DEVELOPMENT OF DRY POWDER ANTIGEN FORMULATIONS FOR PULMONARY VACCINE DELIVERY

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TOMÁS SOU

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Drug Delivery, Disposition and Dynamics

Monash Institute of Pharmaceutical Sciences

Monash University

381 Royal Parade

Parkville, Victoria, 3052

Australia

General Declaration

Monash University

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17 Doctor of Philosophy and Research Master's regulations the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 2 original papers published in peer reviewed journals, 1 accepted manuscript and 2 submitted manuscripts for publication. The core theme of the thesis is the development of dry powder antigen formulations for pulmonary vaccine delivery. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Department of Pharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences under the supervision of Dr Michelle McIntosh, Associate Professor David AV Morton and Dr Lisa Kaminskas.

The inclusion of co-authors reflects the fact that the work came from active collaborations between researcher groups and acknowledges the multi-disciplinary nature of research.

In the case of chapter 2-6 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent of candidate's contribution
2	Investigating the interactions of amino acid components on a mannitol-based spray-dried powder formulation for pulmonary delivery: a design of experiment approach	published	Study initiation and design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion. 70%
3	The effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried formulations for pulmonary delivery of biomacromolecules	published	Study initiation and design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion. 80%
4	Designing a multi-component spray-dried formulation platform for pulmonary delivery of biomacromolecules: the effect of polymers on the formation of an amorphous matrix for glassy state stabilisation of biomacromolecules	published	Study initiation and design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion. 90%

5	Designing a multi-component spray-dried formulation platform for pulmonary delivery of biomacromolecules: the effect of polyol, disaccharide, polysaccharide and synthetic polymer on the solid-state properties of spray-dried formulations	submitted	Study initiation and design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion. 70%
6	Spray-dried influenza vaccine with trehalose and leucine produces a highly aerosolisable powder that induces superior systemic and mucosal immunity after pulmonary administration	submitted	Study initiation and design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion. 80%

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Signed: ……

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Abstract

Pulmonary immunisation has gained increased recognition as a means of triggering both a mucosal and systemic immune response through aerosol delivery to the lungs. The appropriate formulation of antigens in a dry, solid state can result in improved stability, thereby removing cold chain storage complications associated with conventional liquid-based vaccines. Nevertheless, dry powder pulmonary vaccine formulations are not being widely used as yet. This thesis describes the latest developments in pulmonary vaccines, and explores the use of recent advancements in spray-drying technologies for the production of dry powder formulations for pulmonary vaccine delivery.

Dry powder inhaler formulations for pulmonary delivery of antigen should be readily and consistently dispersible and aerosolisable upon inhalation by an appropriate range of patient groups and circumstances for efficient delivery. In addition, the formulation should be capable of stabilising the antigen, which is commonly a potent biomacromolecule (e.g., protein or peptide required in relatively low dose of <1 mg). In contrast to the respiratory delivery of many small molecule drugs which is commonplace via simple ordered mixtures, there is no well-characterised delivery platform that is readily adaptable to the incorporation of potent biomacromolecules. A novel multi-component particulate system suitable for pulmonary delivery of biotherapeutics is therefore highly beneficial.

To investigate the potential benefits from multi-component spray-dried systems, the interaction effects of the amino acids leucine, glycine and alanine on a mannitol-based spray-dried formulation for pulmonary delivery was initially studied using a design of experiment approach. The amino acid leucine was found to facilitate particle

formation and production of aerosolisable multi-component spray-dried powders that would otherwise not be suitable for aerosol application. Further results from X-ray powder diffraction studies suggested the formation of a partially ordered leucine shell resulting from self-assembly on particle surfaces was important in assisting particle separation and powder aerosolisation. The results also suggested the potential benefit of leucine in retarding crystallisation and maintaining amorphicity of the formulation. This has significant implication as an amorphous environment is required for glassy stabilisation of biomacromolecules e.g., vaccine antigens.

The particle formation effect of leucine was found to be transferrable to excipients covering a range of molecular weights from polyol (mannitol), disaccharide (trehalose), polysaccharide (inulin) to synthetic polymer (PVP). These multi-component systems were found to be both amorphous with sufficiently high glass transition temperature for stability at typical ambient condition, while remaining aerosolisable with high fine particle fractions. An in vivo immunisation study using influenza vaccine co-spray-dried with trehalose and leucine was shown to produce highly aerosolisable powders that induced superior systemic and mucosal immunity after pulmonary administration. This formulation strategy was found to be promising for the production of dry powder formulations for pulmonary vaccine delivery.

Multi-component spray-dried delivery systems with leucine and appropriate baseline excipients were therefore shown to produce inhalable dry powder formulations that were aerosolisable with the amorphous environment needed for stabilisation of biomacromolecules. This work presented a novel formulation strategy for the production of dry powder carrier platforms for pulmonary delivery of vaccines, and more broadly for various potent biotherapeutics.

Statement of originality

I hereby certify that the work contained in this thesis has not been submitted by myself or any other person for a degree of Monash University or of any other institution.

Tomás Sou

June 2013

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Tomás Sou

June 2013

Representative publications

JOURNAL PAPERS

- Sou, T., McIntosh, M.P., Kaminskas, L.M., Prankerd, R.J., Morton, D.A.V., 2013. Designing a multicomponent spray-dried formulation platform for pulmonary delivery of biomacromolecules: The effect of polymers on the formation of an amorphous matrix for glassy state stabilization of biomacromolecules. Drying Technology 31, 1451-1458.

- Sou, T., Kaminskas, L.M., Nguyen, T.-H., Carlberg, R., McIntosh, M.P., Morton, D.A.V., 2013. The effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried formulations for pulmonary delivery of biomacromolecules. European Journal of Pharmaceutics and Biopharmaceutics 83, 234-243.

- Sou, T., Orlando, L., McIntosh, M.P., Kaminskas, L.M., Morton, D.A.V., 2011. Investigating the interactions of amino acid components on a mannitol-based spray-dried powder formulation for pulmonary delivery: A design of experiment approach. International Journal of Pharmaceutics 421, 220-229.

- Sou, T., Meeusen, E.N., de Veer, M., Morton, D.A.V., Kaminskas, L.M., McIntosh, M.P., 2011. New developments in dry powder pulmonary vaccine delivery. Trends in Biotechnology 29, 191-198.

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- Tomás Sou, Lisa M. Kaminskas, Michelle P. McIntosh, David A.V. Morton. Investigation of the protective effect of L-leucine on particle formation of hygroscopic excipients during spray-drying. *Drug Delivery to the Lungs 23*, Edinburgh, UK. 2012.

- Tomás Sou, Michelle P. McIntosh, Lisa M. Kaminskas, David A.V. Morton. The effect of L-leucine on particle formation, morphology and hygroscopicity of spray-dried formulations for inhalation. *Globalisation of Pharmaceutical Education Conference 2012*, Melbourne, Australia. 2012.

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- Tomás Sou, Michelle P. McIntosh, Lisa M. Kaminskas, Laurence Orlando, David A.V. Morton. An investigation on the interaction effect of amino acids on the performance of a mannitol dry powder formulation using a design of experiment approach. *Globalisation of Pharmaceutical Education Conference 2010*, North Carolina, USA. 2010.

- David A.V. Morton, Michelle McIntosh, Tomás Sou. Investigating effects of surface modifications on a mannitol dry powder inhaler plume by laser diffraction. *Respiratory Drug Delivery*. 2008.

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CHAPTER ONE

NEW DEVELOPMENTS IN DRY POWDER PULMONARY VACCINE DELIVERY

1. New developments in dry powder pulmonary vaccine delivery

1.1. Statement of the problem

Immunization forms an important public health strategy, and vaccination of susceptible populations is regarded by the World Health Organisation (WHO) as the most effective way to reduce disease and death from infectious diseases (World Health Organisation, 2005). Although vaccines for several diseases are widely available in many developed countries, access and uptake of immunisation programs need improvement, especially in developing countries. The WHO estimates that at least two million children die from vaccine-preventable diseases each year, and millions more suffer from disability and illness because they have not been appropriately immunised (World Health Organisation, 2005). In 2009, it was estimated that more than 23 million children worldwide, mostly in India and Nigeria, did not receive the full three required doses of diphtheria-tetanus-pertussis (DTP) vaccine during their first year of life (World Health Organisation, 2010a); and at least 49 countries, mostly in Africa, were not able to achieve 80% coverage of infants with measles containing vaccines (MCV) (World Health Organisation, 2010b). The rapid mobilisation of mass immunisation campaigns internationally in response to the H1N1 swine influenza pandemic in 2009 reiterates the importance of vaccination in both developing and developed countries (World Health Organisation, 2009).

Most currently available vaccines are administered via a parenteral route as either intramuscular (IM) or subcutaneous (SC) injections. Parenteral administration is typically required because most antigens, such as proteins and polysaccharides, are macromolecules which are unable to penetrate into the systemic circulation via other

less-invasive routes such as oral and transdermal. However, parenteral vaccination has several drawbacks, which are particularly problematic in developing nations, including the requirement for skilled medical personnel for administration, the risk of needle-stick injuries, and consequently the transmission of blood-borne viruses. Furthermore, the majority of vaccine formulations available are dependent on the effective management of uninterrupted refrigerator storage conditions to maintain vaccine integrity. This cold chain requirement poses significant practical difficulties in the effective implementation of immunisation campaigns in third world countries where a reliable supply of electricity and cold-chain storage is often lacking.

Therefore, alternative formulations and routes of administration of vaccines are being explored to circumvent these issues (Carstens, 2009). Pulmonary vaccination utilises the unique physiology of the respiratory system for immunisation and confers a number of advantages over parenteral routes. Nevertheless, there are still no commercially available pulmonary vaccines on the market. Recent advancements in drug delivery and formulation technologies present new opportunities for potential growth in this field. The work described in this thesis aims to address some of the formulation barriers by improving our understanding of dry powder formulations for pulmonary delivery of biomacromolecules and focusses on the utility of such delivery systems for pulmonary immunisation with a model vaccine.

1.2. The immune system and measurement of immune responses

1.2.1. Background

Immunisation utilises the adaptive immune response which retains memory and becomes more powerful upon repeated encounters of the same antigen. Adaptive immunity is mediated by lymphocytes, a specialised group of leukocytes, including B and T lymphocytes (B cells and T cells). Protection is conferred by priming the system to an antigen which is able to induce specific immunity against the target disease prior to the first exposure of the pathogen. The memory of this specific immunity then renders a more powerful immune response upon future encounters of the same pathogen as illustrated in Figure 1. Detailed mechanisms of the underlying immunology are discussed in the immunology literature (Male et al., 2006).

Historically, vaccine studies used neutralising-antibody titres or antibody levels measured after immunisation as a sole comparator of the immune responses induced by different vaccines. However, as the knowledge of the immune system advances, it has been increasingly apparent that cellular immunity plays a critical role, especially in diseases involving intracellular pathogens such as tuberculosis, malaria and HIV (Berzofsky et al., 2001; Carstens, 2009; Fahmy et al., 2008). Therefore, current best practice is to measure both antibody and cytokine titres as markers of immune responses. Furthermore, as the importance of mucosal immunity is increasingly being recognised, measurements for mucosal immunity as well as systemic immunity both need to be addressed.

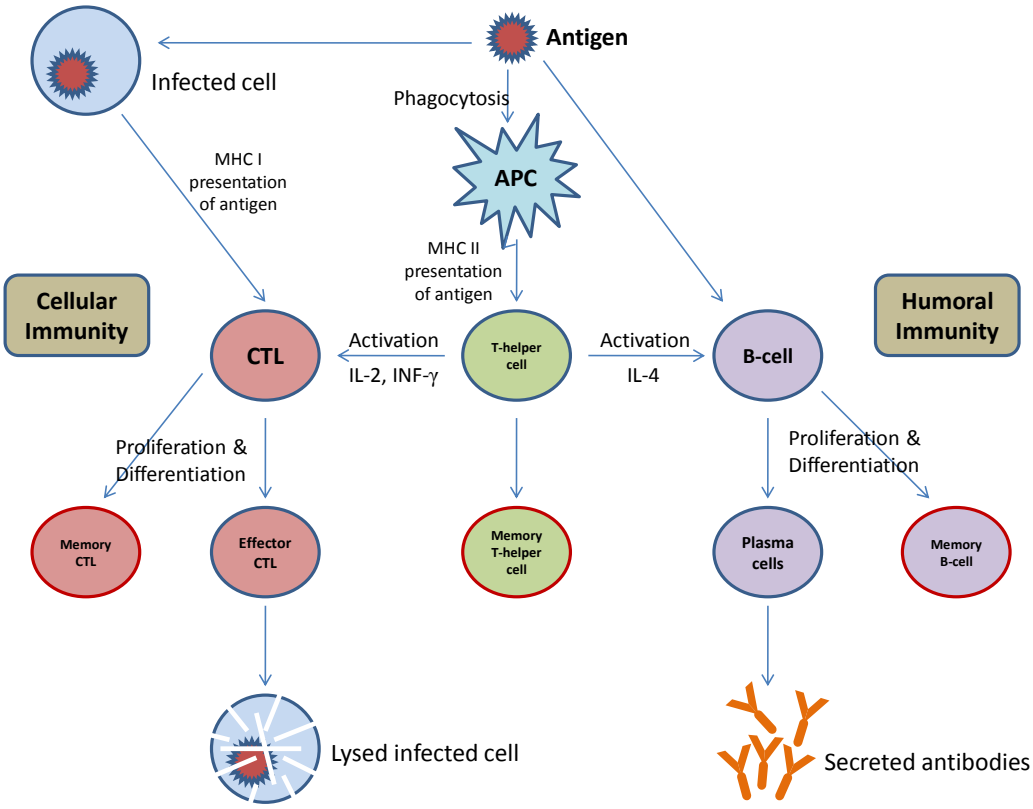

Figure 1. Adaptive immunity. APC: Antigen-presenting cell; CTL: Cytotoxic T-lymphocyte; MHC: Major histocompatibility complex; IL: Interleukin; INF: Interferon.

1.2.2. Humoral immunity

Immunoglobulins (or antibodies as they are more commonly referred) are the single most important component of humoral immunity which is essentially mediated by B-cells and acts primarily against extracellular pathogens. There are at least five main classes of immunoglobulin (Ig) namely IgM, IgG, IgA, IgE and IgD, each displaying different immunological functions as illustrated in Figure 2. IgM is present in human serum as a pentamer and is the predominant antibody in the primary immune response. IgG is the predominant antibody in the secondary immune response and constitutes 75% of the plasma antibody in adults. IgG is further subdivided into four

subclasses and each of these subclasses exhibits a unique profile of biological activities. IgG1 and IgG3, for instance, respond more efficiently in classical complement activation, while IgG2 subclasses have been used as indirect assessment of various cellular immunity pathways (Huang et al., 2004; Male et al., 2006). IgA is a characteristic of secondary immune responses. Its predominant role is in its secretory form in external secretions (e.g., saliva, tears, intestinal and bronchial mucous) as it serves to disable pathogens before they reach the internal environment. IgD is a transmembrane antigen receptor on B-cells and has no known effector functions as a serum protein(Male et al., 2006). IgE is associated with allergic responses since it binds to basophils and tissue mast cells when activated by antigens and releases chemical mediators such as histamine (Male et al., 2006; Silverthorn et al., 2004).

Recent vaccine research often measures several classes of immunoglobulins specific to the antigen at various sites of interest as a comprehensive examination of the immunity induced. To measure systemic immunity, serum IgG is frequently measured as an indicator in both mice and humans (Amorij et al., 2007b; Griffiths et al., 1997; Jiao et al., 2002; Kilhamn et al., 1998; Menzel et al., 2005; Meyer et al., 2006; Minne et al., 2007; Nardelli-Haefliger et al., 2005; Sanders et al., 2009; Wee et al., 2008). IgG1, IgG2a, IgG2b and IgG3 subclasses are reported in some instances to investigate mechanisms of immunity in more detail, such as the extent of humoral and cellular immunity (Amorij et al., 2007b; Huang et al., 2004; Minne et al., 2007; Sanders et al., 2009). IgM, IgE and IgD are measured in some instances in conjunction with other classes as supplementary data (Griffiths et al., 1997; Jiao et al., 2002; Sanders et al., 2009), but do not appear to act as representative indictors alone since they are not primarily responsible for secondary immune responses. Serum IgA is reported to a relatively lesser extent (Amorij et al., 2007b; Griffiths et al., 1997; Jiao et al., 2002;

Kilhamn et al., 1998; Minne et al., 2007; Nardelli-Haefliger et al., 2005; Sanders et al., 2009; Scheerlinck et al., 2006; Wee et al., 2008). For the measurement of local protection provided by mucosal immunity, either or both IgA and secretory IgA (sIgA) measured at the mucosal tissue of interest has almost always been used (Amorij et al., 2007b; Griffiths et al., 1997; Jiao et al., 2002; Kilhamn et al., 1998; Minne et al., 2007; Nardelli-Haefliger et al., 2005; Sanders et al., 2009; Wee et al., 2008), while some investigators also report local IgG levels (Amorij et al., 2007b; Griffiths et al., 1997; Jiao et al., 2002; Menzel et al., 2005; Meyer et al., 2006; Minne et al., 2007; Nardelli-Haefliger et al., 2005; Sanders et al., 2009; Wee et al., 2008).

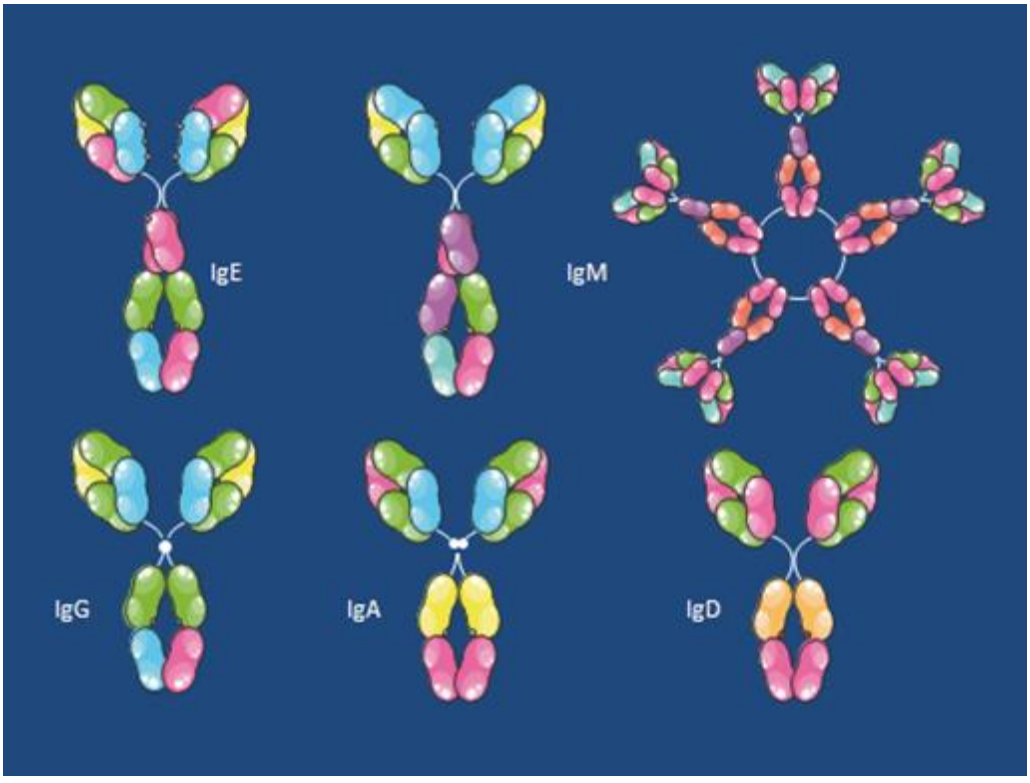

Figure 2. The five main classes of immunoglobulins. [Adapted from Servier Medical Art with permission]

1.2.3. Cellular immunity

Cellular immunity is mediated by T-cells and is mainly responsible for actions against intracellular pathogens and regulations of B-cell responses (Figure 1). Upon invasion of a pathogen, activated T-cells release cytokines to stimulate and amplify immune responses. Many cytokines and subclasses have been identified including various interleukins (ILs), tumour growth factors (TGFs), tumour necrosis factors (TNFs) and interferons (IFNs). Concentrations of these cytokines can be indicative of the magnitude of T-cells activation and cellular responses. For instance, T-helper 1 (Th1) cells secrete IFN-γ, TFN-β and IL-2 to promote macrophage activation, antibody-dependent cell-mediated cytotoxicity and delay-type hypersensitivity; while T-helper 2 (Th2) cells secrete other cytokines including IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 to stimulate humoral immunity pathways such as mucosal immunity, IgA synthesis, IgG1 and IgE isotype switching and mast cell and eosinophil growth and differentiation. Therefore, Th1 cells are associated with cell-mediated inflammatory reactions and Th2 cells are associated with strong antibody and allergic responses. This review by Male et al provides a comprehensive discussion of the complex interplay between these cytokines (Male et al., 2006).

Several cytokines have been reported in recent studies as a measure of cellular immunity. Interferon-γ (IFN-γ) has been reported in several studies as an indicator of Th1 cell response (Amorij et al., 2007b; Bivas-Benita et al., 2004; Matsuoka et al., 2002; Minne et al., 2007). Other Th1 cytokines such as interleukin-2 (IL-2) have been used to a lesser extent (Matsuoka et al., 2002). IL-4 has been reported in a number of studies as an indicator of Th2 cell response (Amorij et al., 2007b; Matsuoka et al., 2002; Minne et

al., 2007). IL-5 has also been used as a marker of Th2 cell response (Matsuoka et al., 2002). In addition to cytokine measurements, IgG1 and IgG2a titres have also been reported to provide an indirect assessment of Th2 and Th1 cells response respectively (Amorij et al., 2007b; Huang et al., 2004; Minne et al., 2007).

1.2.4. Common mucosal immune system (CMIS)

It is well established that there are various mucosa-associated lymphoid tissues (MALT) on different mucosal membranes of the body. These include the gut-associated lymphoid tissue (GALT), nasal-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT) and lymphoid tissues lining other mucosal membranes such as on the genitourinary tract (Bivas-Benita et al., 2005; Holmgren and Czerkinsky, 2005; Male et al., 2006; O'Hagan and Illum, 1990). Several investigators have suggested that the local immune system within these different mucosal surfaces appear to be linked, and immunocytes activated at one site are able to disseminate immunity to other distant and remote mucosal tissues (Holmgren and Czerkinsky, 2005; Minne et al., 2007; Yen et al., 2006). This observation led to the concept of the common mucosal immune system (CMIS). This system involves the detection of an antigen by the immune system and the dissemination of immunity to mucosal membranes distant to the site that is directly exposed to the antigen (Bivas-Benita et al., 2005; Holmgren and Czerkinsky, 2005; O'Hagan and Illum, 1990).

Mucosal immunity is conferred by antibodies on the mucosal site of pathogen invasion. It has been documented that secretory IgA (sIgA) can be detected in bronchial alveolar lavage (BAL) fluid and nasal secretions after oral administration of inactivated influenza virus (Minne et al., 2007; O'Hagan and Illum, 1990). Nasal delivery of human

papillomavirus antigen has also been reported to induce specific antibodies in saliva and cervical secretions (Nardelli-Haefliger et al., 2005). The CMIS appears to be a highly compartmentalised immunological system that functions essentially independent of the systemic immune response (Holmgren and Czerkinsky, 2005). It has been demonstrated that oral immunisation was more effective at inducing immune responses in saliva and vaginal secretions whereas rectal immunisation induced more pronounced responses in nasal secretion and tears (Kantele et al., 1998). The growing understanding of the CMIS is expected to ultimately provide novel opportunities for future optimisation of mucosal immunisations.

1.3. Vaccines

1.3.1. Vaccination

Immunisation provides an important role in public health strategy, which primes the immune system to induce specific immunity to anticipate the pathogen prior to the actual encounter. Vaccination of susceptible populations is regarded by the World Health Organisation (WHO) as the most effective way to reduce disease and death from infectious diseases (World Health Organisation, 2005). While vaccines for a number of diseases are already widely available in many developed countries, access and uptake of immunisation programs, especially in developing countries, remains to be improved. The WHO estimates that at least two million children die from vaccine-preventable diseases each year and millions more suffer disability and illness because they have not been appropriately immunised (World Health Organisation, 2005). These figures highlight the public health importance of vaccine research and development.

Most vaccines currently available are administered via the parenteral route either as intramuscular or subcutaneous injections. Parenteral administration is often required as most antigens are macromolecules, such as proteins and polysaccharides, and are unable to penetrate into the systemic circulation via other administration pathways. However, parenteral vaccination has several drawbacks. Firstly, the procedure is invasive and painful which can reduce adherence to the program. Secondly, the costs and risks associated with handling and disposing of needles such as the necessity of skilled medical personnel for administration, needle-stick injuries, and the consequent transmission of blood-borne viruses, are substantial. Thirdly, due to protein instability, the majority of vaccine formulations available to date are dependent on the effective management of cold chain storage to maintain the integrity of the vaccine. This cold chain requirement poses significant practical difficulties in the effective implementation of immunisation campaigns, especially in third world countries where the reliable supply of electricity and cold-chain storage is difficult. Therefore, alternative formulations and routes of administration of vaccines are being explored to circumvent these issues (Carstens, 2009).

1.3.2. Mucosal immunisation

When examining the dominance of parenteral vaccines on the market, it becomes apparent that most conventional vaccines have been developed based on the principle that the antigen has to penetrate all of the physiological protective barriers to access the immune system in order to induce sufficient immunity. Mucosal immunisation refers to the local delivery of antigen to a mucosal site to induce specific immunity against the invading pathogen, as opposed to the conventional strategy to

deliver antigen into the body and induce immunity from the systemic circulation. Clearly, mucosal immunisation is less invasive and has the potential to remove the complications associated with handling needles. More importantly, it has been suggested that parenteral vaccines are less capable of inducing mucosal immunity than vaccines delivered directly to the mucosal site (Holmgren and Czerkinsky, 2005). Localised application of vaccines is often required to induce a protective response, particularly against pathogens invading through mucosal membranes (Holmgren and Czerkinsky, 2005; O'Hagan and Illum, 1990; Pulliam et al., 2007; Yen et al., 2006).

It has been demonstrated in several studies, preclinical and clinical, that mucosal vaccines, in comparison to parenteral vaccines, induce superior local mucosal immunity and good systemic immunity. Influenza vaccines, for instance, have been shown in a number of studies to induce significantly greater mucosal antibody titres and equivalent or greater serum antibody titres when delivered directly to the respiratory tract compared with delivery via subcutaneous or intramuscular injections (Amorij et al., 2007b; Holmgren and Czerkinsky, 2005; Huang et al., 2004; Minne et al., 2007; Wee et al., 2008). DNA plasmid encoding epitopes of *Mycobacterium tuberculosis* delivered via the endotracheal route has been shown to induce superior IFN-γ levels when compared to the intramuscular route (Bivas-Benita et al., 2004). Other antigens such as measles, respiratory syncytial virus (RSV) and human papillomavirus (HPV) have also been shown to induce a significant immune response when delivered to mucosal membranes (Bivas-Benita et al., 2005; Coates et al., 2006; Matsuoka et al., 2002; Nardelli-Haefliger et al., 2005). These studies demonstrate that mucosal delivery of antigen achieves superior mucosal and systemic immunity against these pathogens in comparison to parenteral immunisation.

Given that many pathogens invade the host after initial presentation to mucosal membranes (e.g., via open wounds or inhaled exposure), mucosal immunity may provide more immediate protection at the site of invasion. Mucosal immunisation, being significantly more effective at inducing mucosal immunity than parenteral immunisation, is likely to be more effective at inducing protection against these mucosal pathogens. In addition, as discussed in previous sections, the CMIS provides novel opportunities for more sophisticated immunisation approaches. Mucosal immunisation may provide 'distant-site' or 'multiple-site' protection with the connection of CMIS disseminating immunity to other mucosal sites. Concurrent immunisation via multiple mucosal sites (e.g., oral and nasal) may produce an additive or synergistic immune response compared to parenteral immunisation.

To this end, a number of mucosal vaccines have been developed and released onto the market. These include the oral cholera vaccine (Dukoral®), the oral typhoid vaccine (Vivotif Oral®) and the intranasal influenza vaccines (Flumist®).

1.3.3. Antigen and adjuvant: important components of vaccine formulations

Antigens and adjuvants are both important components in vaccine formulations. Antigens, as described previously, are immunogenic molecules that initiate adaptive immune responses. Most antigens are macromolecules derived from the target pathogens and are processed to remove the pathogenic properties with the immunogenicity of the molecules retained so that immunity is induced without causing disease. Most current vaccines contain antigens that are derived from live-attenuated pathogens, killed or inactivated whole cell pathogens and immunogenic macromolecules such as proteins, peptides or polysaccharides presented on the surface

of the target pathogens. Each of these of antigens possesses different immunogenic properties which determine the properties of the vaccine formulation.

Adjuvants are substances that when delivered together with the antigen improve the immunogenicity of the vaccine but alone do not produce a significant effect. These substances are advantageous as they can improve the potency and efficacy of the vaccine, thus reducing the dosage and number of booster doses required for a therapeutic effect. For many years, aluminium salt based adjuvants, generally known as 'alum', were the most widely used group of adjuvant and are still employed in several commercially available vaccines (Reed et al., 2009; Tritto et al., 2009). In recent years, new classes of adjuvant systems including toll-like receptor (TRL) ligands such as MPL® and mucosal adjuvants such as cholera toxin B subunit have emerged (Jones, 2008; Reed et al., 2009; Tritto et al., 2009). These adjuvants have been discussed in other reviews (Jones, 2008; Reed et al., 2009; Tritto et al., 2009).

1.3.4. Lymphatic system targeted vaccine delivery

The lymphatic system is an essential component of the immune system. It acts as a central network with other lymphoid organs providing communication channels for immune effector cells to promote an efficient immune response. Interstitial fluid is drained through lymphatic vessels and large particles, such as antigens, are filtered and removed by lymph nodes. The lymphatic system contains a large number of immune effector cells, providing an optimal environment for interactions between these cells and antigens to facilitate an immune response. The lymphatic system plays various important roles in the induction of immune responses. Although a detailed discussion of

the lymphatic system is outside the scope of this thesis, the therapeutic potential of targeted vaccine delivery to the lymphatic system will be addressed further.

To date, vaccines available on the market were designed to deliver antigens to specific tissues, mostly the muscle or subcutaneous tissue, via intramuscular or subcutaneous injections respectively. The administration strategy follows the notion that the antigen will subsequently be processed by immune mechanisms and presented to effector cells for immune response activation. While this approach does induce an immune response, it is possible that a more effective response could result if antigen delivery was specifically targeted to reach the immune system. However, there seems to be little evidence in literature that this potential has been evaluated. Several studies have suggested that nanoparticles tend to migrate into the lymphatic system and accumulate in lymph nodes and thus may bypass the initial uptake process by the dendritic cells (Fahmy et al., 2008; Jones, 2008). With the advancement of drug delivery technology that enables lymphatic system targeting, the development of lymphatic targeted vaccine delivery warrants further attention.

Whether lymphatic system targeted vaccines will induce faster and stronger immune responses compared to conventional vaccines will depend on the efficiency of the inherent antigen uptake mechanisms. If the existing antigen uptake mechanism is highly efficient, then targeting to the lymphoid organs may not render a clinically significant difference. Moreover, as described later in this thesis, direct antigen delivery to constitutively expressed lymphoid organs may not necessarily generate a better immune response and the formation of inducible lymphoid tissues may be triggered by the introduction of antigen. Further studies will be required to explore the value of lymphatic system targeted vaccines.

1.4. Pulmonary immunisation

1.4.1. Background

The use of the pulmonary route as a potential means for vaccine administration has long been recognised (Lu and Hickey, 2007). Aerosol measles vaccination in children in the US has been documented since the early 1960s (Cutts et al., 1997). Successful induction of immune responses after delivery of fluid tetanus toxoid to the pulmonary system was documented in guinea pig in 1965 (Yamashiroya et al., 1966). In 1969, aerosol immunisation with tetanus toxoid in human was published (Wigley et al., 1969). In the same year, the robust immune response and protection induced by pulmonary administration of inactivated influenza vaccine was reported clinically (Waldman et al., 1969). Hence, over the past few decades, researchers have been investigating strategies to utilise the pulmonary system as an alternative approach for immunisation due to its attractive attributes.

Pulmonary vaccination confers a number of advantages over parenteral and other mucosal routes of immunisation. Firstly, it is non-invasive and trained medical personnel are not required for administration. Secondly, it eliminates the complications associated with the use and handling of sharps. Thirdly, it has been demonstrated that local delivery of antigens to the respiratory system induces better local immunity against locally invasive pathogens. It has been shown in mice that superior local mucosal antibody titres in the respiratory system were detected after pulmonary vaccination when compared with intramuscular and oral vaccination with equivalent doses (Amorij et al., 2007b; Minne et al., 2007). The superior immunity induced within the respiratory tract has significant implications as it may provide more immediate

protection compared to other routes of immunisation, particularly against respiratory pathogens such as influenza and tuberculosis (TB), which are currently immunised primarily via either intramuscular, intradermal or subcutaneous injections (Cook et al., 2006; Davids et al., 2006; Hawkridge et al., 2008; Ikeno et al., 2010).

1.4.2. Pulmonary physiology

The respiratory system can be divided into several regions: the nasal cavity, the upper airway, the lower airway and the alveolar regions in the deep lung (Figure 3). The part of the respiratory tract from the nasal cavity to the lower airways including the small bronchioles are categorised as the conducting zone, as the main function of these passages is to transport air from the external environment down to the alveolar regions where gaseous exchange occurs. The conducting zone also serves to warm and moisten the inhaled air making it more compatible to the physiological environment in the lungs (Silverthorn et al., 2004).

The alveolar regions in the deep lung provide an extensive surface area to allow efficient oxygen and carbon dioxide transfer between air and blood. Each alveolus is composed of a single layer of epithelium which consists of two types of epithelial cells. The smaller type II alveolar cells synthesise and secrete surfactant which mixes with the thin fluid lining of the alveoli to reduce surface tension and ease the expansion of the lungs during breathing. The larger type I alveolar cells are very thin so that gases diffuse rapidly through them to the capillaries underneath. About 80-90% of the alveolar surface is covered by blood vessels, forming an extensive blood supply network in close proximity to the air in the alveolus to promote rapid gas exchange (Silverthorn et al., 2004).

