exposed to the PLA microspheres at concentrations of 0.1 and 1 µg/well. Thus 10 µg/well with a cell count of $2 \times 10^4$ cells/ well was chosen as the concentration of PLA microsphere samples for the cellular study.
Figure 5.9. Effect of the concentration of fresh made PLA microspheres containing 4% TDB (w/w) on cytotoxicity using the cell number of $0.2 \times 10^4$ per well (mean ± SD, n=3).

Figure 5.10. Effect of the concentration of fresh made PLA microspheres containing 4% TDB (w/w) on cytotoxicity using the cell number of $0.4 \times 10^4$ per well (mean ± SD, n=3).
Figure 5.11. Effect of the concentration of fresh made PLA microspheres containing 4% TDB (w/w) on cytotoxicity using the cell number of $0.8 \times 10^4$ per well (mean ± SD, n=3).

Figure 5.12. Effect of the concentration of fresh made PLA microspheres containing 4% TDB (w/w) on cytotoxicity using the cell number of $2 \times 10^4$ per well (mean ± SD, n=3).
Figure 5.13. Effect of the concentration of fresh made PLA microspheres containing 4% TDB (w/w) on cytotoxicity using the cell number of $4 \times 10^4$ per well (mean ± SD, n=3).

Figure 5.14. Effect of the concentration of fresh made PLA microspheres containing 4% TDB (w/w) on cytotoxicity using the cell number of $6 \times 10^5$ per well (mean ± SD, n=3).
Figure 5.15. Effect of the concentration of fresh made PLA microspheres containing 4% TDB (w/w) on cytotoxicity using the cell number of $8 \times 10^4$ per well (mean ± SD, n=3).

5.3.4. Cell proliferation and cytotoxicity

To investigate the toxicity of the various microsphere, cell proliferation was determined using the CellTiter 96® AQone® One Solution Cell Proliferation Assay. The results (Figure 5.16) revealed cell proliferation to be consistently high, over ~89% in all cases. The macrophages treated with the non-freeze-dried formulations were shown to have a cell viability of $95.01 \pm 1.06\%$ (PLA+TDB+BSA) to $97.82 \pm 1.70\%$ (PLA+BSA), while values of $89.73 \pm 2.17\%$ to $94.36 \pm 0.86\%$ were obtained for the corresponding freeze-dried formulations (Figure 5.22.). The addition of BSA to the macrophages did not significantly influence cell proliferation, and no significant difference was found between the formulations with or without BSA. The difference in composition between non-freeze-dried and freeze-dried formulations was the
presence of cryoprotectants, trehalose and leucine. According to the results showed in Figure 5.22, leucine did not have any inhibitory effect on the macrophages cell number at a concentration of 1% (w/v). However, the macrophage viability decreased from 99.40 ± 0.83 to 91.43 ± 2.31% when treated with 6% (w/v) trehalose. Similar results were also noted when the systems were in a freeze-dried powder form (98.63 ± 1.70 and 90.82 ± 1.12% for leucine and trehalose, respectively). The results of the proliferation assay showed the PLA microspheres formulations to have a small inhibitory effect on cell viability.

Trehalose has been reported to offer stabilization and protection to cell membranes during the freeze-drying process by binding to the polar head group of the membrane phospholipid (Crowe et al., 1985), and has been used to preserve tissues for organ transplanting (Hirata et al., 1993). It has been reported that oligo- and monosaccharides can inhibit proliferation of human T lymphocytes (Licastro et al., 1987), suggesting that sugars may also have anti-proliferative action on cells. The molecular structure of trehalose is a linkage of two glucose molecules (Birch, 1963), and it does not have the anti-proliferative effect reported for glucose at high concentration on cells, mediated through reduced mitogenic activity of fibroblast growth factor (FGF-2) (Duraisamy et al., 2003). This is thought to be because trehalose is a non-reducing disaccharide which is not hydrolysed into glucose (Elbein, 1974). The safety of trehalose has also been demonstrated in studies using mouse peritoneal macrophages (Taya et al., 2009), who also investigated cell proliferation. In
these studies no suppression on the proliferation of mouse peritoneal macrophages at trehalose concentrations of 0.1, 1, or 10 mM was measured (Taya et al., 2009). Similarly, no significant change to the viability of Chinese hamster ovary cells treated with trehalose at concentrations up to 0.5% (w/v) was measured (Richards et al., 2002). However, the anti-proliferative effect of trehalose reported in Figure 5.16 might be due to hyperosmolarity; though further studies would be required to confirm this.