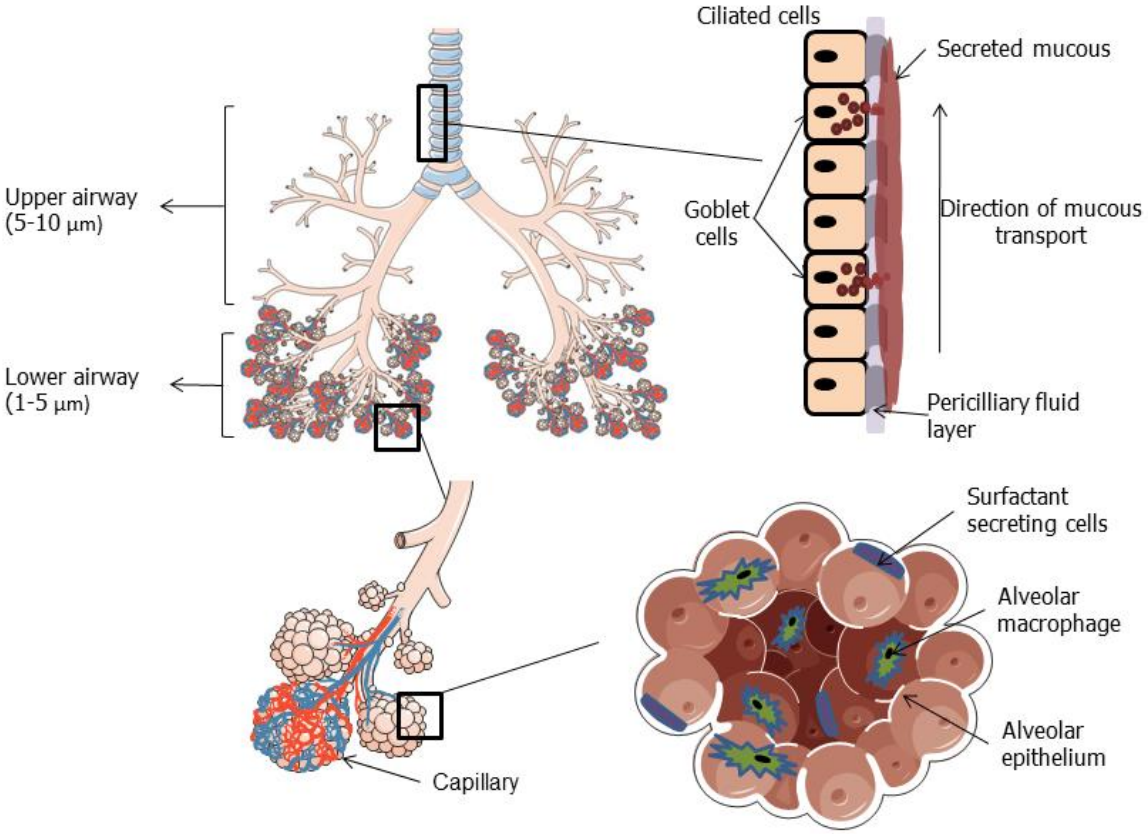

Figure 3. Schematic diagram of the lung and particle size requirements based on intended deposition region in the respiratory tract. [Modified from Servier Medical Art with permission]

The characteristic physiology of the alveolar regions, while allowing rapid gas exchange, also introduces the opportunity for systemic delivery of drug molecules via the pulmonary system. This highly permeable barrier presents the potential to deliver macromolecules such as protein, peptides and polysaccharides, which are generally too large for oral absorption, systemically via non-parenteral route (Bosquillon et al., 2004; Gumbleton and Taylor, 2006; O'Hagan and Illum, 1990; Patton and Platz, 1992; Wee et al., 2008; Weers et al., 2007). Since most antigens are large biological macromolecules derived from the target pathogens, the large surface area in the pulmonary system may

allow more efficient systemic delivery of antigens and thus possibly reduce the dose required, compared to other routes of mucosal vaccination (e.g. oral and buccal), to induce sufficient systemic immunity (Pulliam et al., 2007). Therefore, pulmonary immunisation may not only provide strong mucosal immunity as a mucosal vaccine, it may also provide enhanced systemic immunity due to the effective systemic absorption and thus a more thorough protection against the pathogen. This presents the opportunity of vaccines delivered via the pulmonary route to be used for not only immunisation against diseases of the respiratory system, but also for the systemic immunisation of other diseases outside the respiratory tract.

1.4.3. Regional vaccine delivery in the respiratory tract

In order to exploit the full potential of pulmonary vaccination, it is essential to establish which region of the respiratory tract is the optimal target for antigen delivery and hence immune response. Generally, non-invasive pulmonary drug delivery devices utilise the breathing mechanism of the lung to draw medications into the respiratory tract with the inhaled air stream. During this process, the inhaled drug particles may be deposited on the mucous membrane lining anywhere between the buccal mucosa and the alveolar region. The precise location in the airway where the drug particles will deposit depends on the physicochemical properties of the drug particles and the individual physiology of each patient as well as the inspiratory flow rate and the amount of mucous on the airway.

The delivery of vaccine to the upper respiratory tract has received much interest over the last few decades. Studies on intranasal immunisation have demonstrated the potential utility of this route for various vaccines such as measles, influenza and bacille

Calmette–Guerin (BCG) in different animal models and human (Ainai et al., 2013; Cutts et al., 1997; Huang et al., 2004; Jiao et al., 2002; Major et al., 2002). Intranasal measles immunisation has been performed via various techniques including intranasal instillation, sprays and drops in a number of studies inducing a wide degree of seroresponse ranging from 20-100% (Cernescu and Cajal, 1984; Cutts et al., 1997; Kok et al., 1983). *Mycobacterium bovis* BCG has been shown to induce immune response after intranasal application as demonstrated by the recruitment of dendritic cells and detection of cellular immunity including cytokine production and T-cell activation (Lagranderie et al., 2003; Major et al., 2002).

In particular, intranasal influenza vaccine has been widely studied in a variety of forms in a number of animal models (Ainai et al., 2013; Huang et al., 2004; Sanders et al., 2009; Scheerlinck et al., 2006; Smith et al., 2003). Local IgA antibody response has been detected within one week after nasal immunisation with an adjuvanted influenza vaccine in sheep (Scheerlinck et al., 2006). Intranasal delivery of a dry powder influenza vaccine has been shown to induce systemic and mucosal immune response in rats (Huang et al., 2004). Influenza vaccine administered as an intranasal spray has also be demonstrated to induce serum and mucosal antibody response (Ainai et al., 2013). While direct delivery of antigen to the nasal cavity is relatively straightforward, however, as the first and foremost region of the conducting zone in the respiratory tract to screen the outside environment, the nasal mucosa is physiologically designed to entrap and remove any foreign material, rather than promoting interactions of such material with the host (Silverthorn et al., 2004). In contrast, the lower airway to the deep lung, which is physiologically evolved to be more permeable for rapid gaseous exchange, may potentially provide a more suitable entry route for antigen access to the immune system.

It is logical to hypothesise that direct delivery of the antigen to local lymphoid tissues in the respiratory tract would more effectively induce an immune response. In the human respiratory tract, the presence of the bronchus-associated lymphoid tissue (BALT) in healthy adult lungs is controversial (Bivas-Benita et al., 2005; Moyron-Quiroz et al., 2004; Tschernig and Pabst, 2000). It has been suggested that BALT is most concentrated at the bronchial bifurcations (O'Hagan and Illum, 1990). While this may suggest that delivery of antigens to the upper airway where BALT is most concentrated is a reasonable approach, it is not supported by experimental results as yet.

Several studies have been conducted in mice to establish the best target site for vaccine delivery. After comparing site-specific delivery of influenza vaccine to the nasal cavity, the upper airway, the central airway and the deep lung in mice, Minne and co-workers found that deep lung delivery produced the best serum, mucosal and cell-mediated immunity (Minne et al., 2007). Intranasal administration of influenza virus, regardless of formulation status and the presence of adjuvant, has been shown to be less effective in inducing both humoral and cellular immunity compared to intratracheal administration of the equivalent formulations (Smith et al., 2003). Sanders and co-workers demonstrated in mice that restricted delivery of influenza vaccine to the nasal passage and upper airway did not induce a detectable specific antibody titre and access to the lower respiratory tract and lungs were required to induce a good response (Sanders et al., 2009). Another study comparing formulations with different particle and droplet sizes, and therefore different access to the respiratory tract, also appeared to support the notion that access to the lower airway is required to produce a superior immune response (Amorij et al., 2007b). It is difficult to determine at this stage whether these results from rodents are representative of human situations. However, these results

seem to support the hypothesis that either the entire respiratory tract or the deep lung is the best target for vaccine delivery.

The lack of association between the location of BALT and the magnitude of immune responses induced after vaccine delivery suggests the likely involvement of an antigen uptake mechanism in the lung that is independent of the prior presence of BALT. Recently, the identification of inducible BALT (iBALT), which develops after the onset of infection or inflammation, has been demonstrated in mice (Bivas-Benita et al., 2005; Moyron-Quiroz et al., 2004). It has been shown that the formation of iBALT can be triggered by infection or inflammation in previously healthy mice in the absence of pre-existing BALT. This identification of iBALT effectively diminishes the premise that local lymphoid tissue is the best target for vaccine delivery as the formation of lymphoid tissue may be triggered by the antigen itself (Moyron-Quiroz et al., 2004). Other mechanisms such as the involvement of local antigen presenting cells (APCs), mainly the pulmonary dendritic cells and macrophages (Bivas-Benita et al., 2005), and the subsequent presentation and transport of antigen to immune activator cells in the lymphatic system may also play an important role in inducing immune responses. Hence, the optimal site for antigen delivery and the underlying antigen presentation mechanism remains to be defined.

1.4.4. Antigen for pulmonary delivery

It is important to understand the immune response induced after pulmonary delivery of various antigens, as this can be instrumental in identifying potential candidates that would benefit from the development of pulmonary vaccines. Over the years, several antigens have been studied to establish their suitability for pulmonary

delivery. Influenza has been used as an antigen in various forms in a number of studies in different animal models. Influenza antigens have been prepared as whole or split inactivated or disrupted virus (Minne et al., 2007; Sanders et al., 2009; Wee et al., 2008) or from commercially available vaccines (Amorij et al., 2007b; Waldman et al., 1969). These antigens have been examined in mice, sheep and humans, with promising results (Amorij et al., 2007b; Minne et al., 2007; Sanders et al., 2009; Waldman et al., 1969; Wee et al., 2008). Attenuated measles viruses have also been extensively utilised in human lungs as nebulised vaccines in large-scale immunisation campaigns with good response (Coates et al., 2006; Cutts et al., 1997). Measles vaccination using dry powder inhalation has also been shown to induce specific immunity in macaques (de Swart et al., 2007). Micro-particle aerosol of live attenuated tuberculosis vaccine of bacille Calmette–Guerin (BCG) has been demonstrated to induce good protection in guinea pigs (Garcia-Contreras et al., 2008). These antigens, being transmitted via the pulmonary route, appear to have great potential suitability for pulmonary immunisation.

Other types of antigens have been studied in the pulmonary system. Commercially available pneumococcal polysaccharide vaccine (Pneumovax®) has been trialled in both healthy volunteers and patients with chronic obstructive pulmonary disease (COPD) to determine the feasibility of inhalation vaccination with polysaccharide antigen and has been shown to be safe with rapidly induced serum antibody responses in both populations (Menzel et al., 2005; Meyer et al., 2006). Pulmonary DNA vaccination has been suggested and DNA plasmid encoding epitopes of *Mycobacterium tuberculosis* has been formulated as chitosan encapsulated nanoparticles for pulmonary delivery in mice and has been shown to increase IFN-γ levels (Bivas-Benita et al., 2005; Bivas-Benita et al., 2004). The promising results from

these studies demonstrated the versatility of antigens that can potentially benefit from pulmonary immunisation.

Recently, the potential use of pulmonary immunisation for disease outside the respiratory tract has also been explored. With the knowledge of the CMIS, it is possible that immunity induced in the pulmonary system can disseminate to protect against invasion at other mucosal membranes. In an attempt to seek an alternative route to immunise humans against the cervical cancer associated human papillomavirus type 16 (HPV16), recombinant HPV16 virus-like particle (VLP) vaccine was administered to adult female volunteers via nebulisation and shown to induce not only comparable antibody titres in serum but superior antibody titres in cervical secretions when compared to intramuscular vaccination (Nardelli-Haefliger et al., 2005). Further studies are required to establish the implications of these results on other diseases.

It is worth noting that the properties of the antigen itself will determine whether it is suitable for mucosal immunisation. In general, most antigens are proteins or peptides and often they do not have a specific binding mechanism that will allow them to reside on the mucous membrane. It has been suggested that the low bioavailability of most proteins and peptides administered to the mucous membrane is the result of rapid removal of the molecules from the sites of absorption by mucociliary clearance mechanisms (O'Hagan and Illum, 1990).

Some pathogens that invade the host via the respiratory route employ specific binding mechanisms which allow them to reside on the mucosal membrane and to promote invasion. For instance, the surface glycoprotein haemagglutinin (HA) of the influenza virus binds to sialic acid receptors on human airway epithelial cells to allow invasion of the host (Bardiya and Bae, 2005; Nicholls et al., 2007; Shinya et al., 2006). Since HA is highly antigenic and all influenza vaccines must contain a relevant HA

regardless of the form of whole virus, split virus, subunit virus, live virus or DNA for sufficient immunity to result (Potter and Jennings, 1999), these influenza vaccine antigens are capable of binding to the mucosal membrane lining the human airway to facilitate immune response activation.

Other mechanisms such as the use of bioadhesive gelling systems that can prolong the duration of residence of the antigen on the mucosal site are likely to be beneficial in facilitating antigen uptake and subsequent immune activation (O'Hagan and Illum, 1990; Read et al., 2005).

1.4.5. Adjuvant for pulmonary delivery

There are a limited number of adjuvants that have been studied for use in the pulmonary system. ISCOMATRIX™, a saponin-based adjuvant (Hu et al., 1998; Hu et al., 2001; Reed et al., 2009), has been used in several pulmonary vaccination studies with influenza vaccines in both mice and sheep with good response (Sanders et al., 2009; Wee et al., 2008). Chitosan, a non-toxic biodegradable polymer, has been successfully employed for the encapsulation of DNA plasmid for pulmonary immunisation in mice (Bivas-Benita et al., 2004). Muramyl dipeptide (MDP) and trehalose dibehenate (TDB) have been screened in *in vitro* studies for potential use in pulmonary tuberculosis vaccines (Wang et al., 2009).

However, the development of adjuvants for the respiratory tract is challenging due to toxicity concerns. The thin epithelium lining in regions of the respiratory tract, while providing some permeability to macromolecules, can be vulnerable to external disturbances. For instance, the use of permeability enhancers such as surfactants and bile salts which bind to and open up lipid membranes, may improve penetration and

uptake of the antigen delivered, but also may cause irreversible damage to the epithelium and even short-term loss of integrity of the pulmonary epithelium can have negative implications (Weers et al., 2007). More studies will be required to ascertain the safety profile of new adjuvants before they can be clinically used in human vaccines.

Nevertheless, adjuvants should only be used if the antigen alone is inadequate in inducing sufficient immunity. If the immunogenicity of the antigen alone is sufficient, adjuvants will not be required and the associated complications including the cost and toxicity concerns can be eliminated. Even if an adjuvant is required for sufficient immunity, it will be preferable to use basic compounds with well-known safety profiles to ensure long-term safety of the formulations.

1.5. Pulmonary vaccine development

1.5.1. Animal models for pulmonary vaccine development

Mice have been used as an animal model in several studies for pulmonary delivery of influenza vaccines and other antigens (Amorij et al., 2007b; Bivas-Benita et al., 2004; Minne et al., 2007; Sanders et al., 2009). The main advantage of mice is that it is not resource demanding to maintain and handle and are therefore suitable model for preliminary laboratory scale studies. Furthermore, the ready availability of transgenic mice allows in-depth mechanistic studies which may not be possible without genetically engineered animals. However, it is impossible in many cases to perform repeated dosing and measurement on the same mouse, hence larger sample sizes are needed and biological variability between individual animals cannot be eliminated. The use of other animals such as rats and guinea pigs in pulmonary immunisation studies has also been

reported (Garcia-Contreras et al., 2008; Griffiths et al., 1997; Yamashiroya et al., 1966). However, due to the relatively small size and inability to obtain repeated measures on these animals, they do not appear to provide many practical advantages over mice.

The difference in pulmonary physiology across various species is an important consideration. Small rodents, while providing some practical advantages in early laboratory scale studies, are fundamentally different in lung structure (e.g., size of lungs and branching of airways), respiratory physiology (e.g., breathing patterns and respiratory volume) and development compared to human (Matute-Bello et al., 2008; Wenzel and Holgate, 2006). Large mammals of similar size and with respiratory physiology comparable to human therefore play an important role, especially in later stage of development process and modelling of human diseases. The lungs of sheep, for instance, have tracheobronchial tree with irregular dichotomous and branching pattern similar to human (Kirschvink and Reinhold, 2008; Scheerlinck et al., 2008). Drug delivery in disease models such as asthma, emphysema and chronic bronchitis can also be assessed in these animals (Bischof et al., 2003; Collie et al., 2006; Meeusen et al., 2009; Nikula and Green, 2000; Snibson et al., 2005; Tsai et al., 2007).

In a recent study, sheep were used as a large animal model to evaluate the response of liquid ISCOMATRIX pulmonary influenza vaccine (Wee et al., 2008). In another study, macaques were used to explore the feasibility of inhalable dry powder measles vaccines (de Swart et al., 2007). The use of large animal models provides a number of practical advantages. Repeated dosing and cross-over study design can be performed on the same animals to eliminate individual biological variability. The bigger physical size of the organs will also allow study designs that require sophisticated manipulation of the system. For instance, drug delivery can be restricted to specific regions of the lung to compare the effect of regional delivery to various sites (Meeusen

et al., 2009). Nevertheless, larger animals are more costly to maintain and trained personnel with expertise in handling such animals are required. While experimental work in large animal models was not possible within the scope of this thesis, it represents a likely future direction at a later stage of the development process.

It is worth noting that due to many differences in the structure of bronchus-associated lymphoid tissue (BALT) across species, results from animal studies may not always be directly extrapolated to human situation (Bivas-Benita et al., 2005; Tschernig and Pabst, 2000). Most of the aforementioned studies merely obtained results from the animals with no comparison to human data. Further studies aimed at validating a representative animal model that is applicable to the human situation would be valuable. It will also be beneficial to compare vaccine delivery in disease models at a later stage for respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF) and lung damage resulted from chronic cigarette smoking to investigate the impact of the altered physiology on pulmonary vaccine delivery.

1.5.2. Devices for pulmonary vaccine delivery

Different administration systems have been used for pulmonary drug delivery. Pressurised metered-dose inhalers (pMDIs), dry powder inhalers (DPI)s and nebulisers are all used, with pMDI being the most commonly employed to date. Each of these devices possesses different properties which can influence the efficacy of the drug delivered. The final choice of the device used would depend on the properties of the drug (e.g., stability and physicochemical properties), the dose level required and the dosing regimen, as well as a host of associated practical aspects notably economic and clinical factors. Generally, liquid aerosols, including solutions or suspensions, can be

delivered using nebulisers or pMDIs, while delivery of a dry powder formulation would require utilisation of a different delivery system. It is of particular interest to establish the most efficacious, manufacturable and functional delivery method for pulmonary immunisation.

The use of nebulisers or atomisers, which produce fine droplets of vaccines, for pulmonary immunisation has been reported since studies in the 1960's (Waldman et al., 1969; Yamashiroya et al., 1966). This method of administration was used in measles vaccination programs in children on many occasions in USA, Mexico and Japan (Cutts et al., 1997; Yamashiroya et al., 1966). Furthermore, this method was also used in several recent studies involving pulmonary delivery of human papillomavirus and pneumococcal polysaccharide in both healthy volunteers and patients with chronic obstructive pulmonary disease (COPD) (Menzel et al., 2005; Meyer et al., 2006; Nardelli-Haefliger et al., 2005). Practical use of such devices is therefore supported by experience from these large-scale vaccination campaigns and studies. However, most commonly used nebulisers are generally expensive, inefficient and cumbersome. Although recently portable nebulisers have become available to address some of these issues, power supply is still required for the operation of these devices. In addition, the mechanical stress generated in the atomisation process may impact on the stability of the vaccine formulation (Coates et al., 2006).

Nebulisers operate by atomising solutions or suspensions into a liquid aerosol that can be delivered to the pulmonary system. The success of nebulised vaccines implies that liquid aerosol can be used for antigen delivery to induce immunity effectively. The utilisation of other liquid aerosol generating systems such as pMDI may then be considered, provided the dose required is low enough to be delivered in a metered-dose device. The use of pMDI provides practical advantages over nebulisers as

there is no requirement for a power supply. Furthermore, it is less bulky for distribution to individual vaccine recipients. These practical advantages would allow improved access and implementation of immunisation programs especially in developing countries. However, pMDIs require patient training and proper technique to ensure coordination of breathing and release of dose for optimal delivery. These devices are difficult to provide maximum efficacy for non-chronic therapy such as vaccination, especially for children and patients with minimum experience in using inhaled therapies. In addition, pMDIs are typically designed for multi-dose regimens (e.g., 100 or 200 doses) for ongoing management of chronic respiratory conditions (e.g., asthma, COPD). The relatively complex design of pMDI may not be economically viable for mass delivery of single-dose interventions such as vaccines.

It has been suggested that particulate antigen, as opposed to soluble antigen in solution, is selectively internalised by the microfold (M) cells on the mucosal surface, resulting in more efficient access to antigen-presenting cells (APCs) (Jones, 2008; Sanders et al., 2009), leading to more powerful immune responses. Recently, dry powder aerosols have been explored as a potential medium for vaccine delivery and the feasibility of dry powder aerosol vaccine for pulmonary delivery has been demonstrated in several studies. Dry powder influenza subunit vaccines prepared by spray-freeze drying was shown to induce superior systemic and mucosal humoral and cell-mediated immune responses in mice after pulmonary delivery when compared to the liquid vaccines administered via the pulmonary and intramuscular routes (Amorij et al., 2007b). Specific immune responses were also demonstrated in macaques after dry powder inhalation of measles vaccine (de Swart et al., 2007). Thus, the development of inhalable dry powder vaccine delivery system is of particular interest.

1.6. Dry powder pulmonary vaccine formulation

1.6.1. Background

For many years, most vaccines available on the market have been formulated for parenteral administration either as solution, suspension or dry powder for reconstitution which eventually is administered as a liquid formulation. However, in many instances, liquid formulations are not ideal. Most antigens are biomacromolecules such as protein and peptides derived from the disease causing pathogen. These biomacromolecules are generally at a greater risk of chemical and physical degradation in liquid formulations (Mahler et al., 2005; Schule et al., 2008). In order to maintain the stability of these antigens, most vaccines have to be refrigerated upon storage which introduces the complications of cold chain management (Chen and Kristensen, 2009). There are a limited number of approaches that have been successful in improving the characteristics of these formulations.

Recently, delivery of biomacromolecules in the form of dry powder aerosols to the pulmonary system has gained increasing interest as it appears to be the most promising non-parenteral route (Patton and Platz, 1992; Schule et al., 2008; Wall, 1995; Weers et al., 2007). In addition, powder vaccine formulations have been demonstrated to provide improved stability when compared to conventional liquid formulations (Bosquillon et al., 2004; Chen and Kristensen, 2009; Huang et al., 2004; LiCalsi et al., 2001). Stability issues of proteins in liquid formulations may also be improved by formulating them in dried solid state with glass forming stabilisers such as trehalose and sucrose (Andya et al., 1999; Conrad et al., 2000; Costantino et al., 2002; Liao et al., 2002; Maa et al., 2004; Maury et al., 2005; Schule et al., 2008; Yu, 2001). Moreover, as

mentioned in the previous section, particulate antigens seem to be internalised by immune cells more efficiently and may induce a superior response as opposed to soluble antigens (Bramwell et al., 2005; Jones, 2008; Sanders et al., 2009). Therefore, a dry powder aerosol vaccine delivery system may provide multiple advantages over other conventional approaches.

There are many different methods that may be employed to produce inhalable dry powder drug containing particles. These methods can be divided into two main categories namely (i) the top-down approach and (ii) the bottom-up approach.

In the former category, large solid forms of particulates are broken down into small particles of desired particle size. This category has been used traditionally to produce small particles and include various high-intensity milling and grinding techniques such as air-jet milling and ball milling (Johnson, 1997; Malcolmson and Embleton, 1998). In air-jet-milling, for instance, inter-particle collision and attrition is the principle mechanism of micronisation. Coarse particles are suspended in high velocity turbulent gas stream where inter-particle collision fractures them into smaller particles (Johnson, 1997; Shoyele, 2008).

While relatively straightforward to exercise, there are several pitfalls associated with this approach. Firstly, the large amount of energy and mechanical stress involved in these processes have significant implications on the chemical and physical stability of the compound being processed (Johnson, 1997; Malcolmson and Embleton, 1998). Secondly, the process provides little opportunity for control over various important particle characteristics such as morphology, size distribution and shape (Malcolmson and Embleton, 1998; Shoyele, 2008). In addition, biological molecules such as proteins may be too fragile to be milled and further purification is often required after the milling process to extract the pure compound.

The bottom-up approach is where particle formation starts at the molecular level by nucleation which instigates the subsequent precipitation and growth of particles to the desired particle size. Essentially the compound is first dissolved to produce a uniform molecular dispersion followed by a carefully designed precipitation process to manipulate the properties of the particles formed. The characteristics of the final product will depend on the inherent physicochemical properties of the compound itself as well as the parameters used in the process. By adjusting these parameters, this approach not only produces powders of better uniformity, but also provides greater opportunity for control over particle characteristics including morphology, size distribution and shape that is not be possible with the traditional top-down approach (Malcolmson and Embleton, 1998).

Spray-drying and supercritical fluid precipitation (SCFP) are examples in this bottom-up category (Johnson, 1997; Jovanovic et al., 2004; Malcolmson and Embleton, 1998; Vehring, 2008). In SCFP, for example, the compound of interest is first made into a saturated solution. The supercritical fluid employed can function either as a solvent in this initial solution or as an anti-solvent subsequently introduced, depending on the method used. Precipitation of the compound is induced by deliberately supressing the solvation power of this saturated solution (Shoyele, 2008). In spray-drying, the liquid formulation is sprayed into a drying chamber where the compound of interest precipitates upon solvent evaporation (Johnson, 1997; Vehring, 2008).

The fragility of biomacromolecules for vaccine delivery means that the energy intensive top-down approach powder production methods are unlikely to be favourable due to the high risk of destroying the labile native structure and conformation of these biomacromolecules. Furthermore, the attrition top-down process is not well suited to formation of fine inhalable particles with complex composition comprising typically an

amorphous glass of excipients and bioactive. Being typically amorphous glasses rather than crystalline, these may be less brittle and lack crystal fracture planes and hence less susceptible to milling into the sizes/forms required.

Conversely, the bottom-up approach production methods, in addition to being potentially gentler to the compound of interest, provides the opportunity to produce a custom designed solid matrix environment at the molecular level with various excipients for stabilisation of biomacromolecules. The process may also be optimised to, in principle, manipulate the micro-environment for particle formation to produce a carefully engineered formulation with the most desirable properties. In this bottom-up category, spray-drying is a well-established technology in the food and pharmaceutical industries. However, rational particle design and engineering is a relatively young discipline and the use of spray-drying in this respect for the production of pharmaceutical dry powder aerosols for respiratory drug delivery remains an area of much potential to be explored.

1.6.2. Spray-drying

Spray-drying has gained increasing interest recently due to its relative simplicity, cost effectiveness and scalability. Spray-drying is a process in which the compound of interest is first made into a liquid form (e.g., solution or suspension) which is then atomised and sprayed into a drying chamber where droplets are dried by hot air (Andya et al., 1999; Maa et al., 1998). The process can be designed to produce protein containing powders with high stability and has been frequently used to produce protein powders for inhalation (Andya et al., 1999; Chan et al., 1997; Johnson, 1997; Maa et al., 1998; Maa et al., 1999; Malcolmson and Embleton, 1998; Schule et al., 2008). Since

most antigens are biological macromolecules such as proteins and peptides, spray-drying appears to be an appealing process for the production of dry powder pulmonary vaccines. Furthermore, it also provides practical advantages as it is a single unit continuous closed system that can be easily scaled up for commercial production (Johnson, 1997).

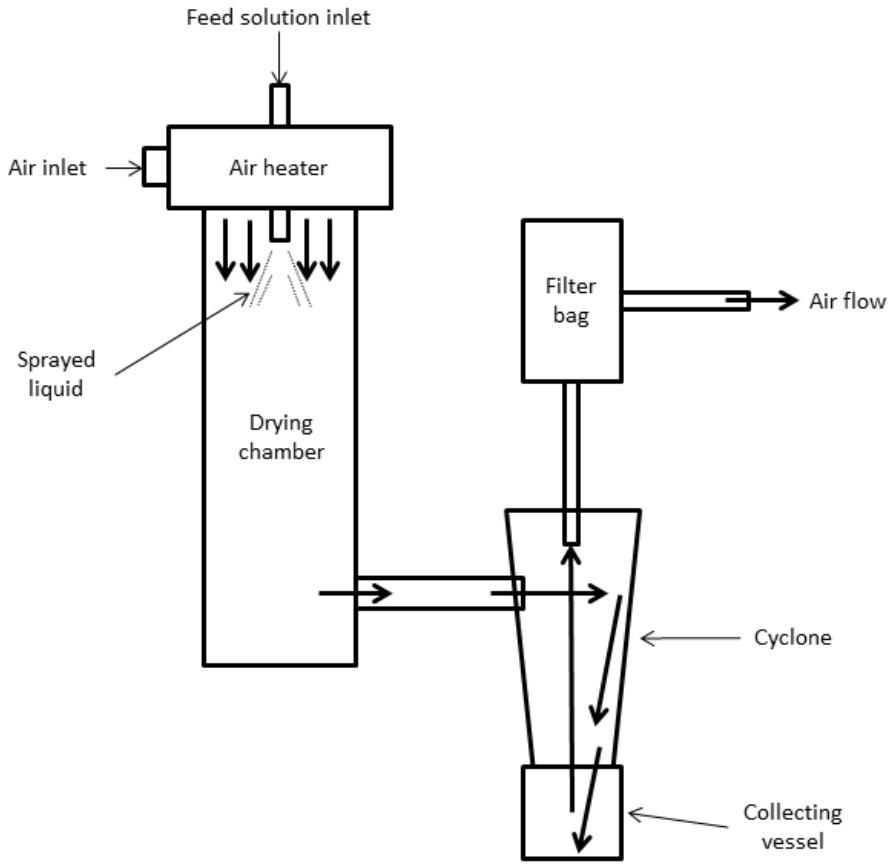

Figure 4. Schematic diagram of a typical spray-dryer showing the operation and basic working principle of spray-drying.

Figure 4 shows as a schematic a typical configuration of a laboratory or pilot scale spray dryer. There are many parameters that can be adjusted in the process to change the behaviour of the final product such as the temperature of the inlet and outlet air, the rate of air flow, the atomisation process and the composition of ingredients and excipients in the feeding solution (Andya et al., 1999; Garcia-Contreras et al., 2008;

Maa et al., 1998; Maury et al., 2005; Namaldi et al., 2006; Tsung and Burgess, 1997; Tzannis and Prestrelski, 1999a, b; Wong et al., 2007). Each of these parameters can be individually adjusted to fine-tune the characteristics of the powder produced until the desired properties for delivery are achieved. For pulmonary delivery, dry powder formulation not only needs to be stable upon storage but also aerosolisable upon inhalation (Maa et al., 1999). Particles with an aerodynamic diameter between 1-10 μm may be desirable depending on the target delivery region in the respiratory tract (Carvalho et al., 2011; Maa et al., 1999; O'Hagan and Illum, 1990). The delivery properties of the final formulations can be manipulated by controlling the properties of the individual particles. Spray-drying appears to be an attractive process which allows the engineering of particles that cannot be easily achieved with other manufacturing processes.

Since the compounds of interest are first formulated into a liquid form, the physical properties of the starting materials are essentially all deconstructed before introduction of the liquid formulation into the spray-dryer. The spray-dryer, subsequently, serves to provide the environment for particle formation, during which allowing the reconstruction of all the physical properties, both at the micro-level with individual particles (e.g., particle size, morphology, composition, moisture content, glass transition temperature, amorphicity, polymorphs) and consequently at the macro-level with the bulk properties of the resultant powder (e.g., particle size distribution, bulk density, flow properties, dispersibility, aerosolisation), most fundamentally from the molecular level. The process can therefore be optimised to produce particles with the desirable properties, by adjusting the spray-drying parameters, hence the conditions of the environment for particle formation.

By processing the same formulation under the same spray-drying condition, the resultant product should, in principle, possess the same physical properties (e.g., particle size, moisture content, amorphicity, T_g) regardless of the physical properties of the starting material. In other words, spray-drying serves as a physical transformation process in which the physical properties of the starting materials are fully transformed into the desirable properties of the resultant product.

1.6.3. Particle size of spray-dried particles

Particle size is an important determinant that dictates the site of deposition of the particles delivered via pulmonary route. In general, particles inhaled through mouth with a diameter larger than 10 μm are deposited at the upper respiratory tract such as the mouth or the pharyngeal region. Smaller particles are deposited in the lower airway to some extent with particles having a diameter of about 1-2 μm being deposited most effectively at the alveolar region. Particles smaller than this size have been argued to be less efficient for alveolar delivery as it has been suggested that they are largely exhaled (Carvalho et al., 2011; Chow et al., 2007; O'Hagan and Illum, 1990). Furthermore, it is well-known that inter-particle cohesive forces become more dominant with decreasing particle size (Forsyth et al., 2001). The magnitude of the inter-particle forces is an important factor that affects the flow and aerosolisable properties of the powder bulk. In addition, as discussed in previous sections, it has been suggested that nanoparticles may be taken up more effectively into the lymphatic system (Fahmy et al., 2008; van der Laan et al., 2008). Therefore, the control of particle size is very important in the development of dry powder formulations.

A key advantage of spray drying is that control of the sprayed droplet size enables control of the dried particle size distribution of resultant product. Spray-dried particles are often spherical and their size can be described by their geometric diameter. The geometric diameter (d_g) of spherical particles is determined by the concentration of the feed solution (C_F) and the diameter of the initial droplet (d_D) which is controlled by the atomisation performance as described in Equation 1 where ρ_P is the estimated density of the particles formed (Maa et al., 1998; Vehring, 2008).

$$d_g = \sqrt[3]{\frac{C_F}{\rho_P}} d_D \qquad \text{(Equation 1)}$$

However, the geometric diameter alone does not provide a complete reflection of the behaviour of the particles since it does not take into account the influence of the density and other morphological features on the aerosolisable performance of the particles. In respiratory delivery, a more important measure of diameter is the aerodynamic diameter which can be defined as the diameter of a unit-density sphere that has the same settling velocity as the measured particle (Vehring, 2008). Particles with the same aerodynamic diameter are likely to deposit at the similar region in the respiratory tract. The aerodynamic diameter of a simple sphere can be described by Equation 2 where ρ^* is the unit-density of the sphere.

$$d_a = \sqrt{\frac{\rho_p}{\rho^*}} d_g \qquad \text{(Equation 2)}$$

Equation 1 and 2 can be combined to form Equation 3 which relates the spray-drying parameters to the aerodynamic diameters of the particles produced.

$$d_a = \sqrt{\frac{\rho_p}{\rho^*}} \sqrt[3]{\frac{C_F}{\rho_P}} d_D \qquad \text{(Equation 3)}$$

As shown in Equation 3, the aerodynamic diameter of spray-dried particles is primarily determined by the feed solution concentration and the initial droplet size

which is determined by the atomiser performance (Vehring, 2008). Therefore, the size distribution of the spray-dried particles can in principle be controlled by carefully manipulating these parameters in the drying process to achieve the desired particle size for the vaccine formulation.

1.6.4. Distributions of compounds in spray-dried particles

The ability to incorporate various compounds in a single particle engineering process is another major advantage of spray-drying. The composition, and thus the physicochemical properties of the powder produced, can be controlled by adjusting the content of the feed solution. However, the architecture and morphology of the resulting powder is not only determined by the ingredients in the feed solution but also the distribution of compounds within the particles which is a function of several complex phenomena. The reported mechanisms of particle formation are reviewed elsewhere (Vehring, 2008).

The movement of molecules within the particles during the drying process may be influenced by several driving forces. Surface activity between the liquid/gas interface of the droplet may lead to preferential adsorption of certain components on the droplet surface and produce a diffusional flux towards the droplet surface. However, as the droplet evaporates, the concentration of the components at the surface will increase and lead to a diffusional flux towards the core of the particles (Kim et al., 2003). The diffusional activities of the components within the droplet must be accurately described to predict the distribution of components within the resulting particles (Vehring, 2008).

The rate of evaporation of the solution droplet is another factor that influences the distribution of compounds and is a process of heat and mass transfer. The rate of

evaporation is determined by the balance of the energy delivered to the droplet surface and the energy required to vaporise the solution (Adhikari et al., 2004; Chen, 2004; Miller et al., 1998). The rate of the diffusional motion of the compound in relation to the radial velocity of the receding droplet surface during the evaporation process has been described by a proposed parameter known as the Peclet number (Tsapis et al., 2002). Where the Peclet number is smaller than 1, the diffusional motion of the solute is fast when compared to the radial velocity of the receding droplet. Surface enrichment of the components is small, thus compounds are evenly distributed in the droplet during evaporation and particle density of particles formed is proposed as likely to be close to the true density (Vehring, 2008). Conversely, where the Peclet number is larger than 1, the droplet surface moves faster than the components in the solution which leads to a high concentration of the compound on the surface of the droplet and the formation of a shell. Hence, Peclet number has been proposed as an approximate predictor of drying influence on the resultant particle form.

For compounds with high Peclet numbers, solid hollow spheres may form if the shell is rigid and does not buckle during the drying process. Otherwise, wrinkled particles may form as the shell collapses (Vehring, 2008). The final morphology of the particle will depend on the properties of a host of complex factors including the compound (e.g., solubility, crystallisation), drying conditions (e.g., feed solution concentration, drying temperature) and the nature of the shell formed (Bain et al., 1999; Elversson and Millqvist-Fureby, 2005; Maa et al., 1997; Raula et al., 2004; Wang and Wang, 2002). In multi-component systems, particle formation would be highly complex, with the influence from the intrinsic properties of the compounds, relative amounts of the materials incorporated and any interaction effects between these components. The resulting morphology of the particles will influence the aerosolisation properties and

thus performance of the formulation. Examples of some typical morphology of spray-dried particles are illustrated in Figure 5.