The MTS measurements showed that the proliferation of macrophages treated with the formulations containing TDB were not significantly different from macrophages treated with PLA microspheres formulated without TDB (Figure 5.16). However, the presence of TDB within the microsphere formulation was shown to influence the cytotoxicity of the formulation when measured the LDH assay (Figure 5.17.). The addition of TDB to the microsphere formulations was shown to significantly increase cytotoxicity; for non-lyophilised microspheres cytotoxicity increased from 4.45 ± 0.86% to 18.35 ± 1.47% with the addition of 4% w/w TDB, and from 3.92 ± 1.01% to 16.11 ± 3.01% to for the PLA microspheres without BSA (Figure 5.17.). A similar trend was also found in freeze-dried formulations with the presence of TDB significantly increasing the measured cytotoxicity.

These results are similar to those of Wang et al. (2009) showing that THP-1 cells exposed to PLGA microspheres containing TDB release less LDH than empty
microspheres. The MTS assay is based on the cell's metabolic and mitochondrial activity, but does not indicate the damage to cell membranes by nitric products secreted during phagocytosis (Yildiz et al., 1999). LDH though will be released upon any damage to cell membranes (Wang et al., 2009), and this might involve oxidative injury induced during the macrophage activation process (Yildiz et al., 1999). Thus the higher cytotoxicity of the formulations without TDB may not be detectable using MTS assay.. The results for macrophage proliferation and cytotoxicity exposed to PLA+TDB+/−BSA non-lyophilised microspheres were in line with these previous reported findings. This indicated that the addition of TDB into the PLA microsphere formulations may provide a “protective effect” on the cell. Alternatively the cytotoxic effect of the TDB-containing microspheres may be due to mitochondrial damage.
Figure 5.16. Cell proliferation of macrophages exposed to PLA microsphere formulations for 24 hours using MTS assay. (Mean ± SD, n=3) * denotes significant difference in cell viability in comparison to the positive control (p<0.05); + denotes significant difference in cell viability in comparison between the two groups (p<0.05).
Figure 5.17. Cytotoxicity study of macrophages after exposed to PLA microsphere formulations for 24 hours using LDH assay. (Mean ± SD, n=3). The value of positive control is 100.21 ± 6.32. * denotes significant difference in cytotoxicity in comparison to the negative control (p<0.05); ** denotes significant difference in cytotoxicity in comparison to the negative control (p<0.001); + denotes significant difference in cytotoxicity in comparison between the two groups (p<0.05).
5.3.5. Phagocytic activities

NAG release, as an indicator of phagocytic activity, in the culture supernatant of macrophages exposed for 24 hours to PLA microspheres formulations was determined using NAG assay. The macrophages exposed to all microsphere formulations tested showed higher NAG release than the untreated macrophages, up to 45% above the negative control (PLA+BSA microspheres) (Figure 5.18.). However, the NAG release from cells treated with TDB containing microspheres was actually statistically lower than their naïve formulations. This may be related to their increased cytotoxicity, rather than an indication of reduced phagocytosis. Further studies would be required to validate this.

According to the research of Howie et al. (1993), particles larger than 50 μm can not be taken up by macrophages, and large particles are likely to attach on the surface of the macrophage rather than to be uptaken, with the upper size limit for macrophage phagocytosis being 5μm (Horisawa et al., 2002). Thus the lower NAG release as a result of the addition of TDB might also be explained by this size-dependent rule. The PLA microspheres and the freeze-dried powders had a mean particle size about 1μm which would readily induce the phagocytic activity. In contrast, the formulations containing TDB with diameters about 2.5-fold greater, may still be within the size limit suggested by the work of Horisawa et al. (2002) but still result in reduced uptake (Figure 5.18.). Phagocytosis has also been shown to be enhanced with particles smaller than 2 μm (Dasai et al., 1996) therefore the difference in size between the two
formulations in addition to the difference in toxicity profile cannot be eliminated as a reason for the differences in phagocytosis. Furthermore, the smaller diameter of non-TDB-containing formulations might led higher cytotoxicity due to they were more easy to be phagocyted than TDB-containing formulations with larger particle size. This could be further validated by producing microspheres of the same size with and without TDB but such formulation work was outside the scope of this project.

In figure 5.18., it also showed that the NAG release induced by the lyophilised microsphere formulations were higher than by the non-lyophilised microspheres. It has been mentioned in cytotoxicity studies that the freeze-dried formulations were more toxic perhaps due to the higher concentrations of trehalose (6%, w/v), thus this increase in NAG release may not be due to reduced phagocytosis but again due to variations in toxicity as also previously reported (Wang et al., 2009).
Figure 5.18. NAG release from macrophages after exposure to PLA microsphere formulations for 24 hours (Mean ± SD, n=3). The value of positive control is 48.39 ± 4.13. * denotes significant difference in NAG release in comparison to the negative control (p<0.05); + denotes significant difference in NAG release in comparison between the two groups (p<0.05).
5.3.6. Macrophage activation

TNF-α activity in the culture supernatant of macrophages exposed for 24 hours to PLA microspheres formulations was determined using TNF-α assay to investigate cell activation. The calibration curves of TNF-α concentration versus mean absorbance and the log value of TNF-α concentration versus the log of optical density were established (Figure 5.19a & b.). The standard curve of the log graph was chosen as the standard curve for calculation showed more linear (Figure 5.19b.).

TNF-α levels released from macrophages after 24 hours exposure to the microsphere formulations of PLA with BSA (178.27 ± 21.38 and 170 ± 10.183 pg/ml, lyophilised and not respectively) and PLA alone (171.86 ± 16.52 and 164.63 ± 22.37 pg/ml) were significantly higher than levels measured with microsphere formulations of PLA+TDB+BSA (106.51 ± 9.14 and 104.25 ± 15.28 pg/ml) and PLA+TDB (100.35 ± 7.54 and 102.54 ± 16.152 pg/ml) (Figure 5.20). The increase in TNF-α release measured in PLA microspheres not containing the immunomodulator TDB might again be related to the reduced toxicity and increased phagocytic activity of macrophages in the presence of PLA microspheres compared to PLA/TDB systems. The macrophages were activated by phagocytosis-induced stimulation and secreted TNF-α, while the phagocytic activity of macrophages would be then increased in presence of TNF-α (Wang et al., 2009). It would also explain why the macrophages treated with TDB-containing formulations released less NAG and TNF-α.
Figure 5.19a. Calibration curve for TNF-α assay calculation. TNF-α concentration versus absorbance (Mean ± SD, n=3).

Figure 5.19b. Calibration curve for TNF-α assay calculation. log(TNF-α concentration) versus log(absorbance at 450nm) (Mean ± SD, n=3).
The macrophages exposed to non-TDB-containing formulations showed higher levels in both of TNF-α and LDH release. It has been reported that TNF-α may induce apoptosis and necrosis (Larrick & Wright, 1988; Larrice & Kunkel, 1990), and lead cytotoxicity due to the nitric oxide production stimulated by TNF-α (Frankova & Zidek, 1998) which may cause cell membrane damage, consequently resulting the release of LDH.
Figure 5.20. TNF-α release from macrophages after exposure to PLA microsphere formulations for 24 hours (Mean ± SD, n=3). * denotes significant difference in NAG release in comparison to the negative control (p<0.05); ** denotes significant difference in NAG release in comparison to the negative control (p<0.001); + denotes significant difference in NAG release in comparison between the two groups (p<0.05).
5.4. Conclusion

Exposing J774 Macrophages to PLA microsphere formulations either freeze-dried or not, could induce cellular responses including cell proliferation, cytotoxicity, phagocytic activity and macrophage activation. These cellular responses could be detected using appropriate method to quantitatively determine the cytokines and enzymes.