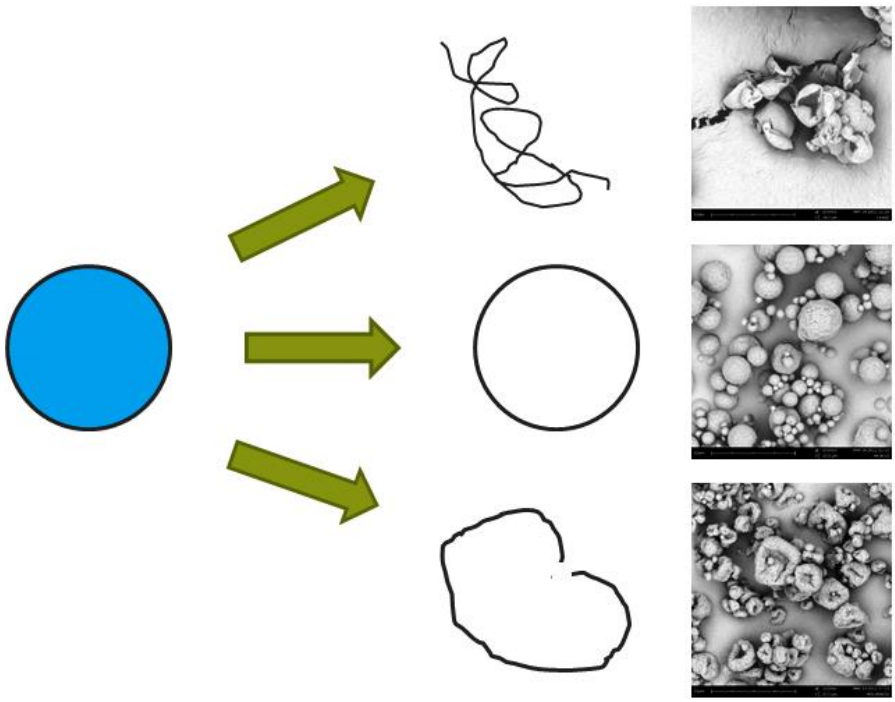

Figure 5. Schematic diagram with scanning electron micrographs showing examples of various morphology of spray-dried particles formed by fragments of broken shells (top), spherical particles (centre) and partially collapsed shells (bottom).

With improved understanding about the particle formation mechanisms and the distribution of compounds within the drying droplets, it may be possible, by using appropriate excipients and spray-drying conditions, to produce particles with a relatively homogenous core for harbouring and stabilisation of the active component while, simultaneously, containing a surface coating for protection of the inner core and provision of the desirable surface properties of the particles. For instance, by co-spray-drying, in the appropriate concentrations, a surface active compound, that would preferentially migrate to particle surfaces, with other easily diffusible excipients that would evenly distribute throughout the core, it may be possible to produce such custom

designed spray-dried particles with multiple functionalities. These carefully designed formulations may potentially provide unprecedented advantages including robust solid-state stability and aerosolisation performance for pulmonary delivery of antigens, and this concept is shown schematically in Figure 6.

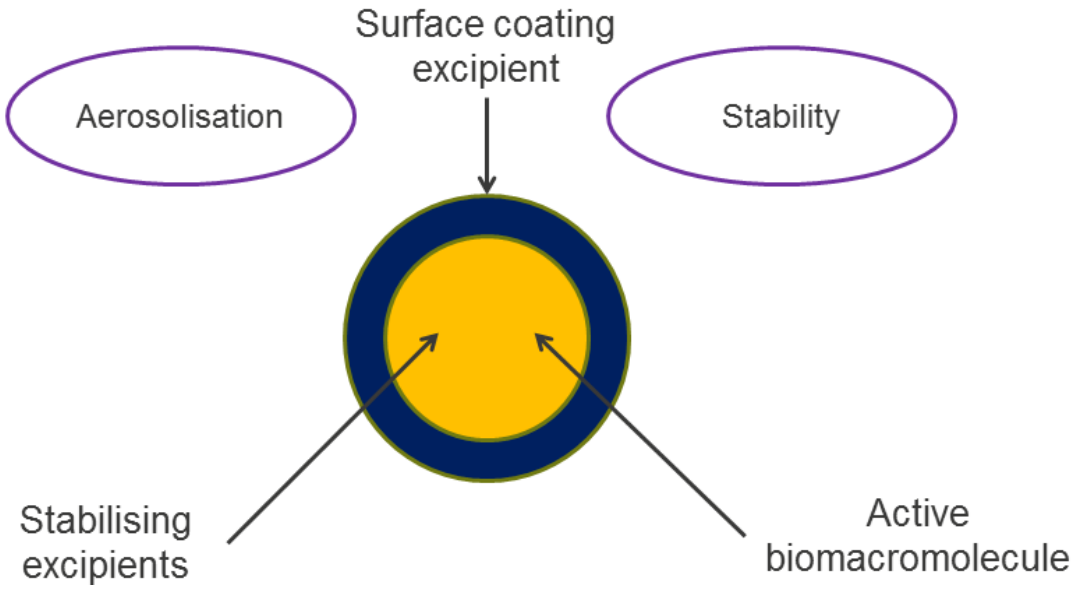

Figure 6. Schematic diagram of an example custom designed spray-dried particle with a surface coating excipient that would protect the inner core and modify surface properties of the dry powder formulations to provide improved aerosolisation and stability.

1.6.5. Aerosolisation of dry powder inhaler formulations

Dry powder inhaler formulations should be readily dispersible and aerosolisable upon inhalation for efficient delivery. However, inter-particulate cohesive forces are particularly dominant in the finely micronised powder (i.e., <5 µm) required for pulmonary delivery (Forsyth et al., 2001). These forces influence flowability and de-agglomeration of micronised dry powders and therefore the aerosolisation properties

and delivery efficiency of dry powder formulations (Ashurst et al., 2000; Prime et al., 1997). Particles can be engineered to contain various ingredients by adjusting the content of the feed solution. Excipients can therefore be incorporated manipulate the properties of the dry powder formulation.

Many excipients have been investigated to improve the aerosolisation properties and performance of inhalable dry powder formulations (Li et al., 2003; Maa and Prestrelski, 2000; Rabbani and Seville, 2005; Staniforth et al., 2002). In particular, leucine has been demonstrated to improve aerosolisation and performance of dry powder inhaler formulations. The inclusion of leucine as an additive in a precursor solution for spray-drying has been shown to improve the aerosolisation of the resulting powders (Kamlag et al., 2004; Li et al., 2003; Li et al., 2005; Lucas et al., 1999; Morton and Kamlag, 2005; Seville et al., 2007). However, this dispersibility enhancing property appears to be specific to leucine and is not necessarily the case with other amino acids (Chew et al., 2005; Minne et al., 2008; Seville et al., 2007).

Studies investigating the aerosolisation enhancing effect of various amino acids including leucine, phenylalanine, tryptophan, methionine, asparagine, arginine, aspartic acid and threonine on the dispersion of spray-dried powders found leucine to be the most effective amino acid that enhanced *in vitro* particle deposition, demonstrating the highest fine particle fractions (FPF) and reproducibly high emitted dose (ED) (Chew et al., 2005; Seville et al., 2007). While effects on particle cohesion and powder aerosolisation produced from the use of a single excipient are more extensively studied, the specific interaction effects resulting from the combined use of different amino acids has not been thoroughly investigated. Furthermore, the specific mechanism of the aerosolisation enhancing effect of leucine remains unclear from the literature.

1.6.6. Stabilisation of spray-dried biological macromolecules

For vaccine delivery, it is critical to preserve the integrity of the antigen used to ensure the efficacy of the vaccine. The stabilisation of proteins and peptides are of particular interest since they are the main class of antigenic biological macromolecules used in vaccines. In the case where the antigen is a live-attenuated pathogen, it is also critical to maintain viability of the antigen to ensure immunogenicity (Garcia-Contreras et al., 2008; Wong et al., 2007). Proteins and peptides are labile macromolecules with complex tertiary and quaternary structures. The maintenance of these macro structures in the native form is essential for the activity of proteins and peptides. However, these macro structures can potentially be damaged by various stresses encountered during the manufacturing process (Maa et al., 1999; Mahler et al., 2005; Wong et al., 2007).

In spray-drying, there are heat stress during the drying process and mechanical stress during the atomisation process that can compromise the integrity and viability of the antigens (Andya et al., 1999; Namaldi et al., 2006; Tzannis and Prestrelski, 1999a, b). Furthermore, dehydration stress caused by the removal of water molecules from the surface of the antigen can also destabilise the protein (Tzannis and Prestrelski, 1999a, b). Ideally, the temperature used in the drying process should be high enough to allow efficient drying but not too high such that the viability and integrity of the antigen is compromised. While the mechanical stress during the atomisation process cannot be avoided, tests should be performed to ensure the loss of activity has not occurred.

The use of excipients to stabilise biomacromolecules in dry solid state is another important consideration. Glass-forming agents such as saccharides, polyols and organic acids have been studied extensively over the years to stabilise spray-dried proteins in the solid state (Amorij et al., 2008; Amorij et al., 2007a; Chang and Pikal, 2009;

Sadrzadeh et al., 2010; Vehring, 2008). These excipients stabilise the macromolecules by two main mechanisms. Firstly, the glass-forming ability of these excipients preserves the structure of proteins by trapping it in a rigid amorphous glass matrix (Conrad et al., 2000; Hancock and Zografi, 1997; Vehring, 2008; Weers et al., 2007; Yu, 2001). An optimal glass-forming agent should be a good glass former that is unreactive with a high glass transition temperature (T_g) (Weers et al., 2007; Yu, 2001). Secondly, these excipients form hydrogen bonds with the proteins in the dry solid state to replace the hydrogen bonds that water forms with the protein in solution, which stabilises the structure (Tzannis and Prestrelski, 1999b; Vehring, 2008; Weers et al., 2007). Common excipients that have been used include sucrose and trehalose (Conrad et al., 2000; Liao et al., 2002; Maury et al., 2005; Tzannis and Prestrelski, 1999a, b; Vehring, 2008; Yu, 2001). Studies are required to establish the implications of these mechanisms on the stabilisation of vaccine formulations.

1.7. Hypothesis and aims

While spray-drying has been a well-established technology in the pharmaceutical and food industry, its specific use to engineer particles with desirable properties (i.e., aerosolisable and capable of stabilising biomacromolecules) for antigen delivery via the pulmonary route is still a relatively young discipline. In particular, the use of spray-drying to combine various excipients for the production of multi-component particles with potentially multiple functionalities (e.g., hydrogen bond formation, amorphous glassy stabilisation, high T_g, moisture protection, particle separation, yield improvement, aerosolisation) remains an area with much potential to be explored.

1.7.1. Hypothesis

With this background, the central hypothesis of this thesis is proposed as:

A novel dry powder delivery system for pulmonary delivery of a model antigen can be developed via the combined use of appropriate excipients and optimisation of spray-drying process

1.7.2. Specific aims

To test this central hypothesis, a set of specific aims have been defined as:

1) To determine the interaction effects of adjusting amino acid excipient compositions on various properties of a mannitol-based spray-dried powder formulation for pulmonary delivery

2) To ascertain the effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried powder formulations for pulmonary delivery of biotherapeutics

3) To identify the effect of polymers on the formation of an amorphous matrix in a multi-component spray-dried particulate platform for pulmonary delivery of biotherapeutics

4) To identify the effect of molecular weights of excipients ranging from polyol, disaccharide, polysaccharide to synthetic polymer on solid-state properties of a multi-component spray-dried particulate platform for pulmonary delivery of biotherapeutics

5) To determine the impact of incorporating a model antigen on physical and aerosolisation properties of a prototype spray-dried particulate platform and the in vivo immunogenicity of the subsequent spray-dried vaccine after pulmonary administration

These specific aims are addressed in Chapters 2 to 6 in this thesis, respectively. As a thesis by publication, Chapter 2 to 6 are presented in their original forms as submitted for publication.

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CHAPTER TWO

INVESTIGATING THE INTERACTIONS OF AMINO ACID COMPONENTS ON A MANNITOL-BASED SPRAY-DRIED POWDER FORMULATION FOR PULMONARY DELIVERY: A DESIGN OF EXPERIMENT APPROACH

Monash University

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study initiation, experimental design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion.	70%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
Laurence Orlando	Experimental design
Michelle P McIntosh	Supervision, manuscript revision
Lisa M Kaminskas	Supervision, manuscript revision
David AV Morton	Supervision, manuscript revision

Candidate's Signature [redacted]

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia

Laurence Orlando	[redacted]	**Date** 7.6.13
Michelle P McIntosh		**Date** 7/6/13
Lisa M Kaminskas		**Date** 6/6/13
David AV Morton		**Date** 7/6/13

2. Investigating the interactions of amino acid components on a mannitol-based spray-dried powder formulation for pulmonary delivery: a design of experiment approach

Tomás Sou, Laurence Orlando, Michelle P. McIntosh, Lisa M. Kaminskas and David A. V. Morton*

Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia

*Corresponding author. [redacted]

Email: [redacted] (D.A.V. Morton)

2.0. Abstract

Combining an amino acid and a sugar is a known strategy in the formulation of spray or freeze dried biomolecule powder formulations. The effect of the amino acid leucine in enhancing performance of spray-dried dry powders has been previously demonstrated, but interaction effects of several constituents which may provide multiple benefits, are less well-understood. A 3 factor 2 level (2^3) factorial design was used to study the effects of leucine, glycine and alanine in a mannitol-based dry powder formulation on particle size, aerosolisation, emitted dose and cohesion. Other qualitative tests including scanning electron microscopy and X-ray powder diffraction were also conducted on the design of experiment (DoE) trials. The results show that the use of glycine and/or alanine, though structurally related to leucine, did not achieve similar aerosol performance enhancing effects, rather the particle formation was hindered. However, when used in appropriate concentrations with leucine, the combination of amino acids produced an enhanced performance regardless of the presence of glycine and/or alanine, yielding significantly modified particle properties. The results from the DoE analyses also revealed the lack of linearity of effects for certain responses with a significant curvature in the model which would otherwise not be discovered using a trial-and-error approach.

2.1. Introduction

Dry powder inhalation has been an attractive delivery method for pulmonary drug administration due to its many advantages: these include ease of administration, convenient portability, relatively simple formulation, low cost and inherent solid state stability, especially for proteins and peptides (Carpenter et al., 1997; Prime et al., 1997; Rave et al., 2004). Dry powder formulations must be readily dispersible and aerosolisable upon inhalation for efficient delivery. However, inter-particulate cohesive forces are particularly dominant in the finely micronised powders (i.e. 1-5 μm) required for pulmonary delivery (Forsyth et al., 2001). These forces influence flowability and de-agglomeration of micronised dry powders and therefore the aerosolisation properties and delivery efficiency of dry powder formulations (Ashurst et al., 2000; Prime et al., 1997).

Spray-drying has gained increased interest for engineering suitable small particles due to its simplicity, adaptability, cost-effectiveness and scalability (Fourie et al., 2008). Spray-drying is a process in which the compound(s) of interest are first prepared in a liquid form, which is then atomised into a drying chamber in which the droplets are dried with heated air. Particle formation is achieved by precipitation of the dissolved compounds as the solvent evaporates from the solution droplets in the drying chamber. The ability to incorporate various ingredients in a single step manufacturing process is a powerful strength of spray-drying. Particles can be engineered to contain various ingredients by adjusting the content of the feed solution. Excipients can therefore be incorporated which may, in principle, manipulate the properties of the dry powder formulation. For example, the amino acid glycine has been added to the sugar mannitol to modify the particle precipitation process, with the benefit of increasing its

resulting solid state particle glass transition temperature (T_g) (Sadrzadeh et al., 2010; Schule et al., 2008).

Many excipients have also been considered to improve the aerosolisation properties and performance of inhalable dry powder formulations (Li et al., 2003; Li et al., 2005a; Maa and Prestrelski, 2000; Rabbani and Seville, 2005; Staniforth et al., 2002). Leucine has been demonstrated to improve aerosolisation and performance of dry powder inhaler formulations and more specifically the inclusion of leucine as an additive in a precursor solution for spray-drying has been shown to improve the aerosolisation of the resulting powders (Kamlag et al., 2004; Li et al., 2003; Li et al., 2005b; Lucas et al., 1999; Morton and Kamlag, 2005; Seville et al., 2007). However, this dispersibility enhancing property appears to be specific to leucine and is not necessarily the case with other amino acids (Chew et al., 2005; Minne et al., 2008; Seville et al., 2007). While effects produced from the use a single amino acid on the performance of a dry powder formulation is more extensively-studied, the specific interaction effects resulting from the combined use of different amino acids is not well understood. Such interactions may allow or prevent multiple powder functionalities being achieved.

Several studies investigating amino acids as spray-drying additives used concentrations based on mass ratios (Chew et al., 2005; Minne et al., 2008; Seville et al., 2007; Shur et al., 2008). However, it is the interactions between the amino acids and other formulation components at the molecular level that eventually determines the behaviour of the final formulation. The extent of molecular interactions may hence relate to the number of molecules, as opposed to mass, which does not provide the same number of molecules of each component for comparability. Compounds with a lower molecular mass obviously contain more molecules than larger compounds in the same

mass. Molar concentrations were therefore used in the present study instead of mass ratio in order to investigate and compare the effects achieved with the same number of molecules of, glycine, alanine and leucine.

Studying the effect of several formulations with multiple excipients in different compositions by trial-and-error or 'changing one separate factor at a time' (COST) approaches are often inefficient as the results from these experiments do not allow the identification of interaction effects between formulation ingredients (Naelapää et al., 2010). A previous study investigating the effect of leucine, glycine and alanine on the performance of dry powder formulations did not provide information on the combination use of these excipients (Minne et al., 2008). Recently, the design of experiment (DoE) approach has been successfully used to optimise spray-drying process conditions by identifying combination of parameters, including drying temperature, airflow rate, pump setting, aspiration setting, feed concentration and solution feed rate, that produced formulations with the best performance (Baldinger et al., 2011; Tajber et al., 2009). The DoE approach was therefore used in the present study to screen in a systematic manner a range of formulations, however, in this case with a focus on various compositions. The study utilised a 2^3 factorial design to investigate the effect of the three amino acids in various compositions.

In the current study, three amino acids, glycine, alanine and leucine were selected specifically with increasing hydrocarbon chain lengths respectively, in order to control specific properties of a mannitol-based dry powder formulation. It was proposed that the hydrocarbon chain length may influence the mass transport and self-assembly of each amino acid upon droplet drying. The subsequent influence of these amino acids on the physical properties of the spray-dried particles including particle size distribution, dispersibility, aerosolisation properties, inter-particle interaction, surface morphology

and crystallinity were therefore investigated. The study aims to screen a range of formulations using a DoE approach to determine the potential utility of these amino acids as performance enhancing excipients for inhalable dry powder formulations. To the best of our knowledge, the effects produced from the combination use of these or similar additives on a spray-dried powder formulation for pulmonary delivery have not previously been investigated.

2.2. Materials and methods

2.2.1. Materials

D-Mannitol was obtained from VWR International Ltd. (Poole, BH15 1TD, England). L-leucine (LEU), glycine (GLY) and L-alanine (ALA) were obtained from Sigma-Aldrich Chemicals (Castle Hill, NSW, Australia).

2.2.2. Preparation of spray-dried powders

Aqueous solutions containing mannitol and selected amino acids (LEU, GLY, ALA) in various compositions as shown in Table 1 were dissolved in 200 mL of Milli-Q water. A small amount of methylene blue dye (10 mg) was incorporated in each formulation to allow a simple quantification of powder by UV-VIS spectrophotometric analysis as described below. The prepared formulations were subsequently spray-dried using a Buchi 190 mini spray-dryer with a 0.5 mm two-fluid nozzle, using the following standard operating conditions: airflow rate, 800 L/h; pump setting, 5 (6.67 mL/min); aspirator setting, 20; outlet temperature, 75 °C .

Table 1. 2^3 Full factorial experimental design: constants, variables and responses.

Factors	**Levels of factors used in the formulation**		
	-1	**0**	**+1**
X_1 = leucine (molar%)	0	15	30
X_2 = glycine (molar%)	0	15	30
X_3 = alanine (molar%)	0	15	30
Responses	**Process and formulation parameters kept constant**		
Y_1 = Mastersizer D_{50} (µm)	Mannitol content: 5 g		
Y_2 = Spraytec D_{50} (µm)	Feed solution volume: 200 mL		
Y_3 = Spraytec ED (mg)	Aspirator setting: 20		
Y_4 = TSI fine particle fraction (%)	Pump setting: 5 (6.67 mL/min)		
Y_5 = TSI ED (%)	Airflow: 800 L/h		
Y_6 = cohesion value (kPa)	Outlet temperature: 75 ºC		

Abbreviation: ED, emitted dose; SD, spray-drying; TSI, twin-stage impinger.

2.2.3. Particle size distribution analysis

The particle size distribution of the powders was determined by laser-light scattering using the Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK) equipped with a Scirocco cell and a Scirocco 2000 dry powder dispersion unit. The powders were dispersed in air at a shear pressure of 3.0 to 4.0 bar, which was selected to achieve suitable de-agglomeration. The average particle size was measured in three replicates for each sample. The volume median diameter (D_{50}) was derived from the diffraction data using the in-built software for each sample.

2.3.4. Powder dispersibility by laser diffraction

A real-time laser-light diffraction particle sizer (Spraytec, Malvern Instruments Ltd, Worcestershire, UK) was used to determine the *in situ* aerosol particle size distribution. The powders were measured with the inhalation cell attachment at a flow rate of 60 L/min using the Monodose inhaler (Miat S.p.A., Milan, Italy) as the aerosol dispersion device. The flow rate was controlled using a Critical Flow Controller Model TPK 2000 & Flow meter model DFM 2000 (Copley Scientific Limited, Nottingham, UK). Approximately 20 mg of each powder was filled into size 3 HPMC capsules (Capsugel, Peapack, NJ, USA) for the tests which were performed over 5 seconds at an air-conditioned laboratory (20 ± 2 ºC, 50 ± 5 % relative humidity). Emitted doses were measured by weighing the inhalers filled with capsules before and after the experiments. The measurements were performed in three replicates for each formulation (n = 3) in a random order to minimise the occurrence of any potential progressive error. The particle size distribution was derived from the laser diffraction data with the in-built software.

2.2.5. In vitro powder aerosolisation and particle deposition

The *in vitro* powder aerosolisation performance and particle deposition was assessed using a twin stage impinger (TSI, Apparatus, A; British Pharmacopoeia, 2000) with the Monodose inhaler (Miat S.p.A., Milan, Italy) as the aerosol dispersion device. The flow rate was adjusted to 60 L/min using a Critical Flow Controller Model TPK 2000 & Flow meter model DFM 2000 (Copley Scientific Limited, Nottingham, UK). Approximately 20 mg of each powder was filled into size 3 HPMC capsules (Capsugel, Peapack, NJ, USA) for the tests which were performed at an air-conditioned laboratory

(20 ± 2 ºC, 50 ± 5 % relative humidity). Each capsule was actuated from the inhaler over 4 seconds for each measurement (n = 5). The amount of powder deposited at different stages was determined using a UV-VIS light spectrophotometer as described below. The cut-off diameter for the TSI at 60 L/min is approximately 6.3 µm (Hallworth and Westmoreland, 1987).

The total amount of powder deposited in the inhaler, stage 1 (S_1) and stage 2 (S_2) was the recovered dose (RD). The amount of powder deposited in stage 1 and 2 was the emitted dose (ED) and it was calculated as the percentage of the RD (Eq. 1). The fine particle fraction (FPF) was defined as the percentage of RD deposited in stage 2 (Eq. 2).

$$ED\% = \frac{(S_1 + S_2) \times 100}{RD} \quad (1)$$

$$FPF\% = \frac{S_2 \times 100}{RD} \quad (2)$$

2.2.6. Inter-particulate cohesion by shear test

Inter-particulate cohesion in the powder samples was characterised by the Freeman FT4 powder rheometer (Freeman Technology, Worcestershire, UK) using its shear cell module configuration as previously described (Zhou et al., 2010). In brief, a shear head was attached to the module driver and shear stresses were measured against a series of normal stress exerted of 7, 6, 5, 4 and 3 kPa. A consolidation stress of 9 kPa was applied to the powder bed prior to each measurement. The shear stress under each normal stress was recorded from which the yield loci were obtained. The cohesion value of each formulation was extrapolated from the yield loci as the shear stress at zero normal stress. The measurements were performed at room temperature and humidity (20 ± 2 ºC, 50 ± 5 % relative humidity). A higher cohesion value represents higher inter-particulate forces and thus a more cohesive powder.

2.2.7. Scanning electron microscopy (SEM)

The morphology of the particles was visualised under a scanning electron microscope (Phanom™, FEI company, USA). Powder samples were gently poured onto a double-sided carbon tape mounted on a sample holder for examination under the SEM. Excessive powder was removed to leave a fine layer of particles on the surface of the tape. The samples were sputter coated with gold using an electrical potential of 2.0 kV at 25mA for 6 minutes with a sputter coater (K550X, EMITECH). SEM micrographs were captured using the in-built image capturing software.

2.2.8. X-ray powder diffraction (XRPD)

Sample powders were sprinkled onto a quartz sample plate smeared with a small amount of Vaseline at room temperature. The sample was then analysed by the X-ray diffractometer (Philips 1140 vertical diffractometer, Philips, Holland) for scanning from 2 to 60°, with an angular increment of 2°/min. The crystalline status of the powders was qualitatively assessed from the diffraction patterns.

2.2.9. UV-VIS spectrophotometric analysis

A UV-VIS light spectrophotometer (Cary 3 Bio, Varian Instruments, Australia) was used to determine the amount of powder recovered from TSI studies using wavelength of 665 nm for detection of methylene blue. Calibration curve was generated for each formulation using linear regression over the range of 0.05-0.80 mg/ml using five concentrations. The regression coefficient (r^2) values were greater than 0.99 for all

formulations demonstrating satisfactory linearity. The amount of powder deposited at each stage was determined from the calibration curve.

2.2.10. Experiment design – 2^3 full factorial design

A 2^3 full factorial design (Design Expert, Version 7.1.3, Stat-Ease Inc., Minneapolis, MN) was used for conducting the experiments. The studied factors were: the amount of leucine (X_1, molar%), glycine (X_2, molar%) and alanine (X_3, molar%). The responses studied were Mastersizer D_{50} (µm, Y_1), Spraytec D_{50} (µm, Y_2), Spraytec ED (mg, Y_3), Twin-stage impinger fine particle fraction (%, Y_4), Twin-stage impinger ED (%,Y_5) and shear test cohesion value (kPa, Y_6). The levels of each variable were designated as -1, 0 and +1, respectively, and the corresponding actual values for each variable are listed in Table 1 and 2. Other process and formulation parameters were kept constant in order to investigate exclusively the effect of the three amino acids on the response variables (Table 1). The influence of factors and their interactions, on each of the response are represented graphically (Figure 1).

Table 2. Formulations as per the 2^3 full factorial experimental design.

Trial number	**Formulation variable – X_1 (leucine, molar%)**	**Formulation variable – X_2 (glycine, molar%)**	**Formulation variable – X_3 (alanine, molar%)**
T1	– 1	– 1	– 1
T2	+1	– 1	– 1
T3	– 1	+1	– 1
T4	+1	+1	– 1
T5	– 1	– 1	+1
T6	+1	– 1	+1
T7	– 1	+1	+1
T8	+1	+1	+1
CP	0	0	0

Abbreviation: CP, centre point; T, trials.

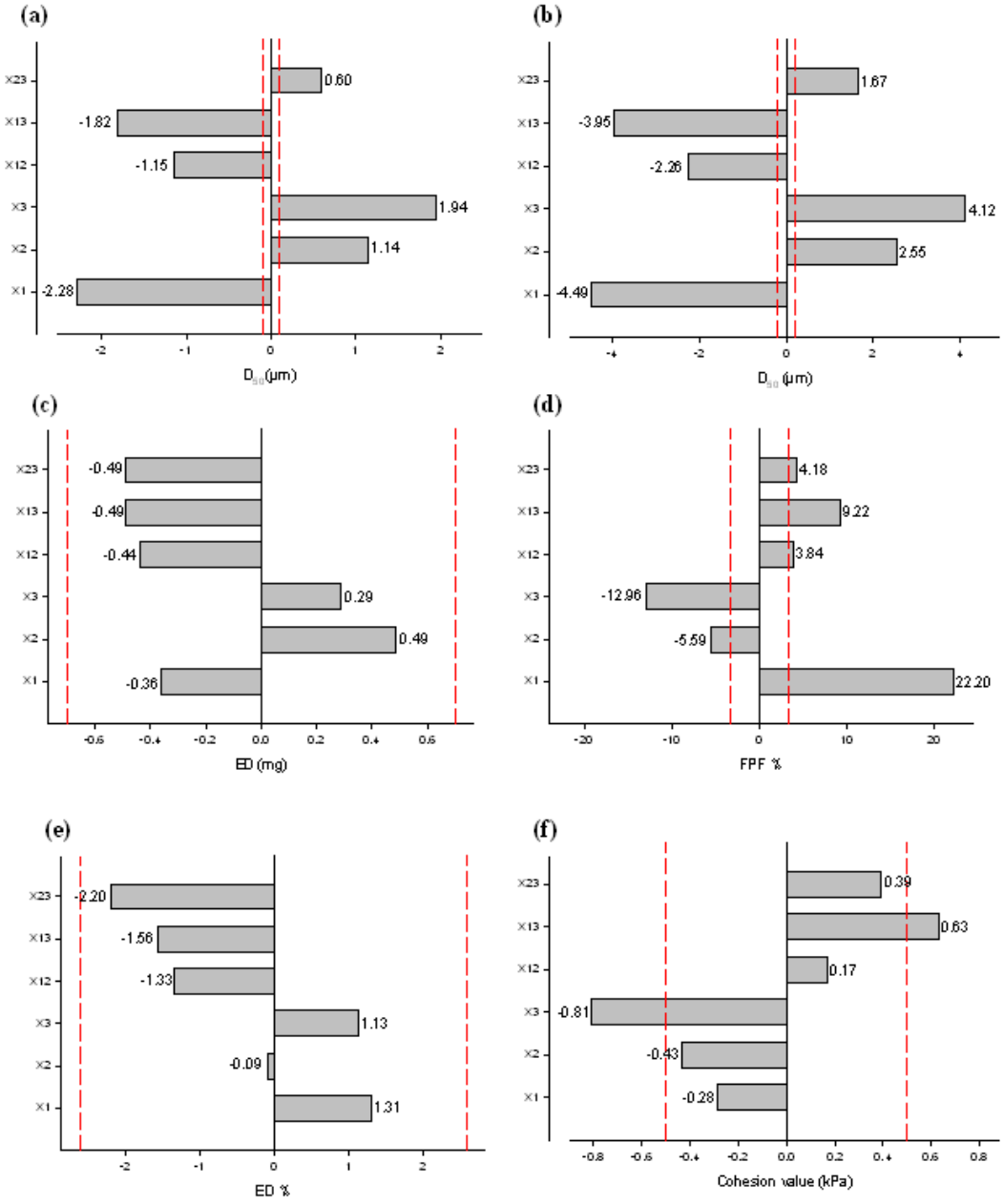

Figure 1. Graphical representation of effect of factors on various responses (*Y*). (a) Mastersizer D_{50} (Y_1), (b) Spraytec D_{50} (Y_2), (c) Spraytec ED (Y_3), (d) fine particle fraction (Y_4), (e) Twin-stage impinger ED (Y_5) and (f) cohesion value (Y_6).

2.3. Results and discussion

2.3.1. Factorial design methodology and analysis

In the present study, the design of experiment (DoE) methodology was employed to systematically evaluate the effect of varying the amount of glycine, alanine and leucine, as well as to identify any interaction among these excipients on the particle size distribution, dispersibility, aerosolisation and inter-particle interaction of the mannitol dry powder formulations. The summary of result data obtained of various responses is presented in Table 3. The DoE approach facilitated the identification of the most significant factors influencing the performance of the formulation.

A mathematical model was generated describing the relationship between the factors and responses for determining the levels of factors which yield optimum responses. For a 2^3 full factorial design, the following first order polynomial equation was fitted to the data:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{123}X_1X_2X_3 \quad (3)$$

where b_0 is the intercept representing the arithmetic averages of all the quantitative responses of eight experimental runs; b_1 to b_{123} are the regression coefficients computed from the observed experimental values (Y); and X_1, X_2 and X_3 are the coded levels of factors. The terms X_iX_j (i and j = 1, 2 and 3) represent the interaction terms. The equation represents the quantitative effect of factors (X_1, X_2 and X_3) upon each of the responses (Y_1 to Y_6). Coefficients b_1 to b_3 represent the effect of factor 1, 2 and 3 while the other coefficients represent the interaction between those factors.

Analysis of variance (ANOVA) was applied for estimating the significance. A *p*-value of less than 0.01 demonstrates the significance of the factor or the interaction (Table 4). In addition, graphical analysis of responses was performed as shown in Figure 1. This analysis allowed the important factors for the considered responses to be identified and an estimated optimum factor level could be selected. The bar graphs were constructed in which the bars that exceed the two lines of limit of significance, calculated according to the experimental variance derived from the centre point results, correspond to the factors that are influential on the response. In particular, the influential factors are those where a level change triggers a response variation which is statistically different from the variation due to the experimental error (Pund et al., 2010).

Table 3. Result data of mean values of various responses: Mastersizer D_{50} (µm, Y_1), Spraytec D_{50} (µm, Y_2), Spraytec ED (mg, Y_3), fine particle fraction (%, Y_4), twin-stage impinger ED (%,Y_5) and cohesion value (kPa, Y_6).

Batch	X_1	X_2	X_3	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
1	0	0	0	1.87	2.83	15.80	66.20	78.04	4.53
2	30	0	0	1.75	2.70	17.50	80.10	91.11	2.21
3	0	30	0	3.75	5.52	19.20	34.62	89.59	2.39
4	30	30	0	2.05	3.50	18.00	72.62	88.00	1.04
5	0	0	30	6.69	12.02	18.90	9.11	92.47	0.71
6	30	0	30	2.27	3.24	17.50	68.64	89.99	1.21
7	0	30	30	13.97	28.55	19.20	2.96	85.90	0.43
8	30	30	30	1.97	3.58	17.20	69.13	87.40	1.32
9[a]	15	15	15	2.05	2.58	16.60	76.84	88.79	1.82
10[a]	15	15	15	1.95	2.49	17.40	76.40	86.97	1.79
11[a]	15	15	15	1.99	2.28	16.40	74.27	88.82	2.11
12[a]	15	15	15	n/a	2.40	17.0	n/a	n/a	1.32

[a] Indicates the centre point of the design. Abbreviation: n/a, not available.

Table 4. A summary of *p*-values for coefficients of factor for response: Mastersizer D_{50} (μm, Y_1), Spraytec D_{50} (μm, Y_2), Spraytec ED (mg, Y_3), fine particle fraction (%, Y_4), twin-stage impinger ED (%,Y_5) and cohesion value (kPa, Y_6).

Coefficients	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6
b_1	**<0.0001**	**<0.0001**	0.1038	**0.0005**	0.0277	0.0470
b_2	**0.0002**	**<0.0001**	0.0529	**0.0075**	0.8008	0.0124
b_3	**<0.0001**	**<0.0001**	0.1641	**0.0014**	0.0409	**0.0013**
b_1b_2	**0.0002**	**<0.0001**	0.0684	0.0156	0.0265	0.1659
b_1b_3	**<0.0001**	**<0.0001**	0.0529	**0.0028**	0.0175	**0.0033**
b_2b_3	**0.0009**	**<0.0001**	0.0529	0.0133	**0.0067**	0.0174
Lack of fit	**0.0002**	**<0.0001**	0.0297	**0.0013**	0.6485	0.8904

Significant effects of factors ($p < 0.01$) on individual responses are shown in bold type.