As the protocols being used within this work had not previously been employed within our research group, a series of studies to establish the protocols were required. In particular, optimising the ratio of cell number versus sample concentration is very important to the cellular response studies. An appropriate concentration of cells and samples kept the optical density of the release of cytokines and enzymes in a linear range, improving data accuracy. In this study, both the CellTiter 96® AQ ueous One Solution and CytoTox 96® Non-Radioactive Cytotoxicity Assay kit provided a protocol for cell number optimisation; and we set up a protocol based on LDH measurement to optimise the concentration of samples. Both the optimised cell number and sample concentration worked well in all the assays employed.

The variance in MTS and LDH assay results suggested that their comparison provides important information such as cell are injury and cell death. In this study, the results of the two assays revealed that macrophage proliferation was inhibited by the formulations. Additionally, cytotoxicity was detected using LDH assay due to damage
of the cell membrane, which was decreased as the addition of TDB into PLA microspheres. Furthermore, the higher level of LDH release caused by freeze-dried formulations might be due to the osmolarity produced by high concentration of cryoprotectant.

NAG release during phagocytosis process indicated that the phagocytic activities of macrophages depend on particle diameter. The uptake of TDB-containing formulations was weaker than the ones without TDB because of their larger size.

TNF-α induced by the phagocysis of macrophage plays an important in some cellular activations which protect host against foreign pathogen (Demirjian et al., 2006), such as inducing Th1 immunoresponse (Wang et al., 2009), leading apoptosis of TB infected macrophages (Arcila et al., 2007) and inhibiting tumour cells. However, TNF-α also improves the secretion of the nitric product which may cause cell injury, and partly contributes to the LDH release. Furthermore, phagocytic activity can be enhanced in the present of TNF-α. The promotional effect between the releases of LDH, NAG and TNF-α was demonstrated in this study.

To summarise, the PLA microsphere formulations had a slight anti-proliferative effect on J774 macrophages, and the high concentration of trehalose used as cryoprotectant was responsible for the extra LDH release. Compared to the formulations without TDB, the addition of TDB had less adjuvant action, based on NAG and TNF-α release,
but a notable decrease in cytotoxicity to macrophages and makes the PLA microspheres safer, and the decrease of BSA loading efficiency was acceptable. However, the particle size increases in presence of TDB, thus an \textit{in vitro} aerosolisation study for TDB-containing formulation is needed to investigate their deposition and aerosol dynamic performance.

In our study, PLA microspheres exhibited a low cytotoxicity while maintaining a particle size less than 3 \textmu m. This PLA microsphere system may be a promising antigen delivery system to the lung and inducing with low cytotoxicity. The addition of TDB may be useful for the vaccine delivery, but its effect on cytotoxicity, phagocytic activity and cell activation needs be investigating further.
Chapter 6

General discussion and conclusions
Delivering drugs via pulmonary route has been used in the modern western medication for the treatment of airway diseases for more than two hundred years (Smith and Bernstein, 1996). It can get direct access to the disease site and result rapid therapeutic response with minimized side effect (Sung et al, 2007). As the end organ of the respiratory track, the lung is also an attractive target for the route of drug delivery for systemic therapies. The lung provides a large absorptive surface and highly permeable membrane in alveoli with a low enzymatic environment compare to gastrointestinal route (Patton et al, 1996 & 2004), the therapeutic activity of drugs are not impaired by the first-pass metabolism in liver. However, the lung is relatively inaccessible, and drug deposition in the lung can be effected by many factors, e.g. particle size, geometry of airway, humidity in the respiratory track, clearance mechanisms and change of architecture of airways due to lung diseases (Labiris & Dolovich, 2003), and the efficacy of drugs can be affected by site of deposition in the lung. Thus, the clinical application for systemic therapies, particularly for controlled drug release formulation via respiratory route is limited, and most researches are still in laboratorial step. Therefore, the development of an effective and therapeutic pulmonary drug delivery system is still a challenging field of inhalation research.

Particulate drug delivery systems play an important role in pulmonary delivery, providing sustained release and reducing systemic side effects (McCalden, 1990). Such drug delivery systems mainly include liposomes (Zaru et al, 2007), microspheres (Newman et al, 1998; Ma et al, 1998; Wang et al, 1999), emulsion and
microemulsions (Sommerville et al, 2000). For vaccine delivery, particulate carriers provide immunological adjuvancy due to the non-specific uptake by antigen presenting cells (Pettis et al, 2000; Elamanchili et al, 2007), and subsequently inducing immune response (Beutler, 1999; Avni et al, 2009). However, particle diameter is critical for effective inhalation, with an aerodynamic size range of 0.5-3μm being required particularly for alveoli deposition.