2.3.2. Particle size analysis

The volume median particle size (D_{50}) of all the formulations measured using Mastersizer 2000 are listed in Table 3. The regression equation for Mastersizer D_{50} is shown as Eq. (4). Spray drying mannitol alone produced small particles with D_{50} of 1.87 μm. This is not surprising as it contains the lowest solid loading. The addition of amino acids in all compositions, other than leucine only, increased the particle size of the mannitol formulation. However, relative changes in sizes did not follow a pattern of solid loading (Table 5). The presence of glycine and alanine, though incurring lower solid loadings owing to their lower molecular weights compared to leucine, increased particle size to the greatest extent as demonstrated by the largest D_{50} of the formulations containing glycine/alanine 30/30%, alanine 30% and glycine 30%. The significant effects of glycine and alanine in increasing D_{50} are evident as shown in graphical

analysis (Fig. 1a). In contrast, leucine was the only amino acid that relatively reduced the D_{50} when added into the mannitol formulation, and all the formulations containing leucine have a D_{50} of < 5 µm. The significant effect of leucine in reducing this particle size measure is evident from the highest negative value of coefficient of term X_1 (Eq. 1) and longest bar length shown in graphical analysis (Fig. 1a).

Interestingly, the interaction terms X_1X_2, X_1X_3 and X_2X_3 all have a significant effect for the Mastersizer D_{50} (Fig. 1a). The term X_2X_3 indicates that the concurrent use of glycine and alanine increases D_{50} significantly in a synergistic manner. However, the negative influence of the terms X_1X_2 and X_1X_3 can be attributed to the initial increase of D_{50} associated with the addition of glycine and alanine, which in turn leads to a more pronounced apparent D_{50} reducing effect when leucine was present. This is consistent with the fact that although glycine and/or alanine increased particle size significantly when used alone, the addition of leucine appeared capable of overwriting the effect of glycine and alanine on D_{50} to produce smaller particles. The enhanced performance resulting from the combined use of these amino acids is demonstrated by the significant lack of fit from analysis of variance and shows the non-linearity effects of these amino acids within the study design space (Table 4). It should especially be noted that the results from the centre point formulations are highly consistent as demonstrated by the narrow experimental variance on graphical analysis (Fig. 1a).

$$Y_1 = 4.29 - 2.28X_1 + 1.14X_2 + 1.94X_3 - 1.14X_1X_2 - 1.82X_1X_3 + 0.60X_2X_3 - 0.75X_1X_2X_3 \quad (4)$$

where $p = 0.0001$ and $r^2 = 0.9168$.

The results are not only consistent with previous findings that leucine is an excipient that can be used to improve aerosolisation of spray-dried particles, but the results show that leucine also assists in the formation of suitable small-sized particles.

However, glycine and alanine, although being structurally similar to leucine, do not achieve similar effects, but instead they significantly increase the particle size of the formulations. It is worth noting that while initial concentration in feed solution is a known determinant of particle size (Vehring, 2008), the range of solid loading used within the study design space did not appear to have a strong influence on geometric particle size as measured by laser diffraction. The total solid loading in the feed solution ranged from 2.50% to 3.72% in the present study (Table 5). It is proposed that the change in particle size within this relatively small range of solid loading was negligible compared to the effects of the formulation excipients on cohesion and shape. The 30% molar ratio of leucine, glycine and alanine used in the present study amounts to mass ratios of 17.8%, 11.0% and 12.7% w/w, respectively, in the final formulations. A previous study using 30% mass ratio of leucine or glycine or alanine as excipients in a spray-dried formulation produced particles with D_{50} within a particle size range of 4.0 to 4.7 µm (Minne et al., 2008). In contrast, the equivalent molar ratio of the three amino acids in the present study when used alone produced particles with very different D_{50} of 1.75, 3.75 and 6.69 µm from leucine, glycine and alanine, respectively. The scanning electron microscope images discussed in section 3.6 confirm that the latter two results show what appear to be fused particles, whereas the leucine formulation results in distinct spherical primary particles (Fig. 2). It is proposed that molar ratios, with respect to surface coverage, may be considered to be more appropriate than mass ratios as we propose that molecules of amino acids are assembling at the surface with their hydrocarbon chains aligning away from the interface in a manner reflecting molecules with surface active properties. This is discussed further in section 3.3. Furthermore, considering the particle sizes produced from the mixed amino acids, it is indicated from this analysis that the combination use of these amino acids with leucine at appropriate

concentrations may also further influence particle size (and form) contrasting that achieved by leucine alone.

Table 5. Table of total solid loading (grams) in each formulation versus Mastersizer D_{50} (µm).

Trial number	Amino acids (grams)			Total solid loading (% w/v)	Mastersizer D_{50} (µm)
	Leucine	Glycine	Alanine		
T1	---	---	---	2.50%	1.87 ± 0.05
T2	1.08 g	---	---	3.04%	1.75 ± 0.01
T3	---	0.62 g	---	2.81%	3.75 ± 0.03
T4	1.08 g	0.62 g	---	3.35%	2.05 ± 0.04
T5	---	---	0.73 g	2.87%	6.69 ± 0.08
T6	1.08 g	---	0.73 g	3.41%	2.27 ± 0.04
T7	---	0.62 g	0.73 g	3.18%	13.97 ± 0.33
T8	1.08 g	0.62 g	0.73 g	3.72%	1.97 ± 0.02
CP1	0.54 g	0.31 g	0.37 g	3.11%	2.05 ± 0.02
CP2	0.54 g	0.31 g	0.37 g	3.11%	1.95 ± 0.04
CP3	0.54 g	0.31 g	0.37 g	3.11%	1.99 ± 0.01

Abbreviation: CP, centre point; T, trials. (Mean ± SD, n = 3)

2.3.3. Powder dispersibility and de-agglomeration

The volume median diameters (D_{50}) measured with the Spraytec are listed in Table 4. The pattern of the results from the Spraytec study correlates well with the results from the Mastersizer study. Apart from mannitol as a foundation material alone, all the formulations without leucine had a D_{50} of greater than 5 µm. Mannitol alone had a D_{50} of 2.83 µm while the formulations containing glycine/alanine 30/30%, alanine

30% or glycine 30% as additives had D_{50} of 28.55 µm, 12.02 µm and 5.52 µm, respectively. Graphical analysis demonstrates the significant effect of leucine in reducing D_{50} and the significant effects of glycine and alanine in increasing D_{50} (Fig. 1b). It is worth noting that the experimental variance of the study, as shown in graphical analysis, is narrow indicating the consistency of the results from the centre point experiments. The regression equation for D_{50} and emitted doses (ED) measured by Spraytec are shown below as Eq. (5) and (6), respectively.

$$Y_2 = 7.74 - 4.49X_1 + 2.55X_2 + 4.11X_3 - 2.26X_1X_2 - 3.95X_1X_3 + 1.67X_2X_3 - 1.79X_1X_2X_3 \quad (5)$$

where p = <0.0001 and r^2 = 0.8820.

$$Y_3 = 17.91 - 0.36X_1 + 0.49X_2 + 0.29X_3 - 0.44X_1X_2 - 0.49X_1X_3 - 0.49X_2X_3 + 0.29X_1X_2X_3 \quad (6)$$

where p = 0.0692 and r^2 = 0.7274.

The interaction effects of the amino acids on Spraytec D_{50} within the design space are significant as shown by the interaction terms X_1X_2, X_1X_3 and X_2X_3 on graphical analysis. This finding is not surprising considering the similar Mastersizer result outcome. These results are consistent with the particle size distribution data from the Mastersizer study and clearly demonstrate the significant effect of leucine on not only the formation of particle size, but also on the aerosolisation of the mannitol dry powder formulations. Interestingly, the four centre point formulations with leucine/glycine/alanine 15/15/15% as additives demonstrated the smallest aerosolised D_{50} of 2.28 µm, 2.49 µm, 2.58 µm and 2.40µm. The curvature is demonstrated by the significant lack of fit from the analysis of variance (Table 4). This result further suggests the interesting finding that the combined use of these amino acids at

appropriate concentrations may improve aerosol dispersibility better than with any of these amino acids alone, and hence deserves further investigation.

Spray-dried mannitol has been shown to be an effective non-reducing sugar stabilising agent to preserve the structure of spray-dried proteins in a number of studies (Andya et al., 1999; Maa et al., 2004; Schule et al., 2008). Spray-dried mannitol produced particles with D_{50} of 2.83 µm which appears to indicate a satisfactory dispersibility for inhalable dry powder formulations, but it should be noted that it also produces the lowest emitted dose (ED) during the Spraytec study (Table 3). The retention of powder in the device after the experiment was visually evident, and suggests a more cohesive powder than the other formulations here. The presence of amino acids in all combinations resulted in improved ED (Table 3) however, considering the experimental variability the effects of these amino acids do not appear to be significant as shown in graphical analysis (Fig. 1c). The ability of leucine to reduce surface cohesiveness resulting in enhanced dispersibility of spray-dried particles has been indicated previously (Muttil et al., 2010; Shur et al., 2008). In the present study, the beneficial effect of leucine was evident in its capacity to offset the effect of the other two amino acids on D_{50} and of improving both de-agglomeration and ED.

The mechanism of the performance enhancing effect of leucine remains unclear from the literature. The results here suggest that the effect of leucine is unlikely to be simply dependent on molecular weight. Leucine has been suggested to have some surface active properties (Glinski et al., 2000) and has a low water solubility of 0.22 mg/mL (Vehring, 2008). Amino acids with non-polar side chain such as leucine, phenylalanine, methionine and tryptophan have been shown to provide higher particle surface coverage after spray-drying than amino acids with polar side chain such as asparagine and arginine (Chew et al., 2005). Therefore, hydrophobicity may play a role

in increasing surface affinity of leucine during droplet drying, leading to modified surface properties of resulting particles. However, the lack of direct correlation between the hydrocarbon chain lengths and effect on aerosolisation from these amino acids suggest that the low solubility and hydrophobicity are not the sole factors causing this. It has also been previously suggested that there is a preferential precipitation of leucine during the drying process and hence the subsequent surface enrichment of leucine on the particles (Kamlag et al., 2004). A lamellar-like self-assembly behaviour of leucine has been reported and this may be a contributing factor to its surface modifying properties (Harding and Howieson, 1976). Though self-packing of molecules has also been reported within the crystal structures of glycine and alanine, these amino acids do not appear to have the same degree of surface affinity compared to leucine (Albrecht and Corey, 1939; Iitaka, 1961; Simpson and Marsh, 1966). It is proposed here that a combination of a high surface affinity during the drying process, mass transport within the droplet, followed by the subsequent self-assembly packing on the particle surface may explain the dispersibility enhancing effect specific to leucine, and further work is ongoing to investigate this hypothesis.

2.3.4. In vitro aerosolisation and particle deposition

While the Spraytec is useful as a high throughput screening tool to study the real-time apparent particle sizing of dry powder formulations, it provides no direct information on the aerodynamic properties upon powder aerosolisation. While particles with a lower D_{50} are likely to deposit in the lower airway, the Spraytec results do not take into account the potential influence of particle density and morphology on aerosolisation and subsequent deposition. The TSI was therefore used as a preliminary

screen of this range of formulations to provide aerodynamic aerosol information complementary to the Spraytec results.

Fine particle fraction (FPF) results correlate well with the Spraytec D_{50} results, as all the formulations containing leucine, with D_{50} below 5 μm demonstrate the highest FPF of greater than 68% (Table 3). Powders containing amino acids without leucine, with D_{50} above 5 μm show significantly lower FPF as demonstrated by formulations containing glycine/alanine 30/30%, alanine 30% or glycine 30%, with FPF of 2.96%, 9.11% and 34.62%, respectively. While mannitol alone shows reasonable FPF of 66.20%, this formulation also demonstrates the lowest ED which is consistent with the Spraytec study findings (Table 3). The regression equations for FPF and ED% from the TSI study are shown as Eqs. (7) and (8), and graphical analyses shown in Fig.(1d) and (1e), respectively. The highly significant effect of leucine (p = 0.0005, Table 4) on improving FPF is demonstrated by the longest bar length in graphical analysis (Fig. 1d) and the highest positive value of coefficient of term X_1 (Eq. 7). The significant negative effects of glycine (p = 0.0075, Table 4) and alanine (p = 0.0014, Table 4) on FPF are also evident on graphical analysis (Fig. 1d). It is interesting to note that the combined use of the three amino acids at 15% were more effective at improving FPF than the combination used at 30% (Table 3). The analysis of variance showed significant lack of fit to the model because of curvature which is consistent with the Mastersizer and Spraytec results (Table 4). Though further investigation is required to define the optimal concentration ranges, these results suggest that the inclusion of glycine and/or alanine with leucine produced the greatest improvement on aerosolisation performance within a critical concentration range and that higher concentration of these excipients may not be appropriate.

$$Y_4 = 50.42 + 22.20X_1 - 5.59X_2 - 12.96X_3 + 3.84X_1X_2 + 9.22X_1X_3 + 4.17X_2X_3 - 2.18X_1X_2X_3 \quad (7)$$

where $p = 0.0020$ and $r^2 = 0.8217$.

$$Y_5 = 87.81 + 1.31X_1 - 0.09X_2 - 1.13X_3 - 1.34X_1X_2 - 1.56X_1X_3 - 2.20X_2X_3 + 2.33X_1X_2X_3 \quad (8)$$

where $p = 0.0128$ and $r^2 = 0.9820$.

The results support previous studies in which leucine was consistently demonstrated to be the amino acid that had the most significant effect on the aerosolisation of dry powder formulations (Chew et al., 2005; Seville et al., 2007). Studies investigating the aerosolisation enhancing effect of various amino acids including leucine, phenylalanine, tryptophan, methionine, asparagine, arginine, aspartic acid and threonine on the dispersion of spray-dried powders found leucine to be the most effective amino acid that enhanced *in vitro* particle deposition, demonstrating the highest FPF and reproducibly high ED (Chew et al., 2005; Seville et al., 2007). The findings of the current study are also consistent with a previous study which demonstrated the effect of leucine, glycine and alanine on FPF with no correlations with hydrophobicity (Minne et al., 2008). However, these previous studies did not investigate the effect achieved from the combination use of these amino acids. The results from the present study suggest that the combination use of leucine with glycine and/or alanine in the appropriate concentrations is able to produce particles within the suitable size range with good aerosolisation properties for pulmonary delivery. In addition, this current study also demonstrates the ability to incorporate multiple components (for multiple potential functionalities within the particle) into an inhalable spray-dried formulation while maintaining good aerosolisation, which might otherwise not be possible without the presence of leucine.

2.3.5. Inter-particle interaction

From the shear cell testing, mannitol alone was the most cohesive powder with a cohesion value of 4.53 kPa (Table 3). The regression equation for cohesion values is shown as Eq. (7). The high r^2 (>0.9) indicates a high correlation between the mathematical model and the experimental results. The presence of amino acids demonstrates a clear trend in reducing inter-particle cohesion of the dry powders as shown by the negative values of coefficients of term X_1, X_2 and X_3 (Eq. 9) and the negative bars in graphical analysis (Fig. 1f). The presence of amino acids in all compositions reduced the cohesion of the mannitol alone formulations (Table 3). The incorporation of leucine, glycine and alanine all reduces inter-particle cohesion as concentration increases. These results indicate that these amino acids as excipients are effective at reducing inter-particle cohesion.

$$Y_6 = 1.73 - 0.29X_1 - 0.44X_2 - 0.81X_3 + 0.17X_1X_2 + 0.63X_1X_3 + 0.39X_2X_3 - 0.07X_1X_2X_3 \quad (9)$$

where $p = 0.0051$ and $r^2 = 0.9741$.

However, leucine was the only amino acid that reduced cohesion without substantially increasing particle size, maintaining the particle size range (1-5 µm), as suitable for pulmonary delivery. Inter-particle forces will increase for a given composition as particle size reduces (Forsyth et al., 2001). Although the inclusion of glycine and alanine reduced cohesion in the absence of leucine, this was evidently attributable to relative increase in particle size. The results suggest that even though glycine and alanine effectively reduced inter-particle cohesion of the mannitol formulations, the increase in particle size means the use of these amino acids is less appropriate for pulmonary delivery (and notably under the process conditions used here)

without inclusion of dispersibility enhancing excipients such as leucine. This finding is further reflected by the poor aerosolisation performance of the formulations containing glycine/alanine 30/30%, alanine 30% and glycine 30% in both the Spraytec and TSI studies. Therefore, it is noted that the presence of leucine is required to maintain a suitable particle size range for inhalation.

2.3.6. Particle morphology and appearance

Spray-dried mannitol as a foundation material alone was observed to form small spherical particles that are heavily agglomerated (Fig. 2a). This result is consistent with the particle size distribution data from the Mastersizer and cohesion study. Upon addition of amino acids, spherical particles were preserved in all formulations containing leucine regardless of the presence of glycine and alanine (Fig. 2b, d, f, h, i). Other formulations containing glycine and/or alanine without the addition of leucine formed much larger particles of irregular shape with rough surfaces (Fig. 2c, e, g). It appears that these particles are created as a result of irreversible fusion of primary structures formed during the drying process of each isolated drying droplet. Amino acids with non-polar side chains including leucine have been shown to have higher affinity to the particle surface (Chew et al., 2005). It is therefore proposed that this fusion is prevented when leucine is present on the surface to form a protective shell. The increased particle size observed is consistent with the Mastersizer and SprayTec size data as discussed above.

So, the result suggests that the presence of leucine assists in the formation of spherical particles by forming a coating on the drying particle surface, hence providing a protective shell which preserves the individual particles as they are collected from the

dryer and preventing any such fusion. In contrast, the presence of glycine and alanine enhanced fusion, and it is suggested that these amino acids therefore increase the relative hygroscopicity, compared to mannitol alone.

Leucine has also been shown to alter particle morphology and may produce either smooth or wrinkled particles depending on the concentration used in the feed solution (Kamlag et al., 2004). Such structures are proposed to result from the formation of hollow particles that are inflated during drying (Vehring, 2008). More recently, co-spray-drying of unfractionated heparin with L-leucine 1% w/w and disodium cromoglycate with L-leucine 5% w/w produced smooth spherical particles (Chew et al., 2005; Shur et al., 2008); while spray-drying of salbutamol sulphate and lactose with L-leucine 12% w/w produced particles of irregular surface (Seville et al., 2007). These results indicate that a relatively high concentration of leucine (i.e. >5% w/w) tends to lead to corrugated particles. The morphology of leucine-containing particles in the present study appears to behave somewhat differently. The concentrations of leucine used within the study design space (15 to 30 molar%), which corresponds to roughly 10 to 18% w/w, did not form corrugated particles. It is therefore speculated that the presence of glycine and/or alanine altered the core structure of the spherical drying particles, while leucine tended to reside on the particle surface, hence not only providing a coating to reduce surface cohesiveness and prevent fusion but also with the net result of reducing tendency to form corrugations in the drying process.

While surface asperities and rugosity on corrugated particles have been demonstrated to reduce powder cohesiveness and therefore improve dispersibility (Chew and Chan, 2001; Lechuga-Ballesteros et al., 2008), in the present study, leucine was able to enhance aerosolisation performance of the mannitol formulations without necessitating the formation of corrugated particles. Furthermore, the FPF results suggest

that this combination may also be advantageous in terms of aerosolisation efficiency. This finding is consistent with previous studies in which spray-dried unfractionated heparin with L-leucine 1% w/w increased FPF by >4.5-fold without alteration of surface morphology (Shur et al., 2008).

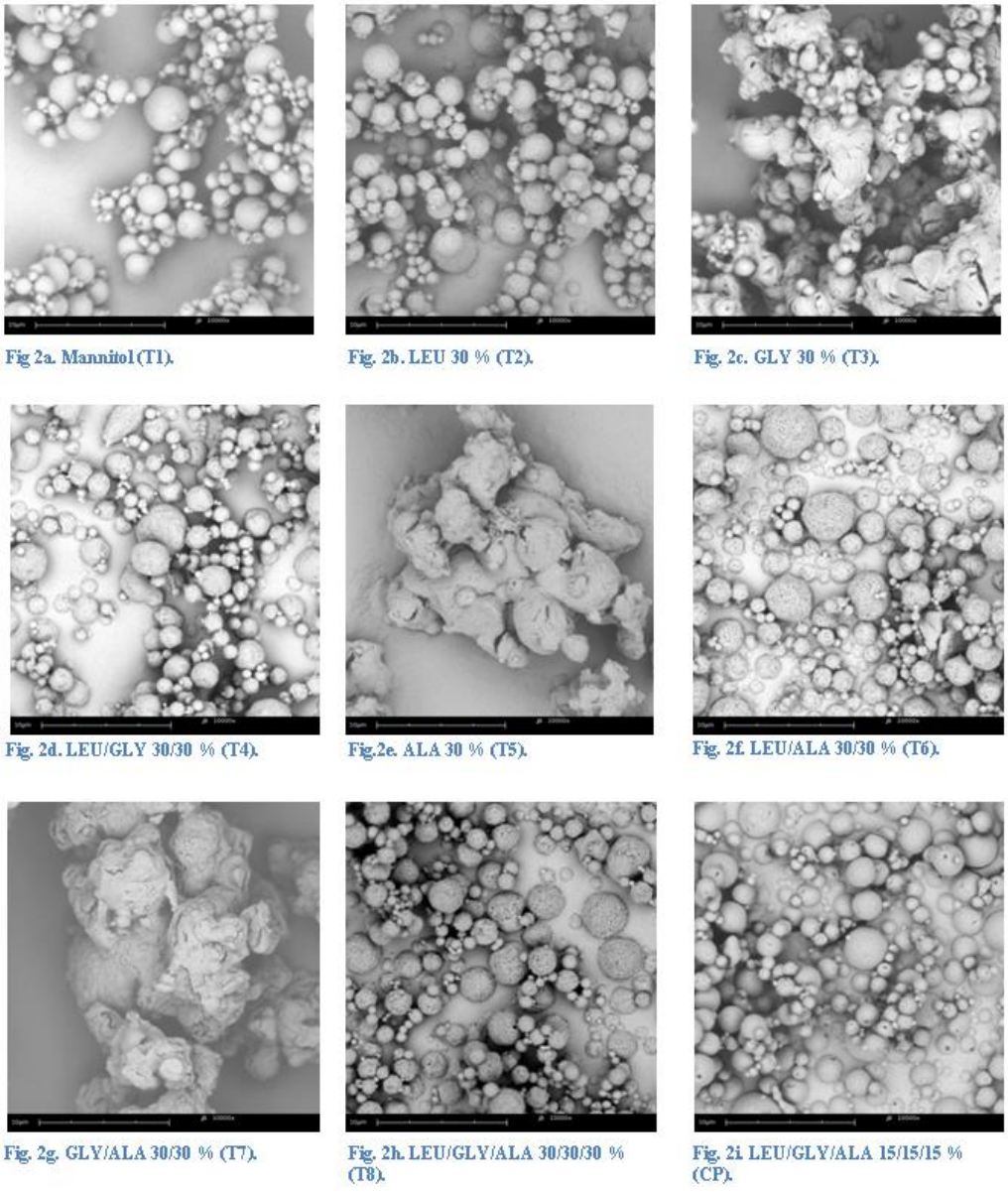

Figure 2. Representative scanning electron micrographs of the spray-dried mannitol formulations containing (a) mannitol (T1), (b) LEU 30% (T2), (c) GLY 30% (T3), (d) LEU/GLY 30/30% (T4), (e) ALA 30% (T5), (f) LEU/ALA 30/30% (T6), (g) GLY/ALA 30/30% (T7), (h) LEU/GLY/ALA 30/30/30% (T8) and (i) LEU/GLY/ALA 15/15/15% (CP) [magnification:10000x].

2.3.7. Crystallinity

To investigate the feasibility of developing a dry powder carrier platform capable of stabilising proteins for pulmonary delivery, the glassy phase (amorphous or crystalline) status of the formulations was investigated by assessing their crystallinity using XRPD. All the formulations were sealed and stored in a refrigerator before the XRPD study. According to the glassy dynamics hypothesis, an amorphous glassy solid should provide a highly viscous "vitreous" environment which restricts the molecular mobility of biomacromolecules and thereby stabilises proteins in a dry solid state (Chang and Pikal, 2009; Weers et al., 2007). Any excipients combination used should ideally be a good glass former with high glass transition temperature (T_g) but otherwise inert. Mannitol alone has a relatively low T_g of 11 ºC (Weers et al., 2007), therefore it is not surprising to find a high level of crystallinity from mannitol as a baseline material alone (Fig. 3). In a study of the salmon calcitonin-mannitol system, the spray-dried powders remained amorphous in formulations containing less than 50% w/w of mannitol (Chan et al., 2004). Therefore, it was hypothesised that the addition of excipients in sufficient quantity could be used to prevent or delay crystallisation of mannitol after spray-drying. It is unclear from the literature how effective small molecule additives such as amino acids, may be at stabilising the amorphous status of mannitol. However, spray-dried powders containing mannitol and glycine have been successfully used to produce inhalable dry powder insulin formulation stable at room temperature without refrigeration (Sadrzadeh et al., 2010; Siekmeier and Scheuch, 2008; White et al., 2005). In particular, a study on spray-dried IgG has established that formulations containing mannitol/glycine combinations produced dry powders with T_g ranging from 14.1 to 53.8 ºC with the 50/50% w/w ratio producing a dry powder with

the highest T_g (Schule et al., 2008). In other words, in the absence of other excipients, a high level of glycine was required to modify the glassy state of a mannitol-based dry powder formulation. The present study examined the effect of leucine, glycine and alanine, both alone and in combination in the proportions defined in the study design space, on the amorphous status of the formulations.

The presence of either a single amino acid or combinations under the experimental conditions used did not appear to have much impact on reducing the crystallinity of the mannitol formulation. The presence of either leucine 30%, glycine 30% or alanine 30% did not appear to reduce the crystallinity profile of the mannitol formulations as demonstrated by the similar XRD profiles (Fig. 3). Similarly combinations of amino acids at other concentrations within the study design space did not appear to have much effect on the crystallinity of the formulations either (Fig. 4 and 5). The formulation containing leucine/glycine/alanine 30/30/30%, provided slightly reduced peaks compared to the background, but this was not quantified and crystalline peaks were still present (Fig. 5). XRPD patterns need to be interpreted with caution when evaluating the crystallinity of small particles. Considering the line broadening effect of particles at this small particle size range, there appears to be little alterations in crystallinity of the formulation examined. While the combination of mannitol/glycine in a 50/50% w/w ratio has been shown to retain high amount of amorphous content (Schule et al., 2008), the result from the present DoE study indicate that the amount of amino acids used within the study design space was not sufficient to prevent crystallisation. Hence, further study using higher concentrations of excipients should be investigated as potential to maintain the amorphous status of spray-dried mannitol based particles.

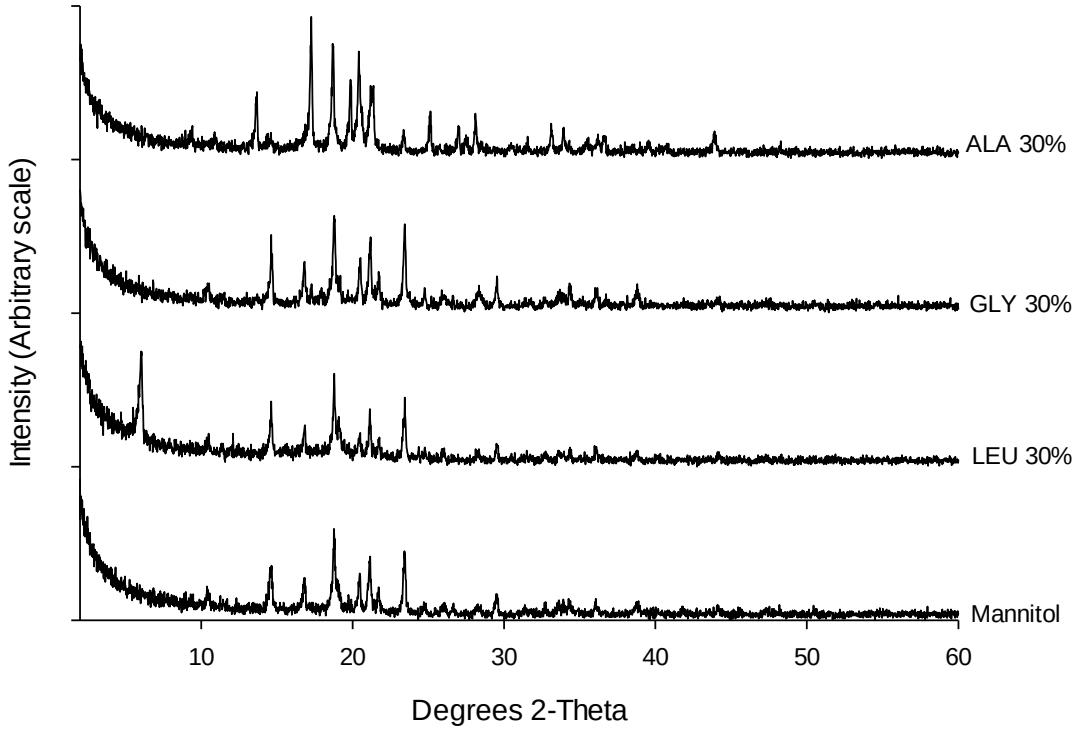

Figure 3. XRPD profiles of mannitol and formulations containing 1 amino acid: mannitol with leucine 30% (molar%), mannitol with glycine 30% (molar%) and mannitol with alanine 30% (molar%).

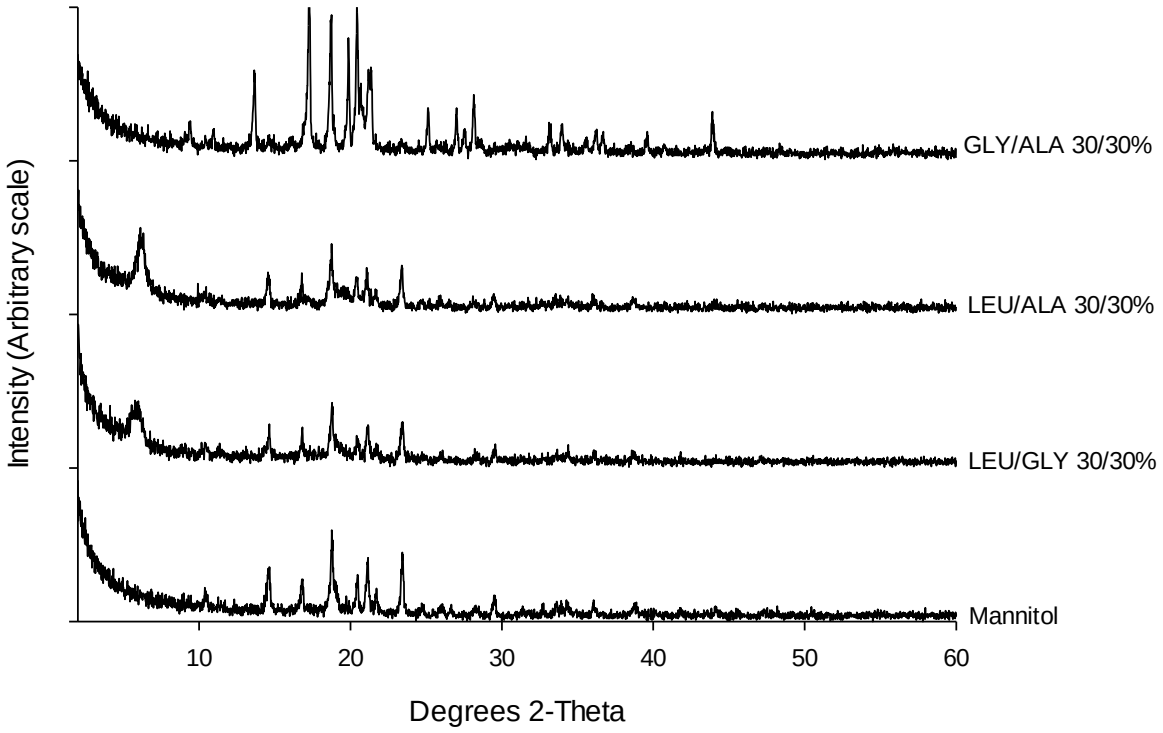

Figure 4. XRPD profiles of mannitol and formulations containing 2 amino acids: mannitol with leucine/glycine 30/30% (molar%) mannitol with leucine/alanine 30/30% (molar%) and mannitol with glycine/alanine 30/30% (molar%).

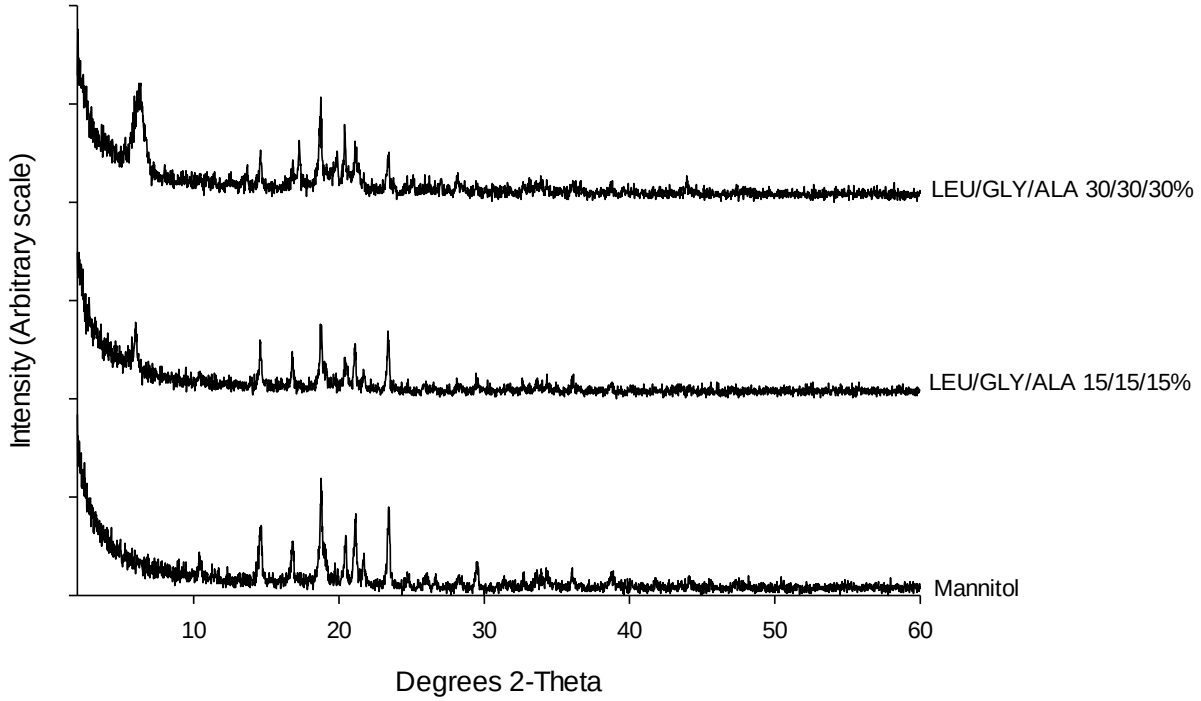

Figure 5. XRPD profiles of mannitol and formulations containing 3 amino acids: mannitol with leucine/glycine/alanine 15/15/15% (molar%) and mannitol with leucine/glycine/alanine 30/30/30% (molar%).

2.4. Conclusion

This study indicates that interactions between combinations of excipients used in spray dried formulations can be identified hence leading to a deeper study of such combination effects and greater understanding of the study design space. The results from the present study show that the use of glycine and/or alanine, though being structurally similar to leucine, provide detrimental rather than beneficial effects on particles both during as well as after spray-drying with mannitol. In addition, the combination of leucine with either (or both) glycine and alanine provides particles with the inherited benefits of the leucine. Although further study is required, this work

indicates there is potential to investigate further ranges of concentrations of combined amino acids that may further lead to added benefits, such as particle morphology control or stable glass formation. The investigation also suggests there are some further aspects regarding the mechanisms leading to these effects that require more study. It is worth noting that the results from the DoE analysis also revealed the lack of linearity of the effects achieved from the combination use of these amino acids across the concentration range within the study design space. This information should be considered in future study design when investigating the optimal concentration and effect of these potential performance enhancing excipients.