Initial studies in this research focused on the formulation optimisation to produce microspheres with appropriate diameter which suitable for alveoli deposition. The investigation of formulation parameters involved in double emulsion solvent evaporation process, such as the molecular weight of the polymer, the concentration of polymer in organic phase and the concentration of emulsifier, provided an understanding of their effect on the diameter and size distribution of the microspheres. The results demonstrated again that the microspheres can be prepared in an appropriate size for pulmonary delivery with high entrapment efficiencies by adjusting such parameters, and resulted in the successful loading of BSA into PLA microspheres, but the addition of emulsifier (PVA) in internal aqueous phase of W/O primary emulsion failed to increase the BSA loading efficiency. From these studies, the optimised formulation for microspheres was identified as:

- Preparation by the W/O/W method.
- Using PLA with molecular weight of 50 kDa.
- Using a PLA concentration of 3% (w/v) for the organic phase.
• Using a PVA concentration of 10% (w/v) for the external aqueous phase.

The storage environments of these PLA microspheres, either in different mediums or in storage temperatures, allows for the comparison between formulations with particle size, surface charge and BSA loading efficiency. The PLA microspheres showed stable in particle size and surface charge, and less BSA leakage when stored in aqueous environments at 4°C in a period of four weeks. While an increased aggregation was found caused by particle aggregation due to the neutral surface when the PLA microsphere suspensions were left at room temperature, and an increased BSA leakage was also noticed in the same condition. The results demonstrated that the PLA microsphere formulations are not stable for long-term storage in an aqueous environment, indicating their instability in pMDI formulations that mainly based on liquid formulations.

As an alternative to the aqueous formulations, investigations then turned into dry powder form. The lead microsphere formulation, made of PLA MW 50 kDa, was subsequently freeze-dried in order to produce dry powders, and a moisture content of ~4% (w/v) were determined using thermo-gravimetric analysis. Cryoprotectants based on disaccharide (sucrose and trehalose) and amino (leucine) were investigated to evaluate their effects on the physico-chemical characters of the PLA microspheres.

Both of the disaccharide-based cryoprotectants, sucrose and trehalose, showed
effective ability of maintaining the particle size of the PLA microspheres. It seemed that the main function of sucrose and trehalose as cryoprotectants is likely to be vitrification to form a glassy layer around the microspheres rather than interaction with the surface forming hydrogen bonds, because the surface charge of the microspheres was not changed in the presence of sucrose and trehalose.

Using leucine as cryoprotectant also effectively prevented microspheres from aggregation, whilst having little effect result of the zeta potential measurement, indicating the main function of leucine as cryoprotectant is particle isolation.

Furthermore, combined use the mixture of trehalose and leucine as cryoprotectant in the formulations resulting a great maintenance in particle size and zeta potential. Although the mechanism of combined use of trehalose and L-leucine as cryoprotectant is uncertain, it did result in a smaller particle size than the formulations cryoprotected with these agents separately suggesting a synergistic action.

Due to in the presence of cryoprotectants, the initial freeze-dried products were cake-like bulk, but can be manually grinded into powders. The size of dry powders was depended on the length of grinding duration.

The resulted freeze-dried powder formulations were then sealed in an anhydrous environment in either at 4°C or room temperature for stability study. All the tested
formulations were stable in terms of retention of physico-chemical attributes, including mean particle size, zeta potential and BSA loading efficiency over four weeks storage both at 4°C or room temperature. The results suggested the PLA microspheres could be used in dry powder inhalation platform, or stored in dry powder form before atomisation.

The results of the subsequent *in vitro* release study showed all the tested formulations had similar release profiles with an initial burst release followed by a gradual release of the rest BSA load. The burst release also supported the need for these formulations to be stored in a dried format, and the following gradual release indicated the potential for sustained drug release of the PLA microspheres.

Overall, the optimised formulation for microspheres used in subsequent studies was identified as:

- Preparation by the W/O/W method followed by freeze-drying.
- Using 6% trehalose and 1% leucine together as cryoprotectants.