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CHAPTER THREE

THE EFFECT OF AMINO ACID EXCIPIENTS ON MORPHOLOGY AND SOLID-STATE PROPERTIES OF MULTI-COMPONENT SPRAY-DRIED FORMULATIONS FOR PULMONARY DELIVERY OF BIOMACROMOLECULES

Monash University

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study initiation, experimental design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
Lisa M Kaminskas	Supervision, manuscript revision
Tri-Hung Nguyen	Assay development
Renée Carlberg	In vitro powder aerosolisation study
Michelle P McIntosh	Supervision, manuscript revision
David AV Morton	Supervision, manuscript revision

Candidate's Signature [redacted]

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia

Lisa M Kaminskas	[redacted]	**Date** 6/6/13
Tri-Hung Nguyen		**Date** 7/6/13
Renée Carlberg		**Date** 7.6.13
Michelle P McIntosh		**Date** 7/6/13
David AV Morton		**Date** 7/6/13

3. The effect of amino acid excipients on morphology and solid-state properties of multi-component spray-dried formulations for pulmonary delivery of biomacromolecules

Tomás Sou, Lisa M. Kaminskas, Tri-Hung Nguyen, Renée Carlberg, Michelle P. McIntosh* and David A. V. Morton*

Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia

*Corresponding authors.

[redacted](D.A.V. Morton)

[redacted]

(M.P. McIntosh)

3.0. Abstract

For a dry powder carrier platform to be suitable for pulmonary delivery of potent biomacromolecules, it has to be aerosolisable and capable of stabilising the biomacromolecules. In the present study, strategies aiming to produce a multi-component spray-dried powder formulation with a stable amorphous glassy matrix containing mannitol, trehalose, glycine and alanine, while using leucine as a particle formation and aerosolisation enhancing agent were investigated. The results from *in-vitro* aerosolisation studies demonstrated high fine particle fractions (FPF) from several formulations. Scanning electron micrographs (SEM) revealed distinct morphological features of these formulations in response to increasing leucine concentration: from the apparent insufficiency for discrete particle formation, to reduced particle agglomeration, to increased surface corrugation. X-ray powder diffraction (XRPD) results indicated that partially-ordered leucine resulting from self-assembly on the particle surface is important for the amino acid to function effectively as an encapsulating agent. This may also play a role in inhibiting crystallisation of other components within the formulation. In conclusion, the results suggest that with suitable particle size, good dispersibility and solid-state properties, selected trehalose/leucine combinations appear to have good potential for development into a universal carrier platform for pulmonary delivery of potent biomacromolecules and the work highlights areas deserving further investigation.

3.1. Introduction

Dry powder inhalation has been widely proposed as an attractive systemic delivery method for pulmonary drug administration of biomolecules including proteins and peptides due to: ease of administration, convenient portability, relatively simple formulation, low cost and inherent solid state stability (Carpenter et al., 1997; Okamoto et al., 2002; Prime et al., 1997; Rave et al., 2004; Weers et al., 2007). There may also be benefits to this route with avoiding first pass metabolism and potentially providing a rapid onset depending on the physicochemical properties of the drug. Dry powder formulations should be readily dispersible and aerosolisable upon inhalation for efficient delivery. However, inter-particulate cohesive forces are particularly dominant in the finely micronised powders i.e. <5 µm required for pulmonary delivery (Forsyth et al., 2001). These forces influence flowability and de-agglomeration of micronised dry powders and therefore the aerosolisation properties and delivery efficiency of dry powder formulations (Ashurst et al., 2000; Prime et al., 1997).

Spray-drying is an established technology which has gained increased interest in this area over the past decade, due to its potential simplicity, adaptability, cost-effectiveness and scalability (Fourie et al., 2008; Lee et al., 2011). Spray-drying is a process in which the compound(s) of interest are prepared in a liquid form, which is then atomised into a drying chamber where the droplets are dried with heated air. Particle formation is achieved through precipitation of the dissolved compounds as the solvent evaporates from the solution droplets in the drying chamber. The ability to incorporate multiple components into a particle at the molecular level in a single step manufacturing process is an appealing aspect of spray-drying. Particles can be engineered to contain various components by adjusting the composition of the feed

solution. In some cases, even colloids or multiphase suspensions such as emulsions or liposomes can be used. Excipients can therefore be selected to manipulate the properties of the dry powder formulation.

Some excipients have been considered as having potential to improve the aerosolisation properties and performance of dry powder formulations (Li et al., 2003; Li et al., 2005a; Maa and Prestrelski, 2000; Rabbani and Seville, 2005; Staniforth et al., 2002). L-leucine has been demonstrated to enhance aerosolisation and performance of dry powder inhaler formulations, either as an additive or more specifically by the inclusion of leucine in a precursor solution for co-spray-drying (Ganderton et al., 2000; Kamlag et al., 2004; Li et al., 2003; Li et al., 2005b; Lucas et al., 1999; Morton and Kamlag, 2005; Seville et al., 2007; Staniforth, 1997). An earlier study has shown that the inclusion of L-leucine was able to assist the production of inhalable mannitol dry powder formulations containing multiple components, namely glycine and alanine, whether alone or in combination, while preserving the aerosolisation properties of the powders (Sou et al., 2011). These multi-component dry powder formulations were otherwise poorly aerosolisable in the absence of the L-leucine. The L-leucine was argued to be responsible for improving the discrete particle formation and preventing solid bridge formation between particles as the powder was collected and recovered. In addition, the same study also demonstrated that the amounts of amino acids used did not prevent the crystallisation of the mannitol in these mannitol-based formulations (Sou et al., 2011).

For a dry powder carrier platform to be suitable for pulmonary delivery of potent biomacromolecules such as proteins, it has to be not only aerosolisable, but also ideally capable of stabilising the biomacromolecules at room temperature. Strategies to produce spray-dried powder formulations with a stable amorphous glass structure are therefore

being investigated. According to the glassy dynamics theory, an amorphous glassy solid should provide a highly viscous "vitreous" environment which restricts the molecular mobility of biomacromolecules and thereby stabilises proteins in a dry solid state (Chang and Pikal, 2009; Weers et al., 2007). Optimal excipient combinations should form a glassy matrix with a high glass transition temperature (T_g) but otherwise be inert with respect to the other components.

Mannitol and trehalose are non-reducing sugars which have the advantage that they will not undergo the Maillard reaction with proteins. Mannitol is classified as a non-hygroscopic compound and its use as a stabilising excipient in dried protein formulations have been widely reviewed (Bakaltcheva et al., 2007; Kibbe, 2000; Schüle et al., 2007; Torrado, 2002). Mannitol in the amorphous state has been shown to be effective in stabilising spray-dried proteins (Costantino et al., 1998). However, mannitol alone has a relatively low T_g of 11 ºC and therefore tends to recrystallise readily during or immediately after spray-drying as a foundation material alone (Weers et al., 2007). Trehalose, which can exhibit a T_g of 117 ºC, depending on the presence of water and other components, tends to form amorphous glasses after spray-drying and has been used to stabilise proteins on storage in several studies (Hulse et al., 2008; Jin et al., 2010; Maa et al., 1997; Ógáin et al., 2011). However, high concentration of trehalose alone may not be ideal for aerosol applications due to the highly hygroscopic and adhesive nature of the amorphous form of this sugar, and subsequently the high degree of particle agglomeration and reported poor aerosolisation (Maa et al., 1997).

Previously, spray-dried powders including mannitol, glycine and sodium citrate have been successfully used to produce inhalable dry powder insulin formulation stable at room temperature without refrigeration (Sadrzadeh et al., 2010; Siekmeier and Scheuch, 2008; White et al., 2005). However, these powders were reputed to be highly

hygroscopic. In addition, another study on spray-dried IgG has found that mannitol/glycine in a 1:1 mass ratio produced dry powder with the highest T_g (Schule et al., 2008). The inclusion of glycine and alanine, individually and in combinations, less than this ratio was reported to be ineffective in terms of preserving amorphous content and improving aerosolisation (Sou et al., 2011). In a study of salmon calcitonin-mannitol systems, the spray-dried powders were reported to remain amorphous in formulations containing less than 50% w/w of mannitol (Chan et al., 2004).

Small molecules and sugars such as glycine, alanine, leucine, sodium citrate, sodium phosphate, trehalose, sucrose and lactose have been previously studied to modify solid-state properties of mannitol carrier systems (Andya et al., 1999; Costantino et al., 1998; Maa et al., 1997; Schule et al., 2008; Sou et al., 2011; White et al., 2005). However, most formulations examined in these studies contained a relatively high proportion of protein to mannitol ratio with 60% to 90% w/w of active protein present in the formulations (Andya et al., 1999; Costantino et al., 1998; Maa et al., 1997; Schule et al., 2008; White et al., 2005). These systems may not be appropriate for more potent active proteins. For example, vaccine antigens which may only be used in the order of < 1% w/w protein loading in the formulation, in which case the low proportion of protein present cannot be relied upon to help maintain the glassy matrix critical for physical stability of the formulation. The present study therefore investigates the feasibility to produce a universal amorphous glassy carrier platform suitable for efficient pulmonary delivery of potent biomacromolecules using a non-reducing sugar as the baseline material in conjunction with various combinations of excipients.

In the present study, mannitol and trehalose are investigated as the model baseline compounds, with a view to developing formulation strategies that may be used to improve aerosolisation and solid-state properties of these dry powder carrier systems

for pulmonary delivery of biomacromolecules. The present study aims to further explore the feasibility of multi-component inhalable dry powder formulations, with potentially multiple functionalities, using a range of concentrations of leucine as the "encapsulating agent" enabling incorporation of various components, including mannitol, trehalose, glycine and alanine in various proportions to modify solid-state properties, while maintaining good aerosolisation of these formulations.

3.2. Materials and methods

3.2.1. Materials

D-Mannitol, D-(+)-trehalose dihydrate, L-leucine (LEU), glycine (GLY) and L-alanine (ALA) were obtained from Sigma-Aldrich Chemicals (Castle Hill, NSW, Australia).

3.2.2. Preparation of spray-dried powders

Aqueous solutions containing the mannitol and/or trehalose and selected amino acids (LEU, GLY, ALA) in various compositions were dissolved in 100 to 200 mL of Milli-Q water. A low load of salbutamol sulphate (SS) (0.1% w/w) was incorporated in selected formulations to allow quantification of powder by liquid chromatograph-mass spectrometry (LC-MS) as described below. The prepared solutions were subsequently spray-dried using a Buchi 190 mini spray-dryer with a 0.5 mm two-fluid nozzle, using the following standard operating conditions: airflow rate, 800 L/h; pump setting, 5 (6.67

mL/min); aspirator setting, 20; outlet temperature, 70 °C . The compositions of the resulting formulations are shown in Table 1.

Table 1. Composition of the spray-dried formulations (% w/w) and summary of results from Mastersizer and Spraytec study (mean ± SD, n = 3).

Powder	Mannitol	Trehalose	Glycine	Alanine	Leucine	Mastersizer D_{50} (µm)
1	100	--	--	--	--	1.96 ± 0.03
2	90	--	--	--	10	2.72 ± 0.01
3	80	--	--	--	20	2.25 ± 0.01
4	50	--	--	--	50	3.01 ± 0.09
5	50	--	50	--	--	13.48 ± 0.05
6	45	--	45	--	10	2.63 ± 0.04
7	40	--	40	--	20	2.58 ± 0.02
8	33	--	33	--	33	2.19 ± 0.01
9	50	--	--	50	--	n/a
10	45	--	--	45	10	2.69 ± 0.05
11	40	--	--	40	20	2.41 ± 0.02
12	33	--	--	33	33	2.41 ± 0.02
13	50	50	--	--	--	4.80 ± 0.10
14	45	45	--	--	10	3.24 ± 0.13
15	40	40	--	--	20	2.11 ± 0.03
16	33	33	--	--	33	3.24 ± 0.09
17	--	100	--	--	--	1.97 ± 0.03
18	--	90	--	--	10	2.36 ± 0.30
19	--	80	--	--	20	2.29 ± 0.01
20	--	50	--	--	50	2.95 ± 0.03

Abbreviation: n/a, not available.

3.2.3. Particle size distribution analysis

The particle size distribution of the powders was determined by laser scattering using the Malvern Mastersizer 2000 (Malvern Instruments Ltd, Worcestershire, UK) equipped with a Scirocco cell and a Scirocco 2000 dry powder dispersion unit. The powders were dispersed in air at a shear pressure of between 3.0 to 4.0 bar, which was selected to achieve optimal de-agglomeration. The average particle size was measured in three replicates for each sample. The volume median diameter (D_{50}) was derived from the diffraction data using the in-built software for each sample.

3.2.4. In vitro powder aerosolisation and particle deposition

The *in vitro* powder aerosolisation performance was determined using a Next Generation Impaction (NGI, Copley Scientific Limited, Nottingham, UK) with the Monodose inhaler (Miat S.p.A., Milan, Italy) as the aerosol dispersion device. The flow rate was adjusted to 60 L/min using a Critical Flow Controller Model TPK 2000 & Flow meter model DFM 2000 (Copley Scientific Limited, Nottingham, UK). Approximately 20 mg of each powder was filled into size 3 HPMC capsules (Capsugel, Peapack, NJ, USA) for the tests which were performed at an air-conditioned laboratory (20 ± 2 ºC, 50 ± 5% relative humidity). Each capsule was actuated from the inhaler over 4 seconds for each measurement (n = 4). The amount of powder deposited at different stages was determined using a LC-MS as described below. Methanol:Milli-Q water 50:50% v/v was used as the collecting solution to recover powders from the NGI stages. The cut-off diameter of each stage is shown in Table 2. Fine particle fraction

(FPF; <5 µm and <3 µm) was calculated as a percentage of emitted dose using the Copley Inhaler Testing Data Analysis Software (CITDAS).

Table 2. D_{50} values for stages of the NGI at 60 L/min.

Stage	D_{50} (µm)
1	8.06
2	4.46
3	2.82
4	1.66
5	0.94
6	0.55
7	0.34
8	n/a

3.2.5. Scanning electron microscopy (SEM)

The morphology of the particles was visualised under a scanning electron microscope (Phenom™, FEI company, USA). Powder samples were gently poured onto a double-sided carbon tape mounted on a sample holder for examination under the SEM. Excessive powder was removed by gentle tapping and air blow to leave a fine layer of particles on the surface of the tape. The samples were sputter coated with gold using an electrical potential of 2.0 kV at 25mA for 6 minutes with a sputter coater (K550X, EMITECH). SEM micrographs were captured using the in-built image capturing software.

3.2.6. X-ray powder diffraction (XRPD)

Sample powders were sprinkled onto a quartz sample plate smeared with a thin layer of Vaseline at room temperature. The sample was then analysed by the X-ray diffractometer (Philips 1140 vertical diffractometer, Philips, Holland) for scanning from 2 to 60° in 2θ, with an angular increment of 2°/min. The crystalline status of the powders was assessed by examination of the resulting diffraction patterns.

3.2.7. Liquid chromatograph-mass spectrometry (LC-MS) analysis

The amount of powder deposited in each stage of NGI was measured using an LCMS/2010EV (Shimadzu Scientific Instruments, Australia). Salbutamol was incorporated into the dry powder formulation as a marker compound to allow quantitation of particle disposition within the NGI because a validated LCMS assay is routinely run in our laboratory. Salmeterol xinafoate was used as an internal standard. 20 µL aliquots of each sample were injected onto a Phenomenex SYNERGI polar-RP column (Phenomenex SYNERGI 4 µm polar-RP 80A, 4.60 mm inner diameter, 150 mm length), and subsequently eluted with gradient method, starting from 35% solvent B to 90% at 0.5 mL/min. The composition of mobile phase A was 95% water, 5% methanol, 0.1% formic acid and mobile phase B was 95% methanol, 5% water, 0.1% formic acid. The column temperature was maintained at 40º C. A single quadrupole mass spectrometer with an electrospray interface in the positive mode was used for selective ion monitoring for 240 (salbutamol sulphate) and 416.2 (salmeterol xinafoate) mass/charge ion peaks.

3.2.8. Statistical analysis

Statistical analysis was performed using student t-test at a *p*-value of 0.05 with SPSS (IBM SPSS Statistics, Version 19, SPSS Inc., USA).

3.3. Results and discussion

3.3.1. Particle size analysis

The Mastersizer D_{50}s of the formulations are listed in Table 1. Spray-dried mannitol as a foundation material alone produced the smallest particle size as demonstrated by the smallest Mastersizer D_{50} of 1.96 µm. Similar to mannitol, spray-dried trehalose alone produced relatively small particles with Mastersizer D_{50} of 1.97 µm. Upon addition of leucine 10% w/w, the Mastersizer D_{50}s of all formulations were found to be within the range of 2.36 to 3.24 µm regardless of particle composition. A graph of Mastersizer D_{50} upon increasing leucine concentration is shown in Figure 1. The effect of leucine in assisting particle formation is most pronounced from the mannitol/glycine formulation as demonstrated by the Mastersizer D_{50} which decreased from 13.48 µm (indicating fused agglomerates) to 2.63 µm upon addition of leucine 10% w/w. The Mastersizer D_{50} of all the formulations slightly decreased as the proportion of leucine increased from 10% to 20% w/w. Further increase in leucine concentration maintained the Mastersizer D_{50}s of these formulations to below 5 µm. However, a higher proportion of leucine above 20% w/w slightly increased the D_{50} in some formulations. Hence, this data suggests leucine should be used in an optimal amount or concentration range to provide the most desirable particle formation effect.

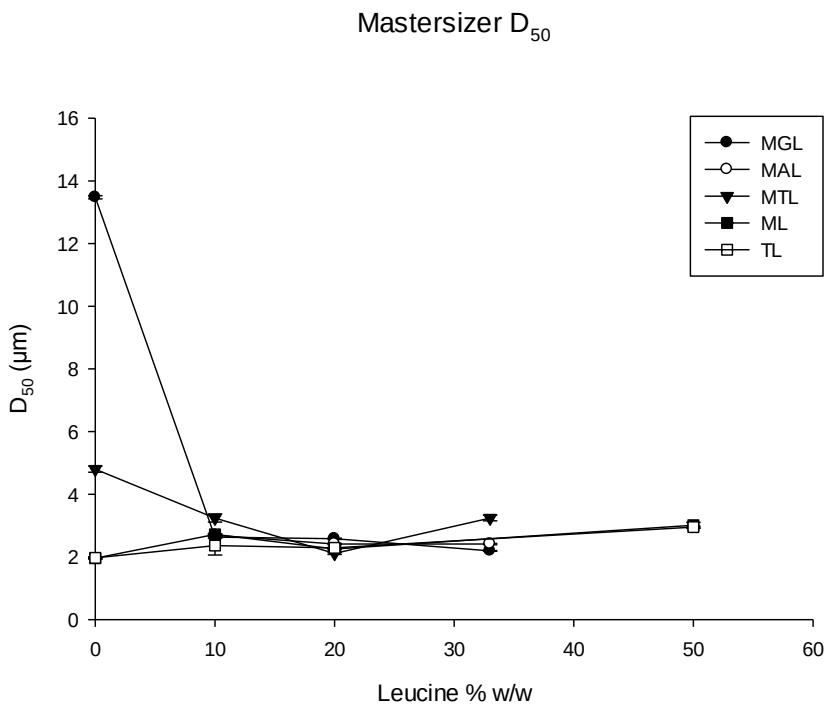


Figure 1. Mastersizer D_{50} (µm) vs. leucine % w/w in the formulations (mean ± s.d., n = 3). Abbreviations: ML, mannitol/leucine; TL, trehalose/leucine; MGL, mannitol/glycine/leucine; MAL, mannitol/alanine/leucine; MTL, mannitol/trehalose/leucine.

3.3.2. In vitro particle aerosolisation and deposition

The NGI deposition profiles and fine particle fractions of the selected spray-dried formulations are shown in Table 3. Apart from the dual sugar combinations containing mannitol/trehalose/leucine, all formulations demonstrated FPFs of higher than 65%. In general, formulations containing20 % w/w leucine produced higher FPFs compared to their counterpart formulations with 10% w/w leucine. The overall retention of powder in device and capsule ranged from 10–20%. The mannitol/leucine 90/10% w/w formulation demonstrated the highest FPF of 80%. The superior aerosolisation performance of this formulation is consistent with our previous study (Sou et al., 2011). The mannitol/trehalose/leucine formulations with either leucine 10 or 20% w/w

demonstrated the lowest FPFs of 26% and 51% respectively. The comparatively lower FPFs of these two formulations correlated with a set of additional unpublished studies where these powders were aerosolised under identical conditions into a Malvern Spraytec system, providing relatively large D_{50} of 7.5 μm and 5.5 μm, respectively. In particular, the mannitol/trehalose/leucine 45/45/10% w/w formulation was poorly dispersible with over 40% of the powder found trapped in the pre-separator of the NGI. The increase of leucine from 10 to 20% w/w improved the FPF of this formulation by almost two-fold. It appeared that these mannitol/trehalose/leucine powders were particularly hygroscopic, in comparison to the other powders produced, and this may explain their relatively reduced aerosolisation efficiency.

The highest aerosolisation performance of the spray-dried mannitol/leucine formulation is consistent with our previous findings (Sou et al., 2011). The dual sugar combination system was tested here to investigate whether the properties of the two sugars, mannitol and trehalose, could be combined to produce a formulation conserving the inherent aerosolisation properties of spray-dried mannitol while providing an amorphous matrix more characteristic of spray-dried trehalose. While the concurrent use of mannitol and trehalose has been shown to be more effective in stabilising a model protein after co-spray-drying, the combined use of these excipients as a carrier platform for respiratory delivery has not been investigated (Hulse et al., 2008). It was interesting to note that the NGI results indicated that the sugars mannitol or trehalose when spray-dried with leucine gave relatively higher FPF than with the mixtures of all three used here. The NGI results demonstrated the potential aerosol application of these combination systems. However, the relatively high retention of powder in the pre-separator and low FPFs of these dual-sugar formulations suggest these systems appear to be more cohesive and difficult to disperse. It is not immediately obvious why this is

the case, and further studies are underway to investigate the relative hygroscopicity as well as glass transition behaviours of such mixed systems.

Table 3. Fine particle fractions of selected formulations from the NGI deposition study. Abbreviations: ML9010, mannitol/leucine 90/10% w/w; MGL454510, mannitol/glycine/leucine 45/45/10% w/w; MGL404020, mannitol/glycine/leucine 40/40/20% w/w; MAL454510, mannitol/alanine/leucine 45/45/10% w/w; MAL404020, mannitol/alanine/leucine 40/40/20% w/w; MTL454510, mannitol/trehalose/leucine 45/45/10% w/w; MGL404020, mannitol/trehalose/leucine 40/40/20% w/w; TL9010, trehalose/leucine 90/10% w/w; TL8020, trehalose/leucine 80/20% w/w.

Formulations	FPF (% < 5 µm)	FPF (% < 3 µm)	ED (mg)	ED (%)	MMAD (µm)
ML9010	80.46 ± 1.89	54.34 ± 3.08	15.15 ± 1.01	85.81 ± 1.66	2.51 ± 0.25
MGL454510	71.26 ± 3.80	53.65 ± 5.88	17.29 ± 2.86	82.44 ± 2.91	1.89 ± 0.63
MGL404020	79.64 ± 1.88	60.95 ± 2.98	19.38 ± 1.79	85.69 ± 2.65	1.77 ± 0.17
MAL454510	74.34 ± 1.75	49.45 ± 1.96	13.56 ± 1.30	79.45 ± 2.60	2.55 ± 0.28
MAL404020	77.37 ± 5.40	44.66 ± 5.54	16.48 ± 0.91	79.86 ± 5.56	3.06 ± 0.17
MTL454510	26.37 ± 4.15	10.63 ± 5.17	15.15 ± 0.71	89.72 ± 1.07	4.99 ± 0.59
MTL404020	50.83 ± 5.57	23.75 ± 9.86	17.81 ± 1.01	90.72 ± 0.31	4.38 ± 0.64
TL9010	66.51 ± 3.32	41.53 ± 9.55	16.41 ± 3.81	84.85 ± 2.79	2.90 ± 0.73
TL8020	66.85 ± 2.91	44.93 ± 7.76	17.24 ± 2.82	82.49 ± 4.31	2.82 ± 0.57

Abbreviations: FPF, fine particle fraction; ED, emitted dose; MMAD, mass median aerodynamic diameter (mean ± s.d., n = 4).

3.3.3. Particle morphology and appearance

The representative scanning electron micrographs of the studied formulations are shown in Figure 2. Spray-dried mannitol alone formed small coherent spherical particles, whereas the mannitol/trehalose mixtures and trehalose alone showed fused

structures suggesting moisture had been taken up to cause solid bridging and particle fusion. The mannitol/trehalose 50/50% w/w formed partially fused particles with extensive bridges formed between the spheres to the extent that some are indistinguishable. Related morphology is observed with spray-dried trehalose alone with which pendular links between adjacent particle surfaces can be seen, however, particle separation is more visually evident here. This is consistent with the observation noted above that the mannitol/trehalose mixture is more hygroscopic than the trehalose alone. Mannitol with either glycine or alanine in 50/50% w/w both produced much larger particles of irregular dendritic shapes with texture evidently suggesting a highly aggressive fusion of primary particles formed during the drying or collecting process. This irreversible fusion of spray-dried mannitol in the presence of glycine and/or alanine in lower concentration has been previously reported (Sou et al., 2011). This effect appears more extensive than previously reported with the higher concentration of glycine and alanine used in the present study as demonstrated by the large particle size and the distinct morphology on the SEM images. This suggests these mixtures are highly hygroscopic and sensitive to moisture, and may not even form as discrete particles from the spray-dryer. In general, this work suggests multiple combinations of small molecule excipients appear more fused and hence hygroscopic than the individual components, and this will be further studied.

The addition of leucine appeared to facilitate particle formation in all the spray-dried formulations. The spherical morphology of spray-dried mannitol is maintained upon addition of leucine up to 20% w/w. With leucine 10% w/w present in the mannitol/glycine and mannitol/alanine formulations, distinct spherical individual particles are evident on the SEM images. However, in contrast to the mannitol formulations, a more wrinkled surface becomes evident when leucine at the increased

level of 20% w/w was present in these combination systems. It may be of interest to note that, in principle, such changes in surface corrugation could either improve dispersibility by reducing contact points between particles, or in contrast reduce dispersibility by an increased interlocking or entrapment phenomena. From the observed small degree of increase in surface corrugation from leucine 10% to 20% w/w, an example of mechanical interlocking/entrapment is evident from the presence of smaller particles embedded in the grooves of some of the larger wrinkles in formulations containing higher concentrations of leucine: as shown in the SEM images. However, on balance it appears that the benefit of reduced contact area outweighed adverse effect resulting interlocking at the higher concentration of leucine > 20% w/w on particle dispersibility, as Table 3 showed MAL404020 and MGL404020 had better dispersion and aerosolisation than MAL454510 and MGL454510. Interestingly, on examination of the mannitol/trehalose formulations, in contrast to the mannitol/glycine and mannitol/alanine formulations, leucine 10% w/w appeared insufficient to achieve good particle formation or maintained particle isolation after production. Increasing leucine concentration to 20% w/w was necessary to provide clear particle separation in these SEM images. Spray-dried trehalose exhibited similar morphology on SEM images to the mannitol/trehalose formulation as partially fused particles remained evident upon addition of leucine 10% w/w. Leucine 20% w/w was required to achieve clear particle separation again in these SEM images. However, the Mastersizer D_{50} of trehalose with leucine 10% and 20% w/w are not significantly different (p = 0.74 and 0.26 respectively) suggesting the partial fusion of these particles with leucine 10% w/w may either be occurring during the SEM sample preparation or alternatively may be reversible upon dispersion. In general, further increase in leucine concentration above 20% w/w produced increasingly wrinkled particles. On examination of the

mannitol/glycine/leucine and mannitol/alanine/leucine formulations containing leucine 33% w/w, the particles appear to be highly wrinkled. Similarly, the mannitol and trehalose formulations with leucine 50% w/w produced particles with comparable wrinkled surfaces.

While previous studies have established the aerosolisation enhancing effect of leucine when used in relatively low concentrations (0.3 to 15% w/w) in dry powder formulations, the modification of particle morphology in response to higher concentrations of leucine has been suggested but not thoroughly investigated (Li et al., 2003; Lucas et al., 1999; Morton and Kamlag, 2005; Seville et al., 2007). In the present study, a transition of morphology upon increasing concentration of leucine from apparent insufficiency to protect particles from moisture induced fusion, to optimal particle separation, to increased surface wrinkles is observed in these formulations. The results show that leucine at 10 or 20% w/w is sufficient to assist particle formation and higher concentrations of leucine beyond 20% w/w appear to provide little benefit in terms of surface moisture protection and particle separation. It has been suggested that at increasing concentrations, leucine produced wrinkled particles resulted from the concentration and precipitation at the surface of drying droplets, forming a partially impervious and rubbery outer shell layer, which then inflates from the internal water vapours on further evaporation, and then subsequently collapses with the wrinkled effect once all vapour is exhausted and the particle is dry (Vehring, 2008; Walton and Mumford, 1999). It is proposed that further investigations using techniques e.g., X-ray photoelectron spectroscopy (XPS) or time-of-flight secondary ion mass spectroscopy (ToF-SIMS) to study surface composition of leucine on these wrinkled particles will be beneficial in confirming this. In particular, it would also be valuable to further assess

the molecular arrangement at the surface, using e.g., X-ray scattering, especially small-angle X-ray scattering (SAXS).

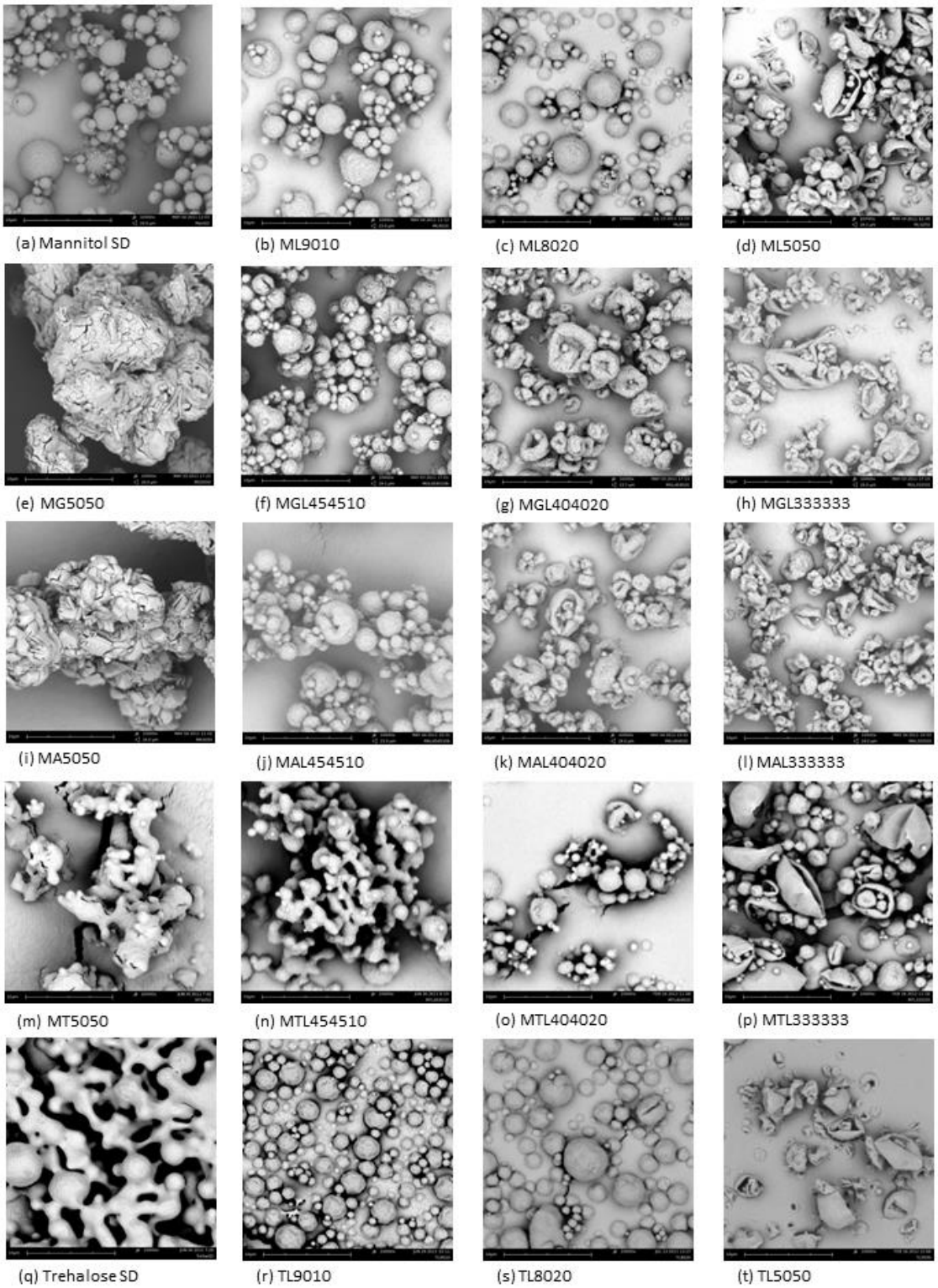
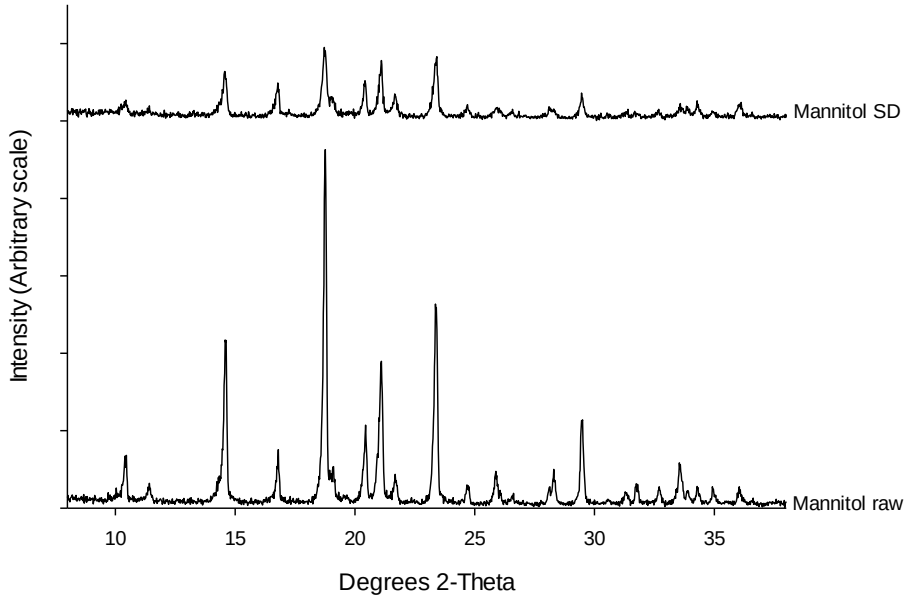


Figure 2. Representative scanning electron micrographs of spray-dried (a) mannitol (Mannitol SD), (b) mannitol/leucine 90/10% w/w (ML9010), (c) mannitol/leucine 80/20% (ML8020), (d) mannitol/leucine 50/50% w/w (ML5050) , (e) mannitol/glycine 50/50% w/w (MG5050), (f) mannitol/glycine/leucine 45/45/10% w/w (MGL454510), (g) mannitol/glycine/leucine 40/40/20% w/w (MGL404020), (h) mannitol/glycine/leucine 33/33/33% w/w (MGL333333), (i) mannitol/alanine 50/50% w/w (MA5050), (j) MAL454510% w/w (MAL454510), (k) mannitol/alanine/leucine 40/40/20% w/w (MAL404020), (l) mannitol/alanine/leucine 33/33/33% w/w (MAL333333), (m) mannitol/trehalose 50/50% w/w (MT5050), (n) mannitol/trehalose/leucine 45/45/10% w/w (MTL454510), (o) mannitol/trehalose/leucine 40/40/20% w/w (MTL404020), (p) mannitol/trehalose/leucine 33/33/33% w/w (MTL333333), (q) trehalose (Trehalose SD), (r) trehalose/leucine 90/10% w/w (TL9010), (s) trehalose/leucine 80/20% w/w (TL8020) and (t) trehalose/leucine 50/50% w/w (TL5050) [magnification: x10000].

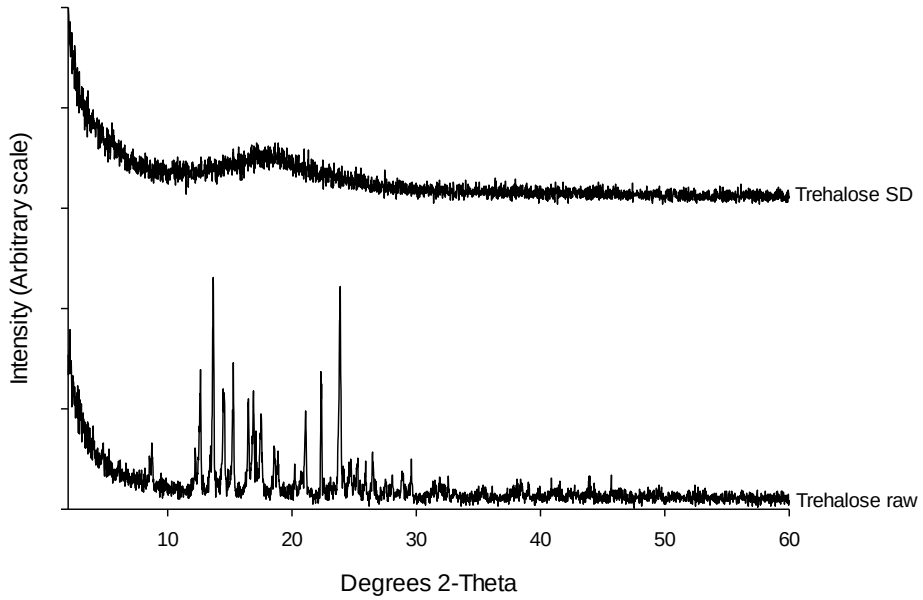
3.3.4. Crystallinity

The XRPD diffractograms from the raw and spray-dried mannitol, trehalose and leucine immediately after spray-drying are shown in Figure 3a-c. The profiles show that the raw mannitol, trehalose and leucine were in crystalline form as received from the manufacturer. While crystalline peaks of spray-dried mannitol are evident from the diffractogram, spray-dried trehalose appears to be largely amorphous as shown by the amorphous halo and absence of distinct crystalline peaks in the diffractogram. The results are consistent with our expectation given the relatively low T_g of mannitol of 11 ºC and high T_g of trehalose of 117 ºC reported (Weers et al., 2007). Three polymorphic forms of crystalline mannitol have been previously reported with unique patterns and peaks on their XRPD diffractograms (Hulse et al., 2009; Lee et al., 2011; Sharma and Kalonia, 2004). The spray-dried mannitol produced using the specified conditions in this study consistently formed the beta polymorph.

(a)


Intensity (Arbitrary scale)
Mannitol SD
Mannitol raw
10
15
20
25
30
35
Degrees 2-Theta

(b)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Trehalose SD
Trehalose raw
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20
30
40
50
60
Degrees 2-Theta

(c)

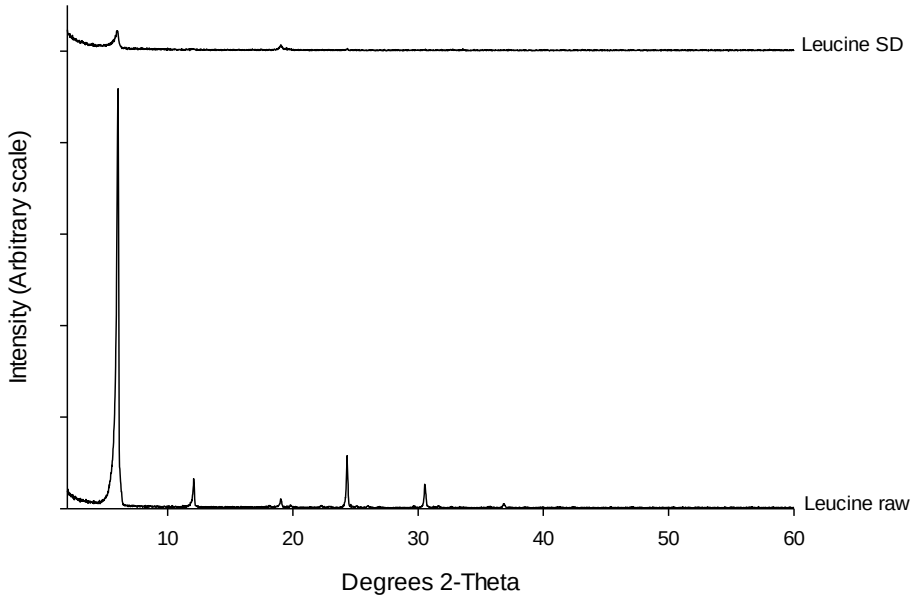

Figure 3. XRPD profiles of raw and spray-dried (SD) (a) mannitol, (b) trehalose and (c) leucine.

Interestingly, we note that the spray-dried leucine exhibits no obvious evidence of amorphous structure but instead only two of the original six diffraction peaks (6.00º and 19.04º 2θ) are evident when compared to the raw material leucine which is clearly crystalline. It is proposed here that the spray-dried leucine, rather than reforming its fully crystalline structure as recently suggested (Feng et al., 2011), instead exhibits a partially ordered molecular arrangement formed as a result of the drying crust, which has strong two dimensional layered order but where complete three dimensional order is not fully achieved. This partially ordered "liquid crystalline"-like status is proposed to result from a lamellar self-assembly of the leucine during droplet drying but has not been previously reported. Furthermore, it is noted that the main peak at 6° 2θ, noted here as the "primary spray-dried leucine peak", corresponds to a d-spacing of 1.47 nm

as derived by using Bragg's law. The unit cell dimension reported for crystalline leucine that corresponds to 2 leucine molecules lying back-to-back in a "lamellar" form is indeed 1.47 nm (Coll et al., 1986). This finding provides robust support for our self-assembly hypothesis.

The XRPD profiles of the formulations containing mannitol and other excipients in a 1:1 ratio are shown in Figure 4. In our previous study, the lower amount of 11–12% w/w of glycine and/or alanine employed did not prevent recrystallisation of the mannitol formulations (Sou et al., 2011). It is worth noting that a broad but intense peak at 6º 2θ is observed from the mannitol/leucine formulation. This peak is strongly suggested to be the primary spray-dried leucine peak and is not present in any other formulations without leucine. The 6º 2θ primary spray-dried leucine peak is evident in all formulations containing leucine (Figures 5-11). The powder XRD is treated here as a qualitative assessment and can provide some semi-quantitative indications in the sense that it allows some comparisons between families of formulations. The 6º 2θ peak appears to become relatively larger as the content of leucine increases. In this context, a possible exception is the trehalose/leucine 90/10% w/w in which the leucine peak is not as fully distinctive, but it is more evident in other combinations at a presence of leucine 10% w/w (Figure 5). The peak is clearly evident in all samples comprising leucine 20% w/w (Figure 6). It is speculated that the trehalose/leucine 90/10% w/w formulation has not achieved an as well-formed leucine surface structure by self-assembly and this is consistent with its noted relative observed fusion. The results also appear to correlate with the observed morphology and particle size studies which had shown that 10% w/w leucine was in some cases insufficient and 20% w/w leucine was necessary for optimal moisture protection and particle separation. Therefore, it is proposed that in the context of the spray-drying conditions in this study, a coherent partially ordered leucine crust

residing on the surface of the particle as detected by XRPD appears to be necessary for leucine to function as a particle formation, moisture protection and aerosolisation enhancing agent effectively. Excipients that affect the assembly of leucine residing on the surface will hence influence the effectiveness and therefore the concentration of leucine required for particle formation.

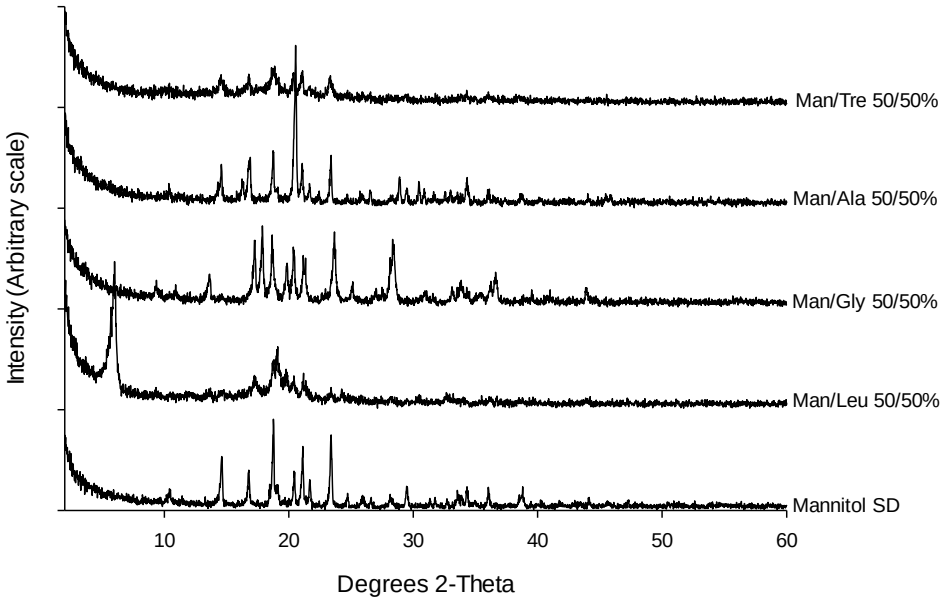

Figure 4. XRPD profiles of spray-dried (SD) mannitol, mannitol/glycine (Man/Gly) 50/50%, mannitol/alanine (Man/Ala) 50/50%, mannitol/leucine (Man/Leu) 50/50% and mannitol/trehalose (Man/Tre) 50/50% w/w.

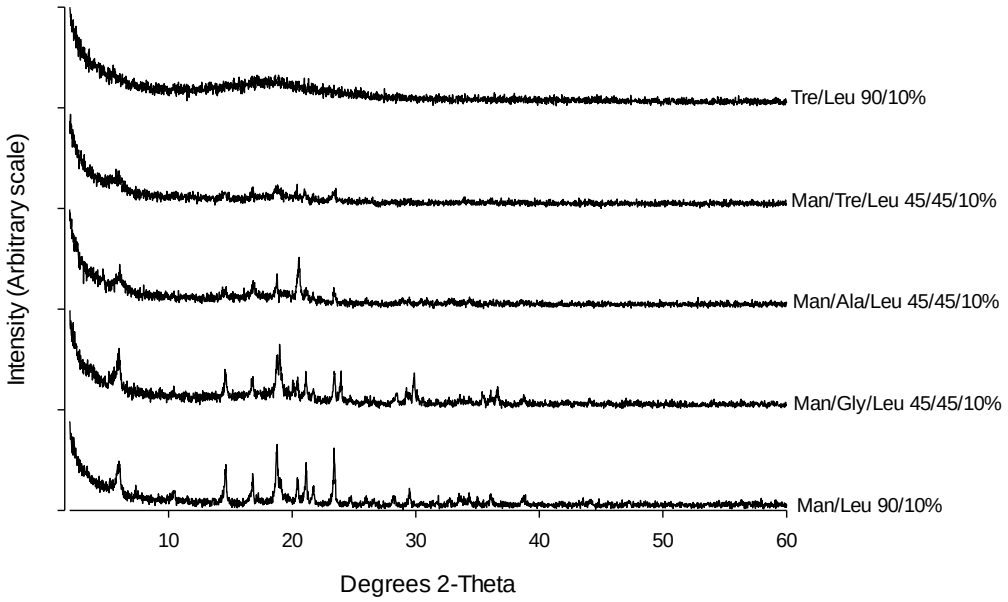

Figure 5. XRPD profiles of spray-dried mannitol/leucine (Man/Leu) 90/10%, mannitol/glycine/leucine (Man/Gly/Leu) 45/45/10%, mannitol/alanine/leucine (Man/Ala/Leu) 45/45/10%, mannitol/trehalose/leucine (Man/Tre/Leu) 45/45/10% and trehalose/leucine 90/10% w/w.

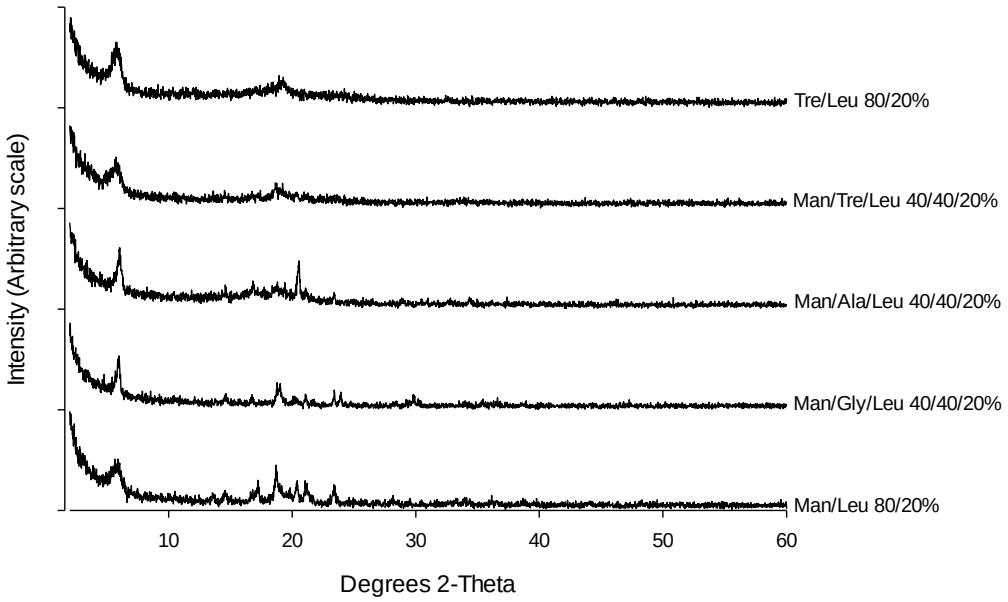

Figure 6. XRPD profiles of spray-dried mannitol/leucine (Man/Leu) 80/20%, mannitol/glycine/leucine (Man/Gly/Leu) 40/40/20%, mannitol/alanine/leucine (Man/Ala/Leu) 40/40/20%, mannitol/trehalose/leucine (Man/Tre/Leu) 40/40/20% and trehalose/leucine 80/20% w/w.

The XRPD profiles of the baseline combinations in response to increasing leucine concentration are shown in Figures 7–11. There is a suggestion from the results that the intensity of all other diffraction peaks of all the formulations appear reduced upon addition of leucine from 0–20% w/w. However, it should also be pointed out that an increase in leucine by mass will reduce the mass of mannitol and this could be a confounding factor that reduces the mannitol diffraction peak size. While the effect of leucine in improving aerosolisation and dispersibility has been demonstrated in a number of studies, the specific effect of leucine on material crystallinity and stabilisation of solid-state properties has not been previously established (Kamlag et al., 2004; Li et al., 2003; Lucas et al., 1999; Morton and Kamlag, 2005; Seville et al., 2007). The results of the present study suggest that the effect of these excipients in preventing

crystallisation of mannitol in these multi-component systems could possibly be dependent on the amount of leucine. The results also suggest that while leucine 10% w/w was sufficient in improving dispersibility of some of the dry powders, leucine 20% w/w might be more effective in reducing material recrystallisation, though this hypothesis would need further verification as the reduced amount of mannitol in these formulations could also reduce the intensity of these crystalline peaks. Further study is ongoing to more fully investigate this behaviour, examining if leucine alters T_g or possibly provides a moisture protecting self-assembled layer.

It has been previously suggested that it is the initial saturation and subsequent supersaturation of leucine in the drying droplet that determines whether the amino acid fully crystallises in the formulation (Vehring, 2008). It was proposed that leucine forms fully crystalline nuclei where their formation at selective concentrations is restrictive to diffusion, hence affects their residing on the particle surface as clusters and creating encapsulating structures (Feng et al., 2011). The results in the present study indicate an alternative mechanism as a result of self-assembly of leucine on the surface and its subsequent effect on the formation of a semi-impervious rubbery layer, dictating a dynamic particle formation, and explaining the wrinkled structures observed. It is further proposed that the presence of leucine in sufficient amount for self-assembly on the particle surface to form a coherent layer, in addition to assisting the formation of spray-dried particles suitable for pulmonary delivery, may also play a role in preserving the amorphous environment for stabilisation of biomacromolecules.

Finally, we also note that even with up to 50% w/w leucine, mannitol shows some evidence of recrystallisation. Glycine and alanine at high levels did not prevent mannitol recrystallisation. In contrast, trehalose remained largely amorphous from 0 to 50% w/w leucine content (Figure 11), suggesting that the trehalose/leucine mixture

appears an attractive combination for more extensive study. For the various combinations containing mannitol and leucine, the diffractograms were less conclusive but suggested a three component of mannitol/trehalose/leucine may be worth further study, although as noted earlier its high hygroscopicity may be a problem.

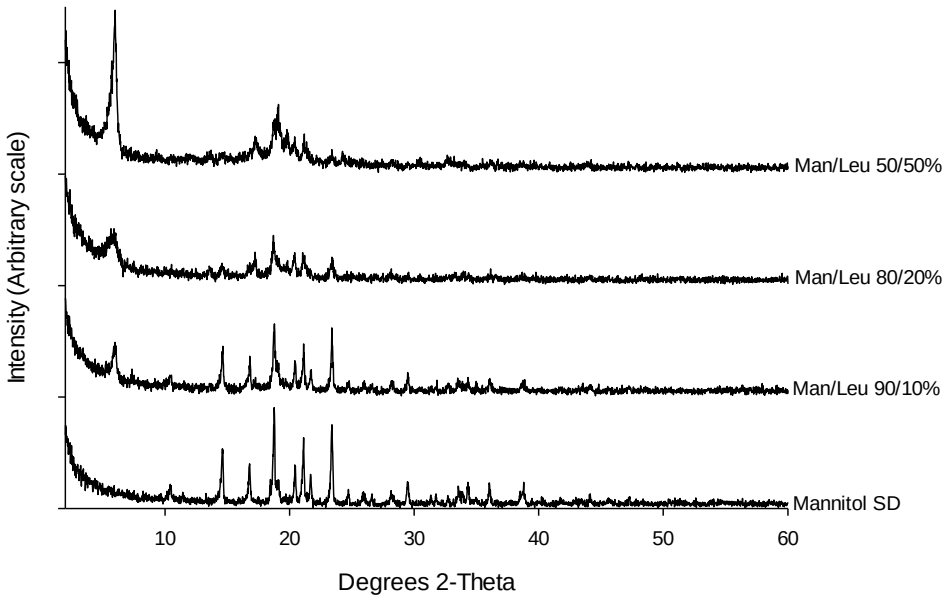

Figure 7. XRPD profiles of spray-dried (SD) mannitol (Mannitol SD) and mannitol/leucine (Man/Leu) in 90/10%, 80/20% and 50/50% w/w.

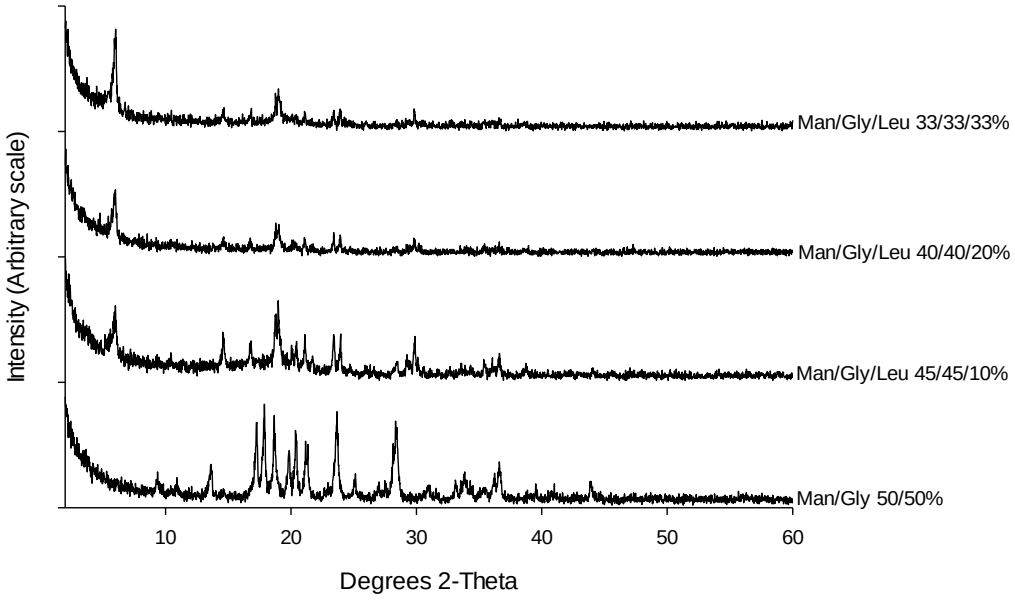

Figure 8. XRPD profiles of spray-dried (SD) mannitol/glycine/leucine (Man/Gly/Leu) in 50/50/0%, 45/45/10%, 40/40/20% and 33/33/33% w/w.

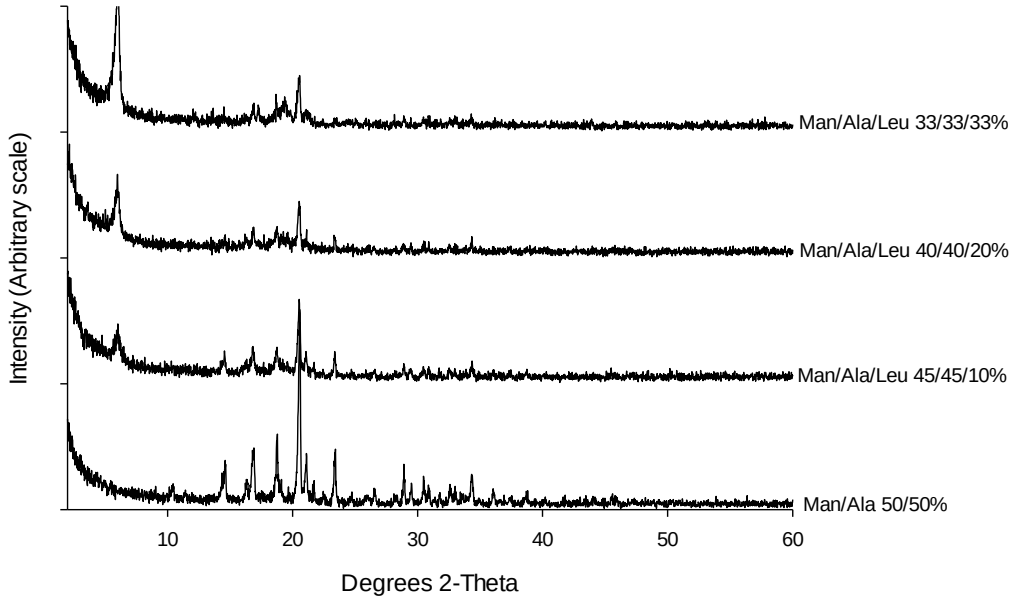

Figure 9. XRPD profiles of spray-dried (SD) mannitol/alanine/leucine (Man/Ala/Leu) in 50/50/0%, 45/45/10%, 40/40/20% and 33/33/33% w/w.

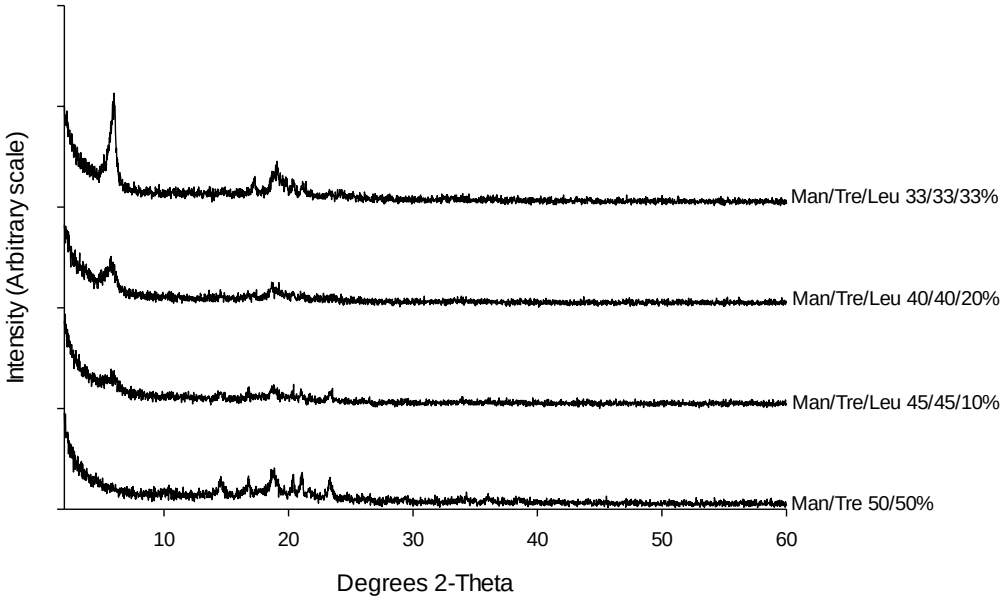

Figure 10. XRPD profiles of spray-dried (SD) mannitol/trehalose/leucine (Man/Tre/Leu) in 50/50/0%, 45/45/10%, 40/40/20% and 33/33/33% w/w.

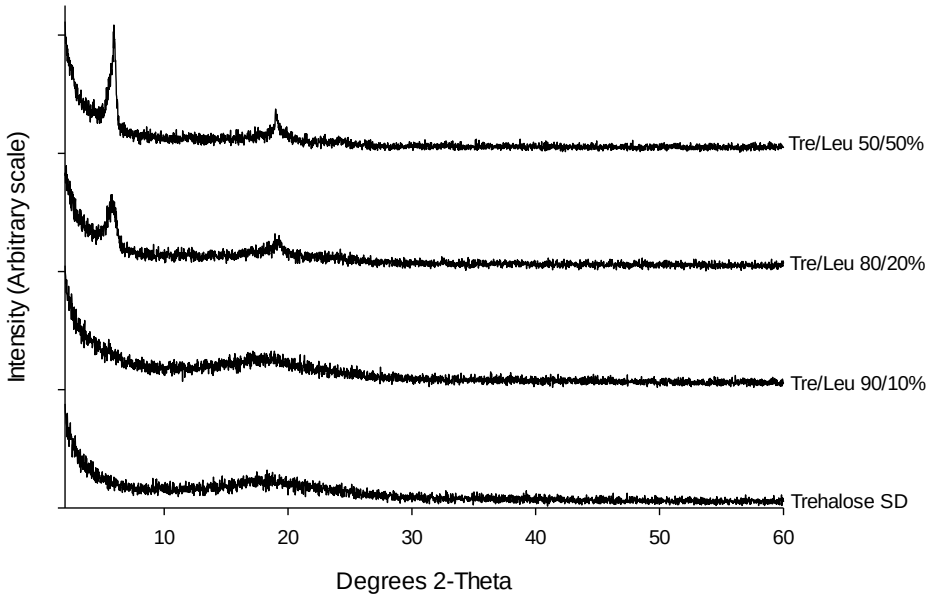

Figure 11. XRPD profiles of spray-dried (SD) trehalose (Trehalose SD) and trehalose/leucine (Tre/Leu) in 90/10%, 80/20% and 50/50% w/w.

3.4. Conclusion

This study has provided some important insights into particle engineering using amino acid and sugar excipients for the development of dry powder carrier platforms for pulmonary delivery of potent biomacromolecules. The results in the present study are consistent with our notion that leucine is able to facilitate particle formation as an encapsulating agent and therefore allows the production of multi-component particles while maintaining good particle size and dispersibility for pulmonary delivery that would otherwise not be possible. Furthermore, the result has also demonstrated that partially ordered leucine resulting from self-assembly on the particle surface is highly important for the amino acid to function effectively as an encapsulating agent, for optimal particle formation and might also play a role in inhibiting crystallisation of other components within the formulation. The results from the present study suggest that, with suitable particle size, good dispersibility and solid-state properties, the trehalose/leucine combination with leucine 10–20% w/w appears to have good potential for development into a universal carrier platform for pulmonary delivery of potent biomacromolecules and this deserves further investigation, especially with respect to moisture sensitivity and a thorough investigation of glass transitions. With the particle engineering strategies utilised in the present study, other baseline material such as other sugar molecules and polymers will be investigated in future studies.

3.5. Acknowledgements

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CHAPTER FOUR

DESIGNING A MULTI-COMPONENT SPRAY-DRIED FORMULATION PLATFORM FOR PULMONARY DELIVERY OF BIOMACROMOLECULES: THE EFFECT OF POLYMERS ON THE FORMATION OF AN AMORPHOUS MATRIX FOR GLASSY STATE STABILISATION OF BIOMACROMOLECULES

Monash University

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study initiation, experimental design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion.	90%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
Michelle P McIntosh	Supervision, manuscript revision
Lisa M Kaminskas	Supervision, manuscript revision
Richard J Prankerd	Manuscript revision
David AV Morton	Supervision, manuscript revision

Candidate's Signature [redacted]

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia

Michelle P McIntosh
Lisa M Kaminskas
Richard J Prankerd
David AV Morton

[redacted]

4. Designing a multi-component spray-dried formulation platform for pulmonary delivery of biomacromolecules: the effect of polymers on the formation of an amorphous matrix for glassy state stabilisation of biomacromolecules

Tomás Sou[a], Michelle P. McIntosh[a], Lisa M. Kaminskas[a], Richard J. Prankerd[a] and David A. V. Morton[a*]

[a] *Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia*

[*]Corresponding author. [redacted] (D.A.V. Morton)

4.0. Abstract

Non-reducing sugars such as mannitol are widely studied as excipients for spray dried pharmaceutical formulations. In contrast, the present study investigated the use of a range of polymers for production of an amorphous glassy carrier platform for pulmonary delivery of potent biomolecules. Two different natural polysaccharides, inulin and dextran, and the synthetic polymer PVP were combined with leucine using spray-drying. In addition, the effect of these in combination with mannitol was studied. The results showed that leucine was a very effective particle formation agent that substantially improved processing yields of the spray-dried polymer/leucine based formulations and formed high rugosity particles with high fine particle fractions. The work indicated the potential utilities of these multi-component systems as a novel dry powder formulation platform for pulmonary delivery of various biomacromolecules.

4.1. Introduction

Dry powder inhalation for pulmonary delivery of biomacromolecules has recently gained increased interest, due to its many advantages including direct lung targeting for local effects, convenient portability, relatively low cost and inherent solid state stability (Carpenter et al., 1997; Prime et al., 1997; Rave et al., 2004). Spray-drying is an attractive method for production of inhalable dry powder formulations due to its adaptability, cost-effectiveness, potential for complex particle engineering and scalability (Fourie et al., 2008). Spray-drying is a process in which the compound(s) of interest are first prepared in a liquid form, which is then sprayed into a drying chamber where the droplets are dried with heated air. Particle formation is achieved by precipitation of the dissolved compounds as the solvent evaporates from the solution droplets in the drying chamber. The ability to incorporate various ingredients in a single step manufacturing process is a powerful strength of spray-drying. Particles can be formulated to contain various ingredients by adjusting the content and nature of the feed solution. Excipients can therefore be incorporated to engineer the properties of the dry powder formulation.

Depending on the characteristics of the compound(s) and the operating conditions used, some compound(s) are intrinsically difficult to process into aerosolisable dry powders by spray-drying. For instance, viscous solutions of compound(s) e.g., polymers can produce unacceptably low yields of 0.5% to 10% after spray-drying where significant levels of the compounds are retained on the wall of the spray-dryer during the drying process. Some hygroscopic compound(s) can produce particles with an unacceptably large particle size distribution for pulmonary delivery due to fusion of primary structures during or after the drying process (Sou et al.; Sou et

al., 2011). Strategies to improve the spray-drying performance and processing yields of these compounds with the addition of appropriate excipients are therefore being actively pursued. The inclusion of L-leucine in the feed solution has been demonstrated to assist production of inhalable mannitol dry powder formulations containing multiple components, e.g., glycine and alanine, whether alone or in combination, while preserving the aerosolisation properties of the powders. These multi-component dry powder formulations were poorly aerosolisable in the absence of leucine (Sou et al., 2011). Similar formulation strategies may be transferrable to the production of other spray-dried powders with different baseline materials that would otherwise be too hygroscopic to be spray-dried.

For a dry powder formulation to be suitable for pulmonary delivery of potent biomacromolecules such as proteins, it has to be not only efficiently and reproducibly aerosolisable, but also ideally capable of stabilising the biomacromolecules at room temperature. Strategies to produce spray-dried powder formulations with predominantly amorphous content are therefore being investigated. According to the glassy dynamics theory, an amorphous glassy solid should provide a highly viscous "vitreous" environment which restricts the molecular mobility of biomacromolecules and degradants such as water or oxygen, thereby stabilising proteins in a dry solid state (Chang and Pikal, 2009; Weers et al., 2007). Any excipient combination used should ideally be a good glass former with a high glass transition temperature (T_g) but otherwise inert. Formulation strategies to produce an amorphous delivery system with high aerosolisation performance are therefore being investigated.

Mannitol is a non-reducing sugar with the advantage that it will not undergo Maillard reaction with proteins. Mannitol is classified as a non-hygroscopic solid and its use as a stabilising excipient in dried protein formulations have been widely reviewed

(Bakaltcheva et al., 2007; Kibbe, 2000; Schüle et al., 2007; Torrado, 2002). Mannitol in the amorphous state has been shown to be effective in stabilising spray-dried proteins (Costantino et al., 1998). However, amorphous mannitol has a relatively low T_g of 11 ºC and therefore tends to recrystallise shortly after spray-drying as a foundation material alone (Sou et al., 2011; Weers et al., 2007). The detrimental effect of mannitol crystallisation on protein stability has been previously demonstrated (Costantino et al., 1998). Most formulations examined in previous studies contained a relatively high protein to mannitol ratio (50% to 90% w/w of active protein present in the formulations) which inhibits crystallisation of mannitol (Andya et al., 1999; Costantino et al., 1998; Hulse et al., 2008; Maa et al., 1997; Schule et al., 2008; White et al., 2005). These systems may not be ideal for more potent active proteins, such as vaccine antigens, for which only a small amount of the active protein is required in the formulation, as the low proportion of protein present, i.e., < 1% w/w protein loading, cannot be relied upon to maintain the glassy matrix critical for physical stability of the formulation. Since the small amount of protein loading may be considered unlikely to impact on the aerosolisation behaviour of the carrier platform, optimisation of the intrinsic performance of the delivery system before addition of an active biomacromolecule is therefore fundamentally important.

The effect of the polysaccharide inulin in stabilising dried protein formulations has been widely studied in the pharmaceutical and food industries (Amorij et al., 2007; de Jonge et al., 2007; Rodríguez Furlán et al., 2011; Rodríguez Furlán et al., 2010; Zijlstra et al., 2009). While spray-dried inulin has been shown to be amorphous and can be used to provide an amorphous matrix for protein stabilisation, the resultant particles have been relatively large (particle sizes >10 µm), due to fusion of the drying droplets as evident from their morphology (Ronkart et al., 2007). These relatively large spray-

dried particles are unlikely to be suitable for respiratory delivery without addition of further excipients. Polyvinylpyrrolidone (PVP) has been previously co-spray-dried with various low water solubility active pharmaceutical ingredients (APIs) to maintain these small molecules in the amorphous state as a strategy to improve their oral bioavailability (Caron et al., 2011; Gupta and Bansal, 2005). It is proposed that similar strategies may be employed to produce an amorphous matrix to stabilise protein pharmaceuticals for pulmonary delivery, provided formation of small particles of the spray-dried product can be suitably controlled with the addition of appropriate excipient. Dextran is a polysaccharide with a reported T_g of 83 °C and has been selected for comparison as it has properties similar to other hydrophilic polymers such as inulin and PVP (Weers et al., 2007).

In contrast to the respiratory delivery of many small molecule drugs which is commonplace *via* simple ordered mixtures, a well-characterised delivery platform that is readily adaptable to incorporation of various biomacromolecules is lacking. The present study is an extension of previous work aiming to investigate the feasibility of a universal amorphous glassy carrier platform for pulmonary delivery of potent biomacromolecules. The present approach uses spray-drying to combine excipients with potentially multiple functionalities (mannitol, inulin, dextran or PVP) and added leucine to facilitate particle formation. In particular, this study focused on the influence of these polymeric compounds on spray-drying yield, the formation of an amorphous glassy matrix after co-spray-drying with mannitol and leucine and the aerosolisation performance of these systems.

4.2. Materials and methods

4.2.1. Materials

D-mannitol, inulin (from dahlia tubers), povidone K30 (PVPK30), L-leucine and glycine were obtained from Sigma-Aldrich Chemicals (Castle Hill, NSW, Australia). Dextran (Dextran T10) was obtained from Pharmacosmos (Holbaek, Denmark).