The subsequent studies was turned towards the evaluation of the aerosolisation performance of the PLA microsphere formulations in both dry powders and dispersion. It became clear that the PLA microspheres were not suitable for pMDI delivery system due to their instability in moisture environment, therefore the *in vitro* deposition studies focused on the DPI and nebulisation platforms. However, the dry
powder formulations delivered by the Eclipse DPI showed a poor aerosolisation performance. This might be because of the large particles due to the insufficient decreasing in size by manual grinding, but the formulations using trehalose and leucine as cryoprotectants showed better aerosolisation performance than using them alone. The results indicated the powders were not particularly respirable via DPI, but it suggested some other methods might work better on decreasing the powders, such like mechanical milling.

As an alternative to DPI, nebulisations of the rehydrated freeze-dried powders using both ultrasonic nebuliser and air-jet nebuliser showed significant increased aerosolisation profiles compared to using DPI, because the cryoprotectants around the microspheres were dissolved in the double distilled water but left the insoluble microspheres suspended in the liquid. The two different type nebulisers showed different atomisation efficacy, the ultrasonic nebuliser was more effective than the air-jet nebuliser in aerosolisation performance of the nebulised suspension. However, the atomisation efficacy of air-jet nebuliser could be improved by diluting the suspension filled in the reservoir.

Finally, murine macrophages (J774 cell line) exposed to the PLA microsphere formulations was investigated to evaluate their in vitro cellular responses in cell proliferation, cytotoxicity, phagocytic activity and macrophage activation.
Chapter 6
General discussion and conclusions

The PLA microsphere formulations showed a slight anti-proliferative effect on the macrophages based on MTS assay, and the results were consistent to the view that PLA has been considered as a non-toxic polymer (El-Baseir & Kellaway, 1998), although the toxicity was estimated based on the lysed cell. Some previous studies used MTS assay to evaluate the cytotoxicity based on the number of dead cells (Case et al, 2008), but these results cannot represent the cytotoxicity due to damage on cell membrane. However, the measurement of LDH released by both lysed and injured cells can fully reveal the cytotoxicity. The result of MTS assay relative to LDH release can be used to estimate the proportion of cytotoxicity caused by lysed cells and the proportion due to the damage on cell membrane. The cryoprotectant, leucine, did not contribute to any cellular response, but trehalose should take the responsibility for the extra anti-proliferative effect and the LDH release which may due to the osmotic stress caused by high concentration of trehalose.

Moreover, the addition of TDB into PLA microsphere formulations showed less adjuvancy, which might due to the particle size of microspheres was increased in the presence of TDB, reducing the phagocytic activity which depends on the particle diameter. The less phagocytic activity decreases the intercellular contact between the microspheres and the macrophages, reducing other cellular responses such like the release of LDH, NAG and TNF-α.

Overall in summary, to achieve the main aim of this thesis - developing an effective polymeric microsphere based drug delivery system for the protein administration via pulmonary route, the PLA microspheres can be prepared in a size range that suitable for pulmonary delivery with high protein loading efficiency using W/O/W double emulsion solvent evaporation method, and the stability of PLA microspheres can be enhanced in dry powder format via a freeze-drying process. The aerosolisation performance estimated through the in vitro deposition of nebulised PLA microspheres is also acceptable due to the maintained physico-chemical properties after rehydration. Despite of the poor aerosolisation profile of the PLA microsphere freeze-dried powders delivered using DPI, all the descriptions above indicated the resulting PLA microsphere formulations are desirable candidates of particulate systems for pulmonary delivery.

6.2. Future prospects for pulmonary drug delivery.

As for the development of inhalation technique and delivery devices, particulate pulmonary drug delivery system may become more and more important for the treatment of systemic diseases due to its advantages compared to other drug delivery systems, particularly for the delivery of protein, peptide and DNA which are potential candidates for gene therapy or vaccination. Unfortunately, the release and subsequent withdrawal of Exubera will undoubtedly have a severe negative impact on all future developments in this area, and it will be difficult to be successfully brought back to
market. However, the future pulmonary delivery systems will keep focusing on systemic drug action, and be recognised as a desirable alternative option to other drug delivery systems.
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