4.2.2. Preparation of spray-dried powders

Aqueous solutions containing the formulations in various compositions were dissolved in 200 mL of Milli-Q water. The prepared formulations were subsequently spray-dried using a Buchi 190 mini spray-dryer with a 0.5 mm two-fluid nozzle, using the following standard operating conditions: airflow, 800 L/h; pump setting, 5 (6.67 mL/min); aspirator setting, 20 (-84 mbar) (Saluja et al., 2010) ; outlet temperature, 70 °C. The compositions of the formulations are shown in Table 1. The processing yields were defined as the percentage of the mass of spray-dried powder recovered (Mrecovered) compared to the mass of total solid loading (Mtotal) in the initial feed solution as shown in Equation 1.

$$Yield\ \% = \frac{M_{recovered}}{M_{total}} \times 100 \quad (1)$$

Table 1. Compositions (% w/w) and processing yields (%) of the spray-dried formulations in the study.

Powder	Inulin	Dextran	PVP	Mannitol	Leucine	Yield (%)
1	100	--	--	--	--	59.5
2	90	--	--	--	10	71.5
3	80	--	--	--	20	67.4
4	--	100	--	--	--	47.2
5	--	90	--	--	10	74.7
6	--	80	--	--	20	71.9
7	--	--	100	--	--	32.2
8	--	--	90	--	10	67.9
9	--	--	80	--	20	70.1
10	50	--	--	50	--	28.6
11	45	--	--	45	10	68.3
12	40	--	--	40	20	71.6
13	33	--	--	33	33	74.1
14	--	--	50	50	--	0.0
15	--	--	45	45	10	42.8
16	--	--	40	40	20	66.5
17	--	--	33	33	33	62.4

4.2.3. X-ray powder diffraction (XRPD)

Sample powders were sprinkled onto a quartz sample plate smeared with a thin layer of Vaseline at room temperature. The sample was then analysed by the X-ray diffractometer (Philips 1140 vertical diffractometer, Philips, Holland) for scanning from 2 to 60° 2θ, with an angular increment of 2°/min. The crystalline status of the powders was assessed qualitatively by examination of the resulting diffraction patterns.

4.2.4. Scanning electron microscopy (SEM)

The morphology of the particles was visualised under a scanning electron microscope (Phenom™, FEI company, USA). Powder samples were gently poured onto a double-sided carbon tape mounted on a sample holder for examination under the SEM. Excessive powder was removed by gentle tapping and air blow to leave a fine layer of particles on the surface of the tape. The samples were sputter coated with gold using an electrical potential of 2.0 kV at 25 mA for 6 minutes with a sputter coater (K550X, EMITECH). SEM micrographs were captured using the in-built image capturing software.

4.2.5. In vitro powder aerosolisation study

The in vitro powder aerosolisation performance was determined using a modified abbreviated Anderson Cascade Impactor (ACI) system configured to simulate the human respiratory tract (HRT) as described and validated previously (Mitchell et al., 2010). The Monodose inhaler (Miat S.p.A., Milan, Italy) was used as the aerosol dispersion device. Briefly, the abbreviated ACI configuration consisted of, from top to bottom, the throat piece, pre-separator, stage 0, a full metal plate coated with a surfactant (Brij-35), stage F containing a filter paper and elastomer gasket, and the rest of the stages. The cut-off aerodynamic diameter of powders deposited on the filter paper using this configuration at a flow rate of 90 L/min is approximately 4.7 μm according to the manufacturer. Approximately 20 mg of samples were weighed and filled into size 3 HPMC capsules (Capsugel, Peapack, NJ, USA) for the tests which were performed in an air-conditioned laboratory (at 20 ± 3 °C, 40 ± 5% relative humidity). Each capsule

was actuated from the inhaler over 6 s for each measurement (n = 3). Fine particle fraction (FPF) was calculated as a percentage of the emitted dose (ED). The ED and amount of powder deposited on the filter paper were determined gravimetrically, an approach which is valid as no separate carrier particles were used in this formulation, and because stage loadings were sufficient to allow suitable measurement.

4.2.6. Statistical analysis

Statistical analysis was performed, where applicable, with SPSS (IBM SPSS Statistics, Version 19, SPSS Inc., USA), using a two-tailed Student's t-test at $p = 0.05$.

4.3. Results and discussion

4.3.1. Effect of excipients on processing yields of the spray-dried formulations

The spray-drying yields of the formulations are shown in Table 1. The yields of inulin, dextran and PVP particles with no other excipients were relatively low, ranging from 32.2% to 59.5%. This was not surprising due to the increased viscosity of the polymer feed solutions. It should be noted that inulin did not completely dissolve in water, but instead formed a very fine opaque suspension. This might explain the relatively higher yield of inulin, compared to the other two polymers in the study, as these dissolved to form true solutions with a higher viscosity. Interestingly, the yields of co-spray-dried mannitol with either inulin or PVP were even lower than the polymer solutions, especially with the mannitol/PVP formulation, which was effectively not

recoverable. It is clear that inulin and PVP produced highly hygroscopic particles after co-spray-drying with mannitol.

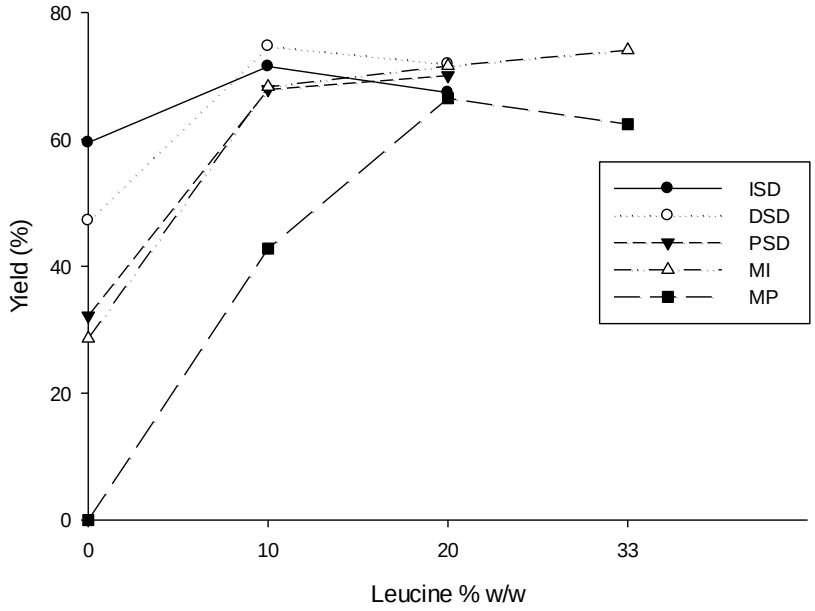

Figure 1. Processing yields (%) of spray-dried formulations in response to increasing concentrations of leucine: spray-dried inulin (ISD), spray-dried dextran (DSD), spray-dried PVP (PSD), spray-dried mannitol/inulin (MI) and spray-dried mannitol/PVP (MP).

The addition of leucine improved the processing yields of all spray-dried formulations (Figure 1). In general, the addition of leucine 10% w/w produced the most prominent yield improvements. Higher concentrations of leucine further improved the yields for some formulations only. The mannitol/polymer formulations were most subject to yield improvements upon addition of leucine, showing relative improvement of at least 139% (Table 2). The ability of leucine to improve spray-drying yield and provide a self-assembled surface coating on resulting particles has been previously demonstrated (Chen et al., 2012; Sou et al.; Tawfeek et al., 2011). The results indicated that the mannitol/polymer combinations were very hygroscopic and were very difficult

to spray-dry without additional excipients. In particular, addition of leucine assisted particle formation and encapsulated the very hygroscopic mannitol/PVP particles into collectable powders. These were non-recoverable from the spray-dryer in the absence of leucine. While the yields for most formulations reached a plateau of about 70% after addition of leucine 10% w/w, the mannitol/PVP formulation showed the greatest yield improvement at leucine 20% w/w before it reached a plateau. It appeared that leucine 20% w/w was required to sufficiently encapsulate the highly hygroscopic mannitol/PVP particles and suggested that this formulation was more hygroscopic than the others in the study.

Table 2. Relative improvement (%) of spray-drying yield in response to increasing concentration of leucine (% w/w) in the formulations.

Formulation	Leucine 10% w/w	Leucine 20% w/w	Leucine 33% w/w
Inulin/Leucine	20.2	13.3	n/a
Dextran/Leucine	58.3	52.3	n/a
PVP/Leucine	110.9	117.7	n/a
Mannitol/Inulin/Leucine	138.8	150.3	159.1
Mannitol/PVP/Leucine	∞	∞	∞

Abbreviations: n/a, not applicable; ∞, infinity.

4.3.2. Amorphicity of inulin, dextran and PVP after co-spray-drying with leucine

The XRPD profiles of inulin, dextran and PVP, both raw and after spray-drying with different concentrations of leucine, are shown in Figure 2a-c. They were largely amorphous, as shown by the typical amorphous halo on the XPRD profiles. Upon

addition of leucine, the spray-dried polymers remained amorphous while the peaks for leucine at 6° and 20° 2θ became increasingly prominent with increased concentrations of leucine. The existence of leucine peaks at 6° and 20° 2θ has been previously suggested to indicate the formation of a self-assembled leucine shell on the particle surface and is consistent with the particle coating effect shown in the present study (Sou et al., 2013). Interestingly, the apparent amorphicity of inulin seems to increase with increasing leucine content. Similar finding has been previously reported with spray-dried mannitol/leucine systems where increasing leucine content appeared to have reduced the crystallinity of mannitol and may be suggestive that the surface coating of leucine is capable of protecting the amorphous matrix (Sou et al., 2013). The leucine peaks were not clear when leucine 10% w/w was present in these polymer-based formulations, especially with the dextran/leucine system, in which the leucine peaks are hardly distinguishable at leucine 10% w/w. Similar observation has been previously reported with spray-dried trehalose/leucine combinations (Sou et al., 2013). The results suggested these amorphous formulations might have retarded complete surface self-assembly of leucine at 10% w/w and hence higher concentrations of leucine were required before the distinctive peak at 6° 2θ could be observed.

Polymeric compounds generally are characterised by a range of molecular weights and a consequent low tendency to crystallisation. Thus, these compounds are appealing candidates to form an amorphous matrix for glassy state stabilisation. Inulin, PVP and dextran have been previously spray-dried with proteins such as recombinant human deoxyribonuclease I and salmon calcitonin for inhalation (Schule et al., 2008; Tewes et al., 2010; Zijlstra et al., 2009). However, the properties of these polymers after co-spray-drying with leucine as standalone delivery platforms for more potent bio-therapeutics have not been previously reported. The results in the present study showed

that these polysaccharides appeared to behave similarly after co-spray-drying with leucine. Although the leucine peak at 6° 2θ was broad and hardly distinctive at leucine 10% w/w, higher concentrations of leucine did not appear to provide additional benefit regarding particle formation and yield improvement in these polymer-based systems.

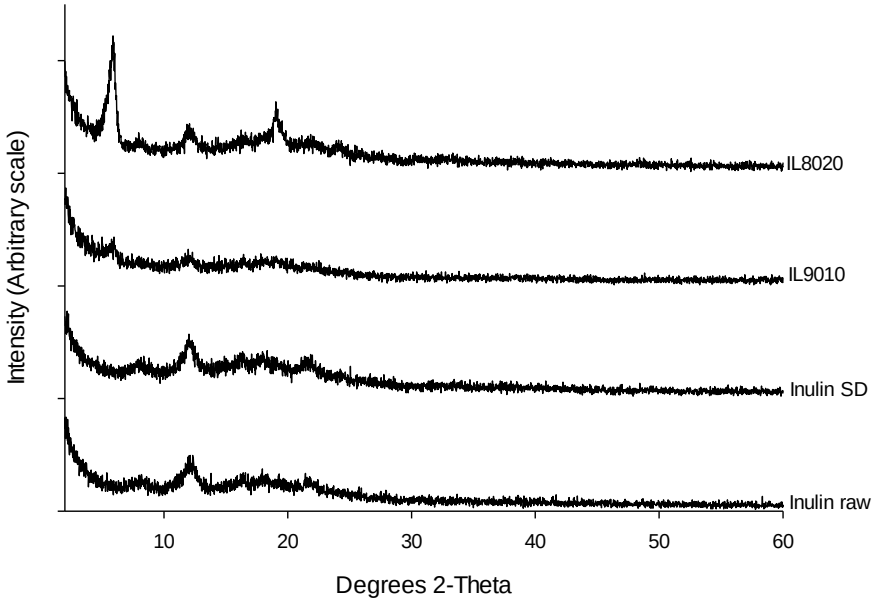

Figure 2a. XRPD profiles of raw inulin (Inulin raw) and spray-dried inulin (Inulin SD), inulin/leucine 90/10% w/w (IL9010) and inulin/leucine 80/20% w/w (IL8020).

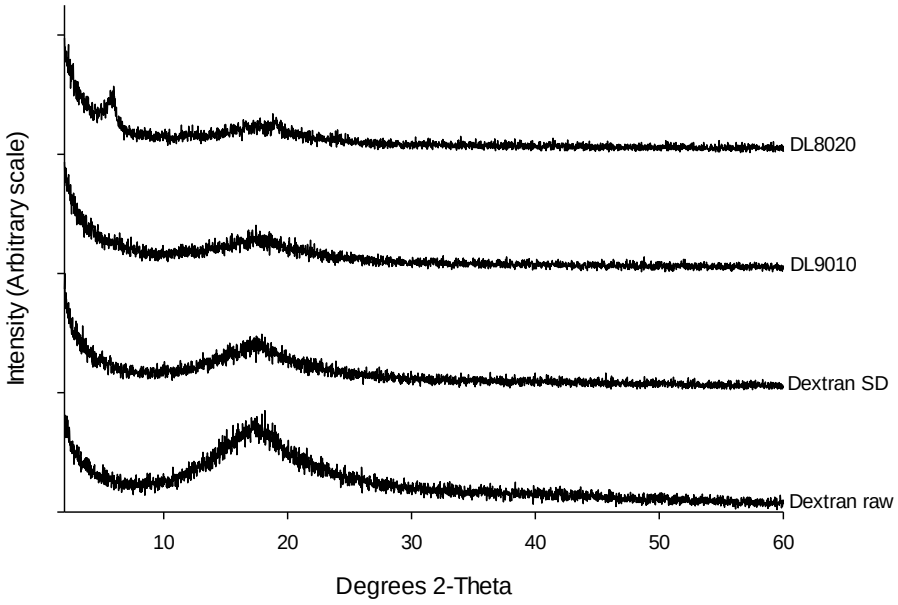

Figure 2b. XRPD profiles of raw dextran (Dextran raw) and spray-dried dextran (Dextran SD), dextran/leucine 90/10% w/w (DL9010) and dextran/leucine 80/20% w/w (DL8020).

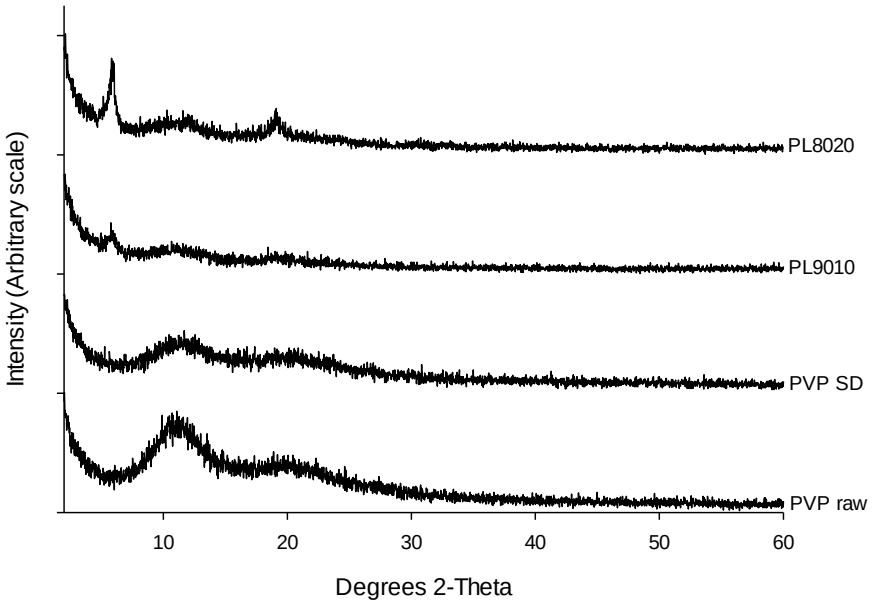

Figure 2c. XRPD profiles of raw PVP (PVP raw) and spray-dried PVP (PVP SD), PVP/leucine 90/10% w/w (PL9010) and PVP/leucine 80/20% w/w (PL8020).

4.3.3. Influence of inulin and PVP on the amorphous status of spray-dried mannitol

The XPRD profiles of the spray-dried mannitol/inulin and mannitol/PVP systems with different concentrations of leucine are shown in Figure 2d-e. The formulations are largely amorphous as demonstrated by the broad halo on the XRPD profiles. Although several low intensity peaks of crystalline mannitol were seen in the MI5050 formulation, these peaks diminished with the introduction of leucine in the formulation. Similar to the polymer/leucine systems, these mannitol/polymer based formulations remained amorphous while the peaks of leucine at 6° and 20° 2θ became increasingly prominent with higher concentrations of leucine. The results indicated that crystallisation of mannitol was retarded when co-spray-dried with these polymers. The hardly distinguishable leucine peaks at 6° 2θ from the XRPD profiles of the MIL454510 and MPL454510 formulations also indicated that self-assembly of leucine at 10% w/w might have been retarded by these amorphous systems, similar to the polymer-based formulations.

Previously, mannitol has been shown to be rapidly crystallised after spray-drying (Sou et al.; Sou et al., 2011). Small molecules (e.g., glycine, alanine, leucine, sodium citrate, sodium phosphate) and sugars (e.g., trehalose, sucrose, lactose) have been previously shown to be insufficient to inhibit crystallisation in the mannitol-based carrier systems without a large proportion of protein (60% to 90% w/w) in the formulations (Andya et al., 1999; Costantino et al., 1998; Ekdawi-Sever et al., 2003; Maa et al., 1997; Ohtake et al., 2004; Schule et al., 2008; Sou et al., 2011; White et al., 2005). The addition of amino acid excipients including glycine, alanine and leucine at high concentrations (> 60% w/w) did not inhibit the crystallisation of spray-dried

mannitol (Sou et al.; Sou et al., 2011). The results in the present study demonstrated that polymers such as inulin and PVP were much more effective in inhibiting the crystallisation of spray-dried mannitol. However, these combination systems containing amorphous mannitol were extremely hygroscopic and particle encapsulating agents such as leucine were required.

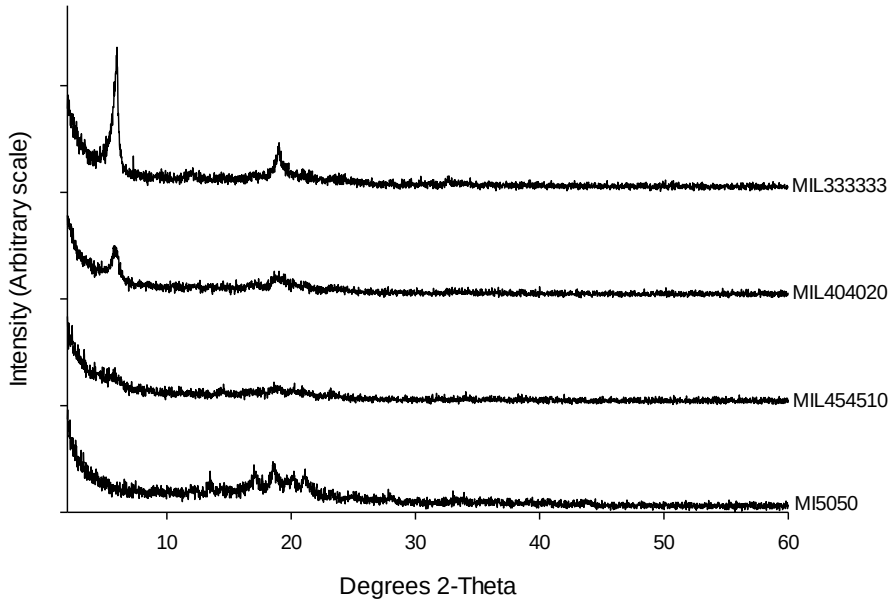

Figure 2d. XRPD profiles of spray-dried mannitol/inulin 50/50% w/w (MI5050) and spray-dried mannitol/inulin/leucine 45/45/10% w/w (MIL454510), 40/40/20% w/w (MIL404020) and 33/33/33% w/w (MIL333333).

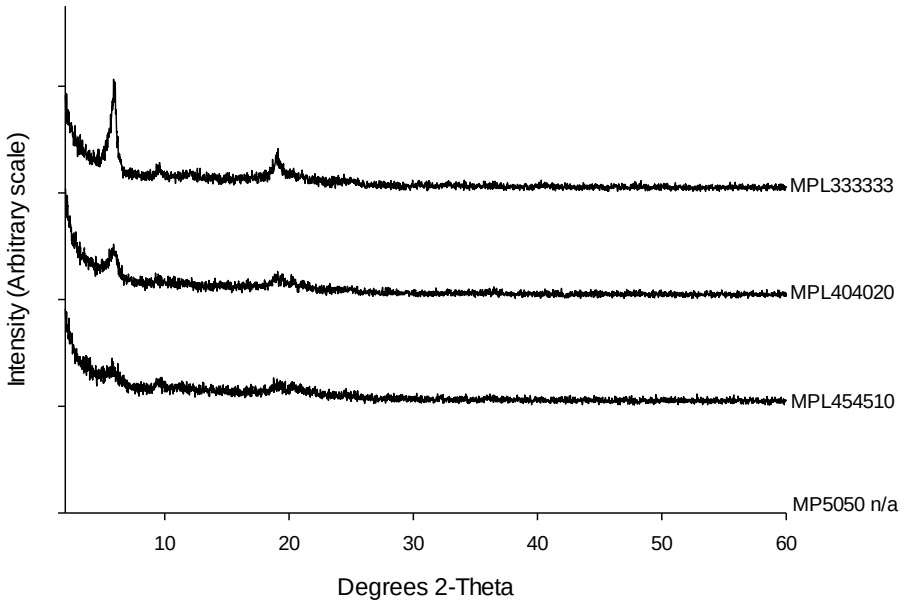

Figure 2e. XRPD profiles of spray-dried mannitol/PVP/leucine 45/45/10% w/w (MPL454510), 40/40/20% w/w (MPL404020) and 33/33/33% w/w (MPL333333). The result of spray-dried mannitol/PVP 50/50% w/w (MP5050) is not available (n/a) as the formulation could not be recovered from the spray-dryer.

4.3.4. Particle morphology and appearance

Scanning electron micrographs of the polymer/leucine formulations with different amounts of leucine are shown in Figure 3. These polymer-based formulations formed dimpled particles with prominent surface corrugations after spray-drying. When comparing the effect of different amounts of leucine on the morphology of various spray-dried polymer particles, the results showed that the amount of leucine at this concentration range did not substantially influence their morphology. The morphology of these spray-dried particles was largely dependent on the choice of polymer. While more subtle surface dimples were observed with inulin-based particles, more prominent surface wrinkles were observed with PVP-based particles. Dextran formed particles of intermediate morphology with more defined surface dimples than inulin but less evident surface wrinkles than PVP.

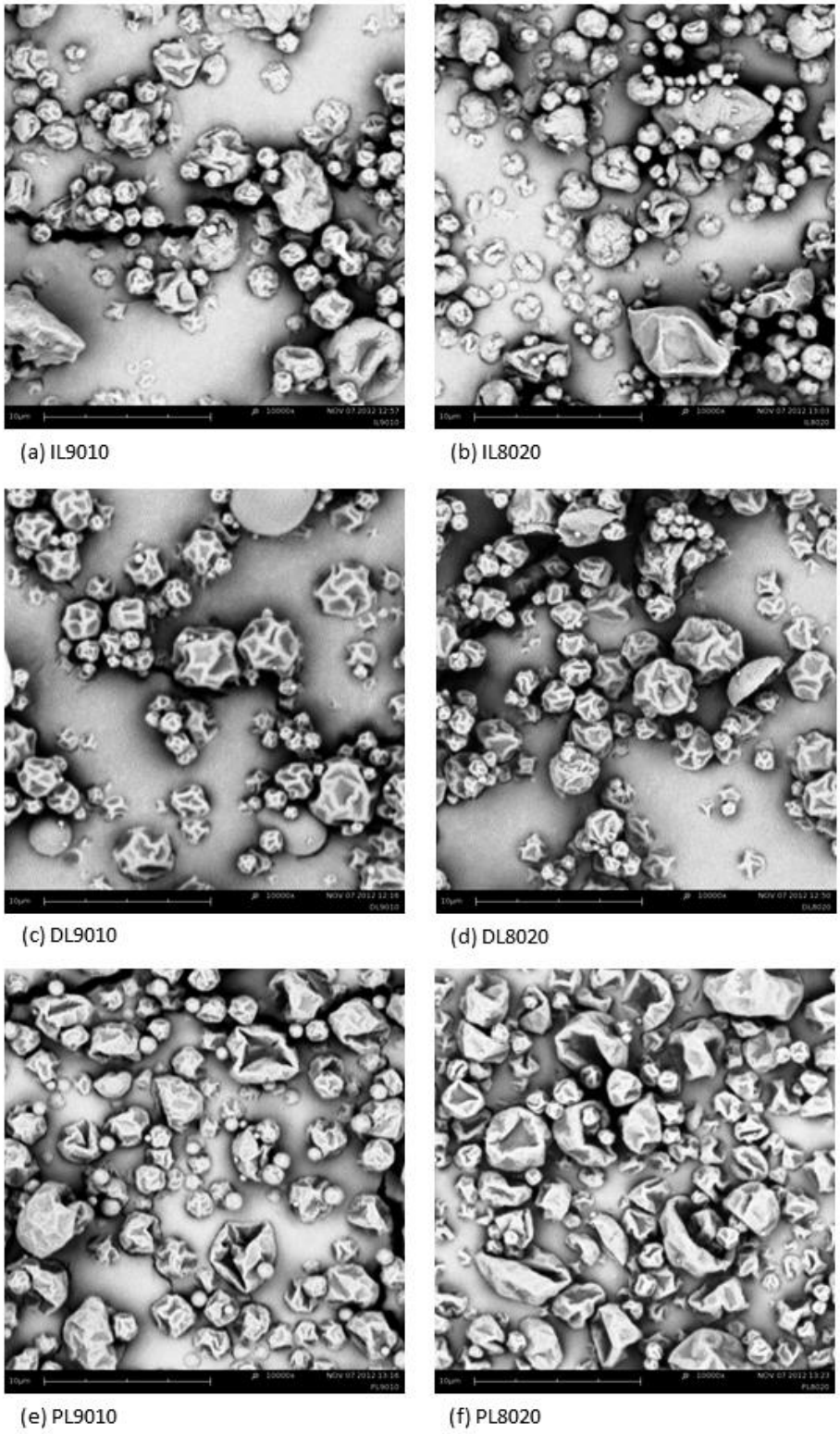

Figure 3. Representative scanning electron micrographs of (a) inulin/leucine 90/10% w/w (IL9010), (b) inulin/leucine 80/20% w/w (IL8020), (c) dextran/leucine 90/10% w/w (DL9010), (d) dextran/leucine 80/20% w/w (DL8020), (e) PVP/leucine 90/10% w/w (PL9010) and (f) PVP/leucine 80/20% w/w (PL8020).

Scanning electron micrographs of the mannitol/polymer/leucine formulations with different amounts of leucine are shown in Figure 4. In general, the results were consistent with our previous studies, in that the presence of leucine helped formation of these multi-component particles, with more surface corrugations and collapsed shells observed at higher concentrations of leucine (Sou et al.; Sou et al., 2011). While both polymers inhibited crystallisation of spray-dried mannitol, these amorphous mannitol-containing particles assumed very different morphologies depending on the polymer used. The PVP-based particles formed substantially more inter-particulate bridges that disappeared with increased leucine concentrations. This observation explained the low spray-dried yield of these particles and that a higher leucine concentration of 20% w/w was required before the maximum yield was achieved, as discussed above.

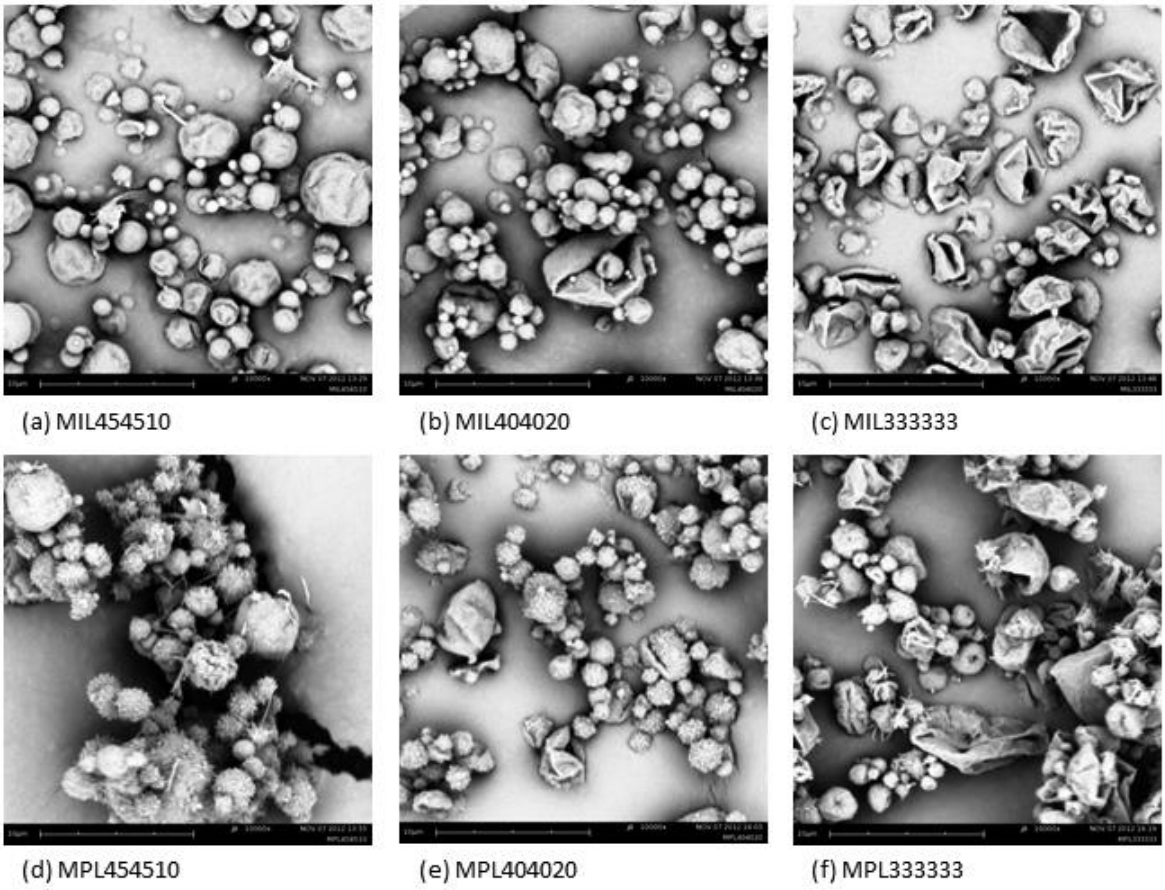

Figure 4. Representative scanning electron micrographs of (a) mannitol/inulin/leucine 45/45/10% w/w (MIL454510), (b) 40/40/20% w/w (MIL404020) and (c) 33/33/33% w/w (MIL333333) and (d) mannitol/PVP/leucine 45/45/10% w/w (MPL454510), (e) 40/40/20% w/w (MPL404020) and (f) 33/33/33% w/w (MPL333333).

4.3.5. In vitro powder aerosolisation and deposition

Given the amorphous nature and the more complete leucine coating as demonstrated by the XRPD profiles, the polymer/leucine 80/20% w/w formulations were selected for an in vitro aerosolisation study to examine their suitability as a pulmonary delivery platform. The fine particle fractions (FPFs) of these formulations are shown in Table 3. The three formulations demonstrated FPFs of 42% to 46%. Considering the FPFs of typical inhaled products are generally around 20% (Hindle and

Gad, 2010), these are relatively high aerosolisation performances which further indicate their suitability as a promising platform vehicle for pulmonary delivery.

Table 3. Fine particle fractions (FPFs, %) of the polymer/leucine 80/20% w/w formulations. (Mean ± s.d., n = 3)

Formulation	FPF% (<4.7 µm)
Inulin/Leucine 80/20% w/w	41.6 ± 4.5
Dextran/Leucine 80/20% w/w	42.4 ± 1.2
PVP/Leucine 80/20% w/w	45.5 ± 1.3

Spray-dried polymers such chitosan (a polyaminosugar) and polylactide-co-glycolide have been previously studied in the use of controlled-release formulations (Beck-Broichsitter et al., 2012; Hamishehkar et al., 2010; Pourshahab et al., 2011; Tawfeek et al., 2011). However, the utility of such spray-dried polymeric delivery systems for pulmonary delivery of biomacromolecules is less well-understood. PVP has previously been co-spray-dried with PEG to stabilise salmon calcitonin for pulmonary delivery (Tewes et al., 2010). The formulations used in that study, however, were only able to provide FPFs of about 30%. The spray-dried polymer systems in the present study produced substantially higher FPFs and appeared to be a more promising platform for pulmonary delivery.

4.4. Conclusion

The results in the present study revealed appealing properties of several novel co-spray-dried polymer/leucine delivery systems. These have the potential to be developed into a dry powder platform for pulmonary delivery of numerous different

biomacromolecules. While polymers are typically difficult to spray-dry, the addition of leucine as a particle formation agent has been shown to be a promising approach to substantially improve yield. The XRPD results suggested formation of a self-assembled leucine shell coating the surface of these amorphous particles, as previously reported. These polymer/leucine formulations gave particles with high surface rugosity; this assisted dispersion and was shown to be suitable for pulmonary delivery with high FPFs. In addition, these polymers effectively inhibited the crystallisation of the model sugar after co-spray-drying. However, these amorphous mannitol-containing particles were highly hygroscopic and recovery of these particles from the spray-dryer was unacceptably low without addition of leucine. SEM images revealed the differing morphologies of these particles, depending on the polymer incorporated, and the formation of extensive bridges between mannitol/PVP-based particles. Further investigations on the utility of these amorphous formulation platforms for pulmonary delivery of various biomacromolecules are being undertaken.

4.5. Acknowledgements

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CHAPTER FIVE

DESIGNING A MULTI-COMPONENT SPRAY-DRIED FORMULATION PLATFORM FOR PULMONARY DELIVERY OF BIOMACROMOLECULES: THE EFFECT OF POLYOL, DISACCHARIDE, POLYSACCHARIDE AND SYNTHETIC POLYMER ON THE SOLID-STATE PROPERTIES OF SPRAY-DRIED FORMULATIONS

Monash University

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study initiation, experimental design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion.	70%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
David AV Morton	Supervision, manuscript revision
Robert T Forbes	Thermal analysis advice
Jason Gray	X-ray powder diffraction study advice
Lisa M Kaminskas	Supervision, manuscript revision
Richard J Prankerd	Manuscript revision
Michelle P McIntosh	Supervision, manuscript revision

Candidate's Signature [redacted]

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia

David AV Morton
Robert T Forbes
Jason Gray
Lisa M Kaminskas
Richard J Prankerd
Michelle P McIntosh

[redacted]

5. Designing a multi-component spray-dried formulation platform for pulmonary delivery of biomacromolecules: the effect of polyol, disaccharide, polysaccharide and synthetic polymer on the solid-state properties of spray-dried formulations

Tomás Sou[a], David A. V. Morton[a], Robert T. Forbes[b], Jason Gray[b], Richard J. Prankerd[a], Lisa M. Kaminskas[a] and Michelle P. McIntosh[a]*

[a] *Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia*

[b] *PI Research Focus Group, School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK*

*Corresponding author. [redacted]

[redacted] (M.P. McIntosh)

5.0. Abstract

For a dry powder formulation platform to be suitable for pulmonary delivery of potent biomacromolecules e.g., proteins and peptides, it has to be not only efficiently and reproducibly aerosolisable, but also capable of stabilising the relevant biomacromolecules at temperatures appropriate for storage and distribution. This study systematically evaluated the use of excipient compounds covering a range of molecular sizes: i.e., from polyol (mannitol) and disaccharide (trehalose), to polysaccharide (inulin) and synthetic polymer (PVP), in conjunction with other small molecule excipients. It is recognised that larger molecular weight excipients with higher T_g values are less prone to recrystallization, however there is limited data around the potential for the inclusion of these compounds in inhalable dry powder delivery systems. The results demonstrated that polymer/leucine systems retained an appropriately high T_g in spite of the relatively high moisture content after spray-drying. The results also show that sodium citrate, in contrast to glycine and leucine, was effective in inhibiting crystallisation of spray-dried mannitol. The findings also demonstrated the synergistic benefits achieved from the concurrent use of several excipients on spray-dried mannitol which have not been previously reported: leucine as a particle formation agent, sodium citrate as a glass-forming agent, and glycine as a morphological modifier.

5.1. Introduction

Dry powder inhalation is an attractive delivery method for pulmonary delivery of biomacromolecules due to its many advantages over other delivery systems, including direct targeting for local effect, ease of administration, convenient portability, relatively low product cost and inherent stability in the solid state (Carpenter et al., 1997; Prime et al., 1997; Rave et al., 2004). Spray-drying is a favoured method for production of such inhalable dry powder formulations due to its adaptability, cost-effectiveness, scalability and potential for complex particle engineering (Fourie et al., 2008). Particles can be formulated to contain various ingredients by adjusting content of the feed solution and excipients can, therefore, be incorporated to engineer properties of the dry powder formulation. According to the glassy dynamics theory, an amorphous glassy solid should provide a highly viscous vitreous environment which restricts the molecular mobility of biomacromolecules and thereby stabilises proteins in a dry solid state (Chang and Pikal, 2009; Weers et al., 2007). Hence, excipient combinations should be selected to provide an amorphous matrix with high glass transition temperature (T_g), but otherwise inert.

Sugars and polyols are commonly used in the stabilisation of biomacromolecules due to their ability to form hydrogen bonds (Andya et al., 1999; Costantino et al., 1998; Maa et al., 1997; Sou et al., 2011a; Vehring, 2008). Mannitol is classified as a non-hygroscopic compound and the use of which as a stabilising excipient in dried protein formulations have been widely reviewed (Bakaltcheva et al., 2007; Kibbe, 2000; Schüle et al., 2007; Torrado, 2002). However, mannitol has a relatively low T_g of 11 ºC and therefore tends to recrystallise shortly after spray-drying (Sou et al., 2011b; Weers et al., 2007). Trehalose, which has a T_g of 117 ºC, tends to

form an amorphous glass after spray-drying and has been used to stabilise proteins upon storage in several studies (Jin et al., 2010; Maa et al., 1997; Ógáin et al., 2011). However, high concentrations of trehalose (i.e., >50% w/w) without additional excipients are not ideal for aerosol applications due to the adhesive nature, hygroscopicity and consequently high degree of particle agglomeration and poor aerosolisation of spray-dried trehalose (Maa et al., 1997).

Sugars (e.g., trehalose, sucrose and lactose) and other small molecules (e.g., glycine, alanine, leucine, sodium citrate and sodium phosphate) have previously been studied to modify solid-state properties of mannitol-based formulations (Andya et al., 1999; Costantino et al., 1998; Ekdawi-Sever et al., 2003; Maa et al., 1997; Ohtake et al., 2004; Schule et al., 2008; Sou et al., 2011b; White et al., 2005). However, most formulations examined in these studies contained relatively high proportions of protein ranging from 50% to 90% w/w (Andya et al., 1999; Costantino et al., 1998; Hulse et al., 2008; Maa et al., 1997; Schule et al., 2008; White et al., 2005). These formulations may not be ideal for highly potent proteins, such as vaccine antigens, since the maintenance of amorphous matrix critical for protein stability may differ with low protein concentration (e.g., < 1% w/w).

Unlike small molecules, polymeric compounds have, by definition, a distribution of higher molecular weights and as a result there is a lower tendency to crystallise. This makes these polymers appealing excipients to generate an amorphous delivery system. For example, spray-dried inulin has been shown to be amorphous (Ronkart et al., 2007). However, the resultant particles are relatively large (i.e., >10 μm) due to fusion of the drying droplets, and are therefore not suitable for respiratory delivery (Ronkart et al., 2007). Polyvinylpyrrolidone (PVP) has previously been co-spray-dried with a range of poorly water soluble drugs in order to maintain these small

molecules in an amorphous state to improve oral bioavailability (Caron et al., 2011; Gupta and Bansal, 2005). It is proposed here that, provided particle size is suitably controlled, similar strategies may be employed to produce an amorphous matrix to stabilise protein pharmaceuticals for pulmonary delivery.

Some compounds are intrinsically difficult to spray-dry into aerosolisable dry powders, regardless of the operating parameters. For instance, viscous solutions of polymers can produce unacceptably low yield (e.g., 0.5% to 10%), due to high retention of starting material on the wall of spray-dryer. Some hygroscopic compounds can produce particles with unacceptably large particle size, due to fusion of primary structures during or after the drying process (Sou et al., 2013; Sou et al., 2011b). The inclusion of L-leucine in feed solutions has been shown to assist production of inhalable spray-dried powders containing multiple components, while improving aerosolisation properties of the powders. These multi-component formulations were otherwise poorly aerosolisable in the absence of leucine (Li et al., 2003; Najafabadi et al., 2004; Rabbani and Seville, 2005; Seville et al., 2007; Sou et al., 2011b). Similar strategies may be used in the production of other spray-dried powders with materials that would otherwise be too hygroscopic for efficient spray-drying.

The present study aims to explore the use of multi-component spray-dried formulation, using leucine as a particle formation agent, for production of a universal particulate platform with an amorphous matrix and appropriate T_g for pulmonary delivery of various potent biomacromolecules. The model compounds were selected to investigate the influence of molecular size, using excipients across the range of polyol (mannitol), disaccharide (trehalose), polysaccharide (inulin) and synthetic polymer (PVP). The influence of change in molecular size on the resultant properties of spray-dried formulations has not previously been reported in this context. In addition, the

inhibition of crystallisation of spray-dried mannitol using additional small molecule excipients (i.e., glycine and sodium citrate) in the absence of protein, and the properties of these multi-component formulations, were also investigated for comparison. This study reports on the influence of these excipients on processing yields, solid-state properties and morphology of spray-dried formulations which are relevant to their performance in stabilising biomacromolecules as a pulmonary delivery platform.

5.2. Materials and methods

5.2.1. Materials

D-mannitol, D-(+)-trehalose dihydrate, inulin (from dahlia tubers), povidone K30 (PVPK30), L-leucine and glycine were obtained from Sigma-Aldrich Chemicals (Castle Hill, NSW, Australia). Tri-sodium citrate was obtained from Ajax Finechem (Seven Hills, NSW, Australia).

5.2.2. Preparation of spray-dried powders

Aqueous solutions containing the formulations in various compositions were dissolved in 200 mL of Milli-Q water. The prepared formulations were subsequently spray-dried using a Buchi 190 mini spray-dryer with a 0.5 mm two-fluid nozzle, using the following standard operating conditions: airflow, 800 L/h; pump setting, 5 (6.67 mL/min); aspirator setting, 20; outlet temperature, 70 °C. The compositions of the formulations are shown in Table 1. The processing yields were defined as the

percentage of mass of spray-dried powder recovered ($M_{recovered}$) to the mass of total solid loading (M_{total}) in the initial feed solution as shown in Equation 1.

$$Yield\ \% = \frac{M_{recovered}}{M_{total}} \times 100 \qquad (1)$$

Table 1. Compositions (% w/w) and processing yields (%) of the spray-dried formulations.

Powder	Mannitol	Trehalose	Inulin	PVP	NaCitrate	Glycine	Leucine	Yield (%)
1	100	--	--	--	--	--	--	63.1
2	90	--	--	--	--	--	10	78.6
3	50	--	--	--	50	--	--	1.0
4	45	--	--	--	45	--	10	77.2
5	50	--	--	--	--	50	--	57.5
6	45	--	--	--	--	45	10	79.6
7	33	--	--	--	33	33	--	0.0
8	30	--	--	--	30	30	10	74.9
9	--	100	--	--	--	--	--	36.9
10	--	90	--	--	--	--	10	65.9
11	--	--	100	--	--	--	--	59.5
12	--	--	90	--	--	--	10	71.5
13	--	--	--	100	--	--	--	32.2
14	--	--	--	90	--	--	10	67.9

Abbreviations: NaCitrate, sodium citrate.

5.2.3. X-ray powder diffraction (XRPD)

Samples were analysed approximately 2 weeks after storage at room temperature in sealed containers. Sample powders were manually compacted at room temperature in silicon sample holders to obtain a level surface for analysis. The samples

were then analysed by X-ray diffractometry (Bruker D8 Diffractometer, Bruker, Germany) for scanning from 2θ = 2 to 60°; step-wise scanning mode with step size 2θ = 0.02°; step time = 1 s. The amorphous nature of the powders was assessed qualitatively from the diffraction patterns.

5.2.4. Differential scanning calorimetry (DSC)

DSC was performed using a TA DSC Q2000 (TA Instruments, UK). Samples (2 to 5 mg) were sealed in Tzero aluminium DSC pans for measurements. Samples were scanned from -50 to 250 ºC at 200 ºC/min. The glass transition temperature (Tg) was defined as the mid-point of the glass transition event.

5.2.5. Modulated differential scanning calorimetry (MDSC)

MDSC was performed using a TA DSC Q2000 (TA Instruments, UK). Samples (1.9 to 4.1 mg) were sealed in Tzero aluminium DSC pans. Measurements were recorded with a modulation amplitude of ±1 °C, a modulation period of 60 s, and a heating rate of 4 ºC/min from -5 to 200 ºC. The glass transition temperature (Tg) was defined as the mid-point of the glass transition event.

5.2.6. Thermogravimetric analysis (TGA)

TGA was employed to measure temperature-dependent mass changes to estimate the sample water content. Measurements were carried out using a TA TGA Q5000 (TA Instruments, UK). Solid samples (1 to 8 mg) were loaded onto an open

platinum sample pan suspended from the microbalance and heated from 10 to 300 ºC at 10 ºC/min.

5.2.7. Scanning electron microscopy (SEM)

The morphology of the particles was visualised with a bench-top scanning electron microscope (Phenom™, FEI Company, USA). Powder samples were gently poured onto double-sided carbon tape mounted on a sample holder for examination with the SEM. Excess powder was removed by gentle tapping and air blowing to leave a fine layer of particles on the surface of the tape. The samples were sputter coated with gold using an electrical potential of 2.0 kV at 25 mA for 6 minutes with a sputter coater (K550X, EMITECH). SEM micrographs were captured using the proprietary image capture software.

5.2.8. Statistical analysis

Statistical analysis was performed using a two-tailed Student's t-test at $p = 0.05$ where applicable with SPSS (IBM SPSS Statistics, Version 19, SPSS Inc., USA).

5.3. Results and discussion

5.3.1. Influence of formulation components on processing yield

The spray-drying yields and their corresponding formulation compositions are listed in Table 1. The yields of various single-component formulations ranged from

32.2% to 63.1%. Mannitol alone demonstrated the highest processing yield under the experimental spray-drying conditions. The addition of glycine and/or sodium citrate decreased the processing yield. In particular, the inclusion of sodium citrate reduced powder recovery most significantly. The two spray-dried formulations containing mannitol and sodium citrate without leucine (powder 3 and 7) were effectively non-recoverable from the system used.

The inclusion of leucine improved both the absolute magnitude and uniformity of the spray-drying yields for all formulations (Figure 1). The processing yields of all leucine containing formulations ranged from 65.9% to 78.6%. Notably, the inclusion of leucine improved the yields of the formulations containing mannitol and sodium citrate by more than 70-fold, from practically non-recoverable to greater than 75%, as shown in Table 2. The processing yield of spray-dried PVP was also improved by more than 2-fold with the addition of leucine.

Table 2. Relative improvement of spray-drying yield with leucine 10% w/w in the formulations (%).

Formulation	Relative yield improvement (%)
Mannitol/Leucine	24.5
Mannitol/Glycine/Leucine	38.4
Mannitol/NaCitrate/Leucine	7463.7
Mannitol/NaCitrate/Glycine/Leucine	∞
Trehalose/Leucine	78.5
Inulin/Leucine	20.1
PVP/Leucine	110.8

Abbreviations: NaCitrate, sodium citrate; SD, spray-dried; ∞, infinity.

Product yield is an important factor in pharmaceutical manufacturing processes. The effect of leucine in improving yields of spray-dried powders has been reported previously (Chen et al., 2012; Maury et al., 2005; Mishra and Mishra, 2011). However, there has not been a systematic evaluation for inhalation formulations, comparing the particle formation effect of leucine on a series of stabilising excipients ranging from simple sugar to polymers.

It has been reported that leucine improves spray-drying yields by decreasing inter-particulate cohesive interactions (Mishra and Mishra, 2011). While the reduction of these cohesive forces is likely to be an important factor for yield improvement, it is proposed here that this is not the sole mechanism to account for the dramatic effect seen. The present study demonstrated that mannitol-based formulations containing sodium citrate were highly hygroscopic in the absence of leucine, as evidenced by the deposition of resultant products on the wall of the cyclone and collecting vessel of the spray-dryer as a wet film. The addition of leucine transformed these particles into collectable powders.

The ability of leucine to assist formation of spray-dried particles containing multiple components by self-assembly and formation of a protective shell on particle surfaces has been reported previously (Sou et al., 2013; Sou et al., 2011b). This surface forming feature of leucine is proposed here as the key feature across the formulations investigated, which enabled hygroscopic materials to be formulated as powders that can be collected and handled. The leucine-containing formulations with high spray-drying yields were subsequently advanced for further investigation on solid-state properties.

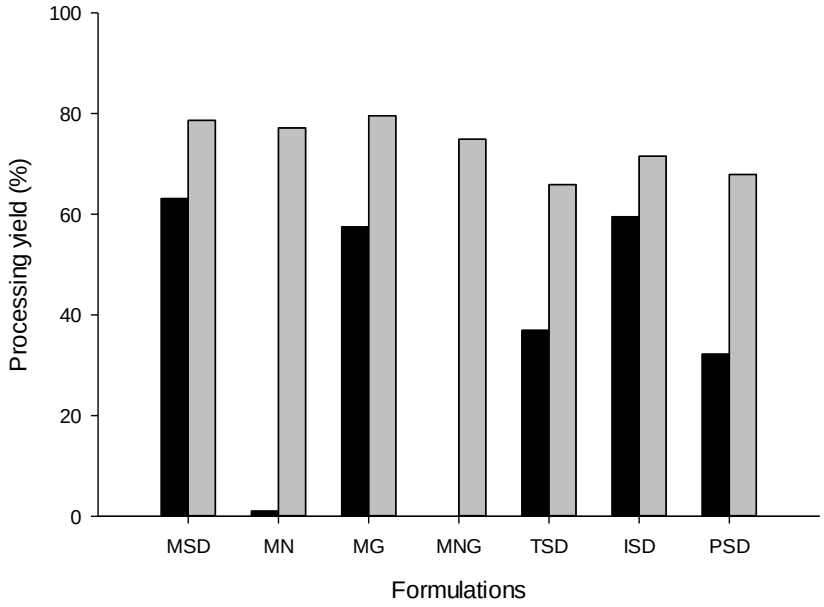

Figure 1. Comparison of spray-drying yields (%) between various baseline formulations without leucine (dark bar) and with leucine (grey bar). Abbreviations: MSD, spray-dried mannitol; MN, mannitol/sodium citrate; MG, mannitol/glycine; MNG, mannitol/sodium citrate/glycine; TSD, spray-dried trehalose; ISD, spray-dried inulin; PSD, spray-dried PVP.

5.3.2. Amorphous status of formulations

The XRPD profiles of the mannitol-based spray-dried formulations are shown in Figure 2. The results demonstrated the influence of various excipients on crystallisation of mannitol. The detrimental effect of mannitol crystallisation on protein stability has been previously reported (Costantino et al., 1998). Previous studies have explored the influence of various small molecule excipients on the crystallinity of spray-dried and freeze-dried mannitol. Freeze-dried mannitol was found in a highly crystalline form when mixed with 25-75% w/w of acetylsalicylic acid as a co-solute (Torrado, 2002). Spray-dried mannitol recrystallised rapidly after spray-drying and crystallisation was not inhibited by the addition of various amino acids and sugars (i.e., glycine, alanine,

leucine and trehalose), either alone or in combination (Sou et al., 2013; Sou et al., 2011b). Consistent with these previous studies, the addition of glycine and/or leucine, did not inhibit crystallisation of mannitol after co-spray-drying (Figure 2). In contrast, the addition of sodium citrate inhibited crystallisation of mannitol regardless of the presence of glycine. However, it did not fully inhibit crystallisation of glycine, as evident from the additional peaks in the MNGL formulation when compared to the MNL formulation.

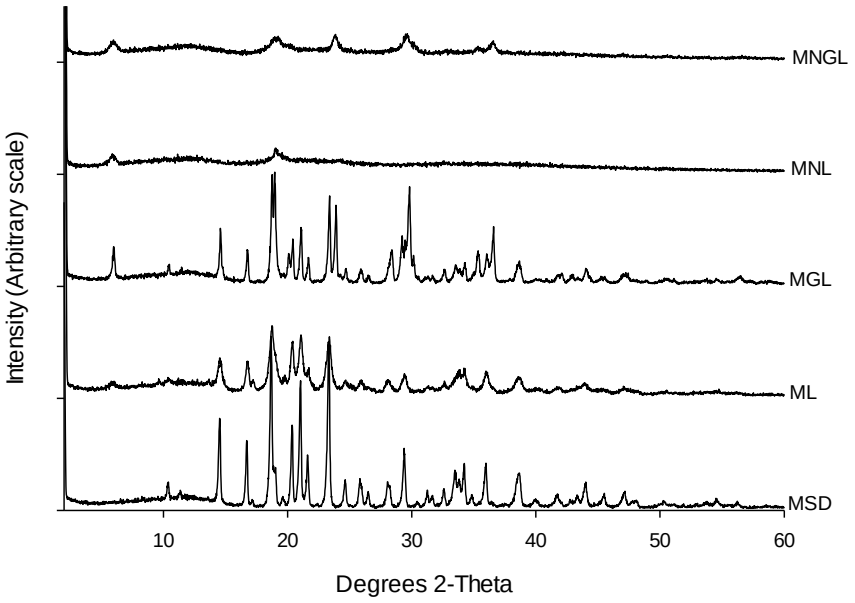

Figure 2. XRPD profiles of the mannitol-based spray-dried formulations: mannitol (MSD), mannitol/leucine (ML), mannitol/glycine/leucine (MGL), mannitol/sodium citrate/leucine (MNL) and mannitol/sodium citrate/glycine/leucine (MNGL).

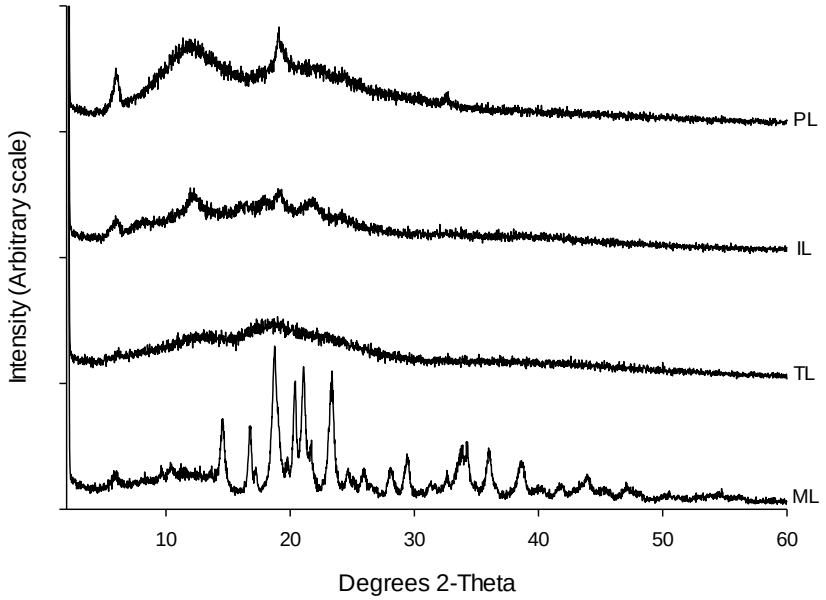

Figure 3. XRPD profiles of spray-dried formulations: mannitol/leucine (ML), trehalose/leucine (TL), inulin/leucine (IL) and PVP/leucine (PL).

Sodium citrate and sodium phosphate have been used with mannitol to produce amorphous spray-dried protein formulations. However, these formulations contained high protein loadings (i.e., greater than 60% w/w) which may have maintained the amorphous matrix (Costantino et al., 1998; Sadrzadeh et al., 2010; White et al., 2005). In contrast, the results in the present study demonstrate that the inclusion of sodium citrate is sufficient to inhibit crystallisation of mannitol in the absence of high protein loading. This composition, however, was extremely hygroscopic and recovery from the spray-dryer was practically impossible without leucine as discussed above.

The XRPD profiles of the mannitol, trehalose, inulin and PVP formulations co-spray-dried with leucine are shown in Figure 3. The trehalose-, inulin- and PVP-based formulations were largely amorphous, as shown by the amorphous halo in the diffractograms. All the powder formulations containing leucine show a distinctive diffraction peak at 6° 2θ that appears relatively broad (Figure 2 and 3). This low angle

peak has been previously identified as distinctive of the formation of a self-assembled leucine structure on particle surfaces (Sou et al., 2013). Nevertheless, this peak is hardly detectable in the trehalose-based formulation, which is consistent with a previous study and may suggest that amorphous trehalose inhibited complete leucine shell formation during spray-drying, relative to other formulations (Sou et al., 2013).

5.3.3. Glass transition temperature

The glass transition temperatures (Tg) of the formulations are listed in Table 3. Consistent with expectations, a Tg value was not detectable in crystalline formulations. Instead, melting endotherms for crystalline mannitol with onset temperatures between 152 to 164 °C were observed (Figure 4). These results correspond to previously reported values of formulations containing various proportions of mannitol (Torrado, 2002). Substitution of glycine with sodium citrate yielded a formulation with Tg = 49.2 °C, which was sufficiently above room temperature to inhibit immediate recrystallisation of spray-dried mannitol. The addition of equivalent amounts of glycine and sodium citrate reduced Tg of the mannitol-based formulation to 32.9 °C.

With the spray-drying conditions used, a trend of increasing Tg in response to increasing molecular sizes of the baseline material was observed: mannitol (polyol) < trehalose (disaccharide) < inulin (polysaccharide) < PVP (synthetic polymer). This finding is consistent with a recent report on the effect of glucose, sucrose and inulin in freeze-dried protein formulations, where the Tg increased with increasing molecular weights of the saccharides (Rodríguez Furlán et al., 2011).

Table 3. Water content and glass transition temperature (T_g) of the spray-dried formulations. The T_g of the baseline compounds without leucine i.e. mannitol, trehalose, inulin and PVP as reported in literature are listed for comparison.

Formulation	Water content (%)	T_g (ºC)	T_g of baseline compound in literature (ºC)
Mannitol raw	n/d	n/d	n/a
Mannitol SD	0.15	n/d	n/a
Mannitol/Leucine 90/10% w/w	0.44	n/d	11 (Weers et al., 2007)
Mannitol/Glycine/Leucine 45/45/10% w/w	0.04	n/d	n/a
Mannitol/NaCitrate/Leucine 45/45/10% w/w	10.84	49.2	n/a
Mannitol/NaCitrate/Glycine/Leucine 30/30/30/10% w/w	7.17	32.9	n/a
Trehalose/Leucine 90/1 % w/w	5.32	65.4	117 (Miller and de Pablo, 2000)
Inulin/Leucine 90/10% w/w	7.85	67.8	120 (Zimeri and Kokini, 2002)
PVP/Leucine 90/10% w/w	11.16	90.3	161 (Chokshi et al., 2008)

Abbreviations: n/a, not applicable; n/d, not detectable; NaCitrate, sodium citrate; SD, spray-dried; T_g, glass transition temperature.

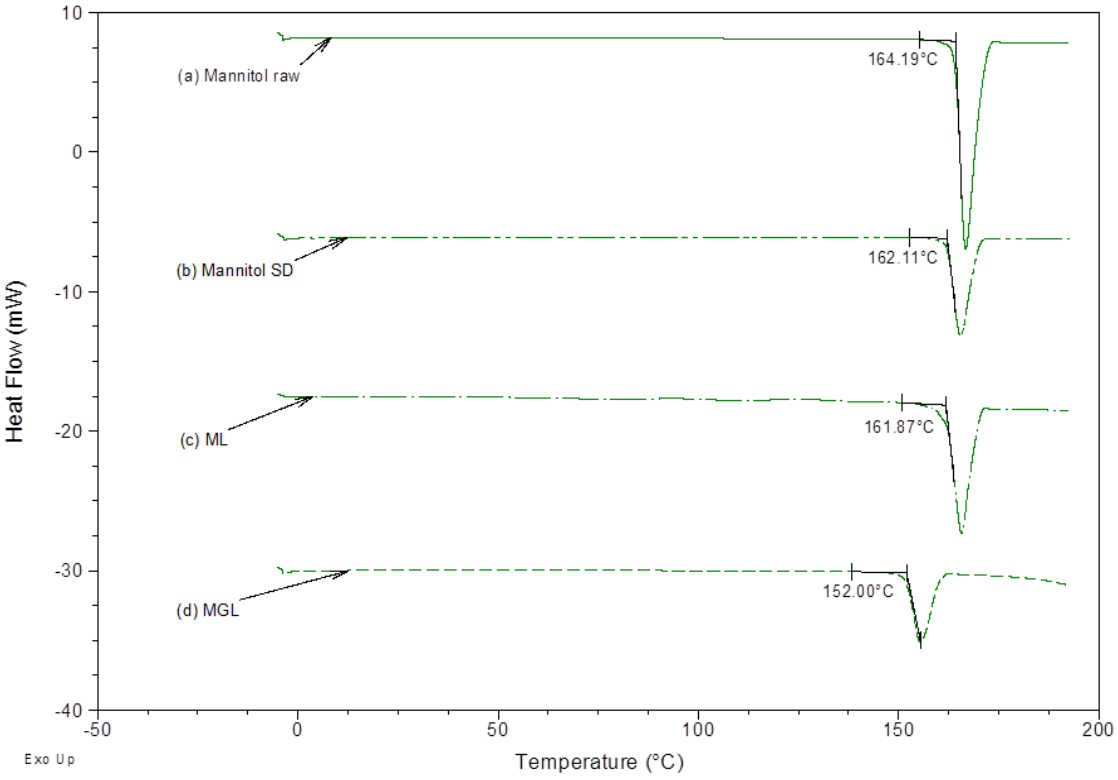

Figure 4. MDSC thermograms showing the onset melting points for (a) mannitol before spray-drying (Mannitol raw), (b) spray-dried mannitol (Mannitol SD), (c) mannitol/leucine (ML) and (d) mannitol/glycine/leucine (MGL).

The relatively low Tg of mannitol (11 °C) indicates that spray-dried mannitol has a high molecular mobility at ambient temperature such that it rapidly recrystallises after spray-drying (Weers et al., 2007). Previous studies have demonstrated the use of small molecules in conjunction with mannitol to produce powders with sufficiently high Tg to remain amorphous at ambient conditions. However, as mentioned above, these formulations also contained high protein loadings. Spray-dried powders containing mannitol and glycine have been successfully used to produce amorphous formulations to stabilise insulin and IgG in dried solid state at room temperature (Sadrzadeh et al., 2010; Schule et al., 2008; Siekmeier and Scheuch, 2008; White et al., 2005). However,

similar amino acid containing formulations without high protein loadings did not raise Tg sufficiently to inhibit crystallisation of spray-dried mannitol (Sou et al., 2013; Sou et al., 2011b).

It has been suggested that inorganic salts, including sodium phosphates and citrates, elevated Tg and prevented crystallisation of mannitol in frozen solutions and freeze-dried solids by reducing molecular mobility as a result of strong hydrogen bonding (Izutsu et al., 2007). However, the influence of these inorganic salts on the solid-state properties of spray-dried materials is less well understood. The intermolecular mobility of compounds in the hydrodynamic environment during spray-drying is greater than for freeze-drying and hence the effect of these excipients may be reduced. The results in the present study have therefore demonstrated the potential functionality of these inorganic salts in raising Tg of mannitol-based spray-dried formulations in the absence of proteins.

The use of polymeric compounds such as inulin in stabilising dried protein formulations has been widely studied in pharmaceutical and food industries (Amorij et al., 2007; de Jonge et al., 2007; Rodríguez Furlán et al., 2011; Rodríguez Furlán et al., 2010). However, the use of spray-dried polymeric system as a standalone particulate delivery platform for aerosol application has not been extensively evaluated. The higher Tg of the inulin- and PVP-based formulations in the present study, and their relatively low hygroscopicity, mean that the potential benefit of these polymer-based spray-dried formulations in stabilising biomacromolecules for pulmonary administration warrants further investigation.

5.3.4. Water content of the spray-dried formulations

The water contents of the formulations are listed in Table 3. The crystalline mannitol-based formulations had a water content below 0.4% w/w, whereas the amorphous mannitol-based formulations with sodium citrate contained significantly more water (i.e., up to 11% w/w). The high water contents in these formulations reflected the greater hygroscopicity of amorphous mannitol. It was interesting to note that the water content in the amorphous mannitol-based formulations correlated with the amount of sodium citrate. The results suggested that while the increased hydrogen bonding potential of sodium citrate might be highly effective in maintaining the amorphous nature of mannitol, it also substantially increased hygroscopicity and retention of water. Water is a well-known plasticiser that reduces Tg dramatically (Hancock and Zografi, 1994; Heljo et al., 2011). The Tg of these formulations could potentially be raised substantially with more efficient drying or a secondary drying process, provided the subsequent process does not cause major disturbances to the architecture of these spray-dried particles.

There was a trend of increasing water content in response to increasing molecular sizes of the baseline material in the formulations. The disaccharide-based (trehalose/leucine), polysaccharide-based (inulin/leucine) and synthetic polymer-based (PVP/leucine) formulations had water contents of 5%, 8% and 11% w/w, respectively. However, the higher molecular weight formulations produced higher Tg values in spite of this relatively increased water content. The potential benefits of residual water in solid-state protein stabilisation have been previously demonstrated (Ekdawi-Sever et al., 2003; Ohtake et al., 2004). The ability to accommodate these water molecules within a rigid amorphous matrix of sufficiently high Tg might diminish the importance of incorporating additional excipients as replacement of water for hydrogen bonding with the biomacromolecules. The additional water molecules captured in the formulation

may serve to reduce the dehydration stress in solid-state to stabilise proteins in conjunction with the limited intermolecular mobility provided by the rigid amorphous matrix. Further investigation to explore the benefits of these formulations in this regard is being undertaken.

5.3.5. Particle morphology and appearance

SEM images of the mannitol-based spray-dried formulations are shown in Figure 5. The highly crystalline mannitol-based formulations (ML and MGL) formed spherical particles in the presence of leucine as previously reported (Sou et al., 2013; Sou et al., 2011b). The amorphous mannitol-based formulation produced by substituting glycine with sodium citrate (MNL) formed hygroscopic particles, as evident by the formation of solid bridges and fusion of small particles on the SEM image. Interestingly, the concurrent use of sodium citrate and glycine with mannitol (MNGL) produced particles with more surface wrinkles than particles with either of these excipients alone. The surface wrinkles have been suggested to result from a collapsed leucine shell which was initially inflated, due to internal vapour formation during the drying process (Sou et al., 2011b). The effect of surface rugosity on reducing inter-particle forces has been previously reported (Chew et al., 2005; Chew and Chan, 2001). These particles with more surface corrugations were less cohesive and more easily handled as mentioned above.

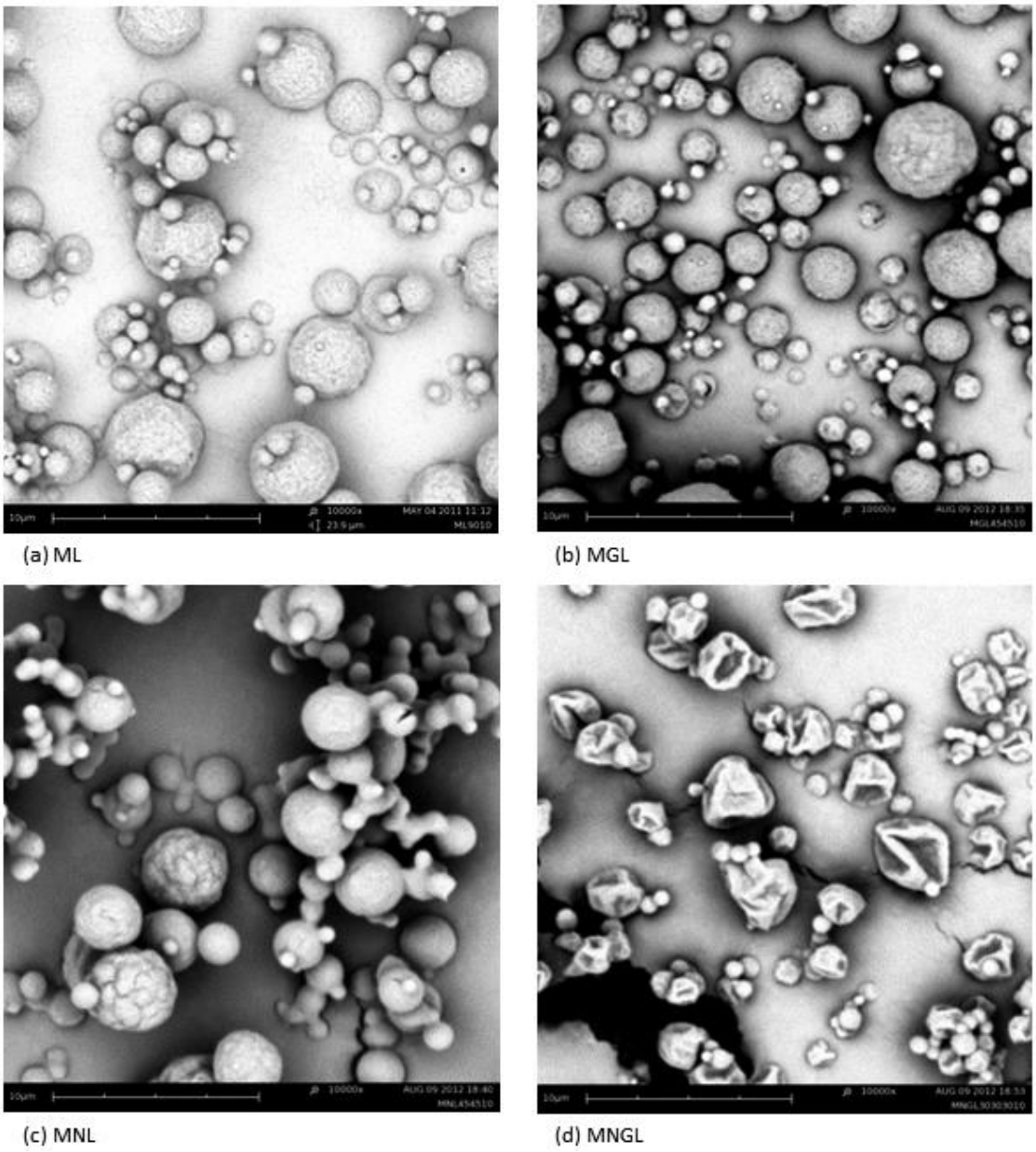

Figure 5. Representative scanning electron micrographs of (a) mannitol/leucine (ML), (b) mannitol/glycine/leucine (MGL), (c) mannitol/sodium citrate/leucine (MNL) and (d) mannitol/sodium citrate/glycine/leucine (MNGL). [Magnification: 10000x]

SEM images comparing the influence of polyol (ML), disaccharide (TL), polysaccharide (IL) and synthetic polymer (PL) as baseline material on morphology of the spray-dried particles with equivalent amounts of leucine are shown in Figure 6. It is clear that under the experimental conditions, the polymeric compounds (IL and PL) produced particles with greater surface corrugations than their counterpart formulations containing either polyol or disaccharide (ML and TL). Polymers have been reported as film-forming agents which enrich surfaces and form spray-dried particles with wrinkled or dimpled morphologies (Vehring, 2008). The concurrent use of leucine and polymer might have a synergistic effect on particle rugosity. The surface corrugations and small particle size, as shown on the SEM images, in conjunction with the enhanced Tg of these polymer-based formulations (IL and PL) suggest that the potential utilities of these compounds for pulmonary delivery of biomacromolecules deserves further investigation.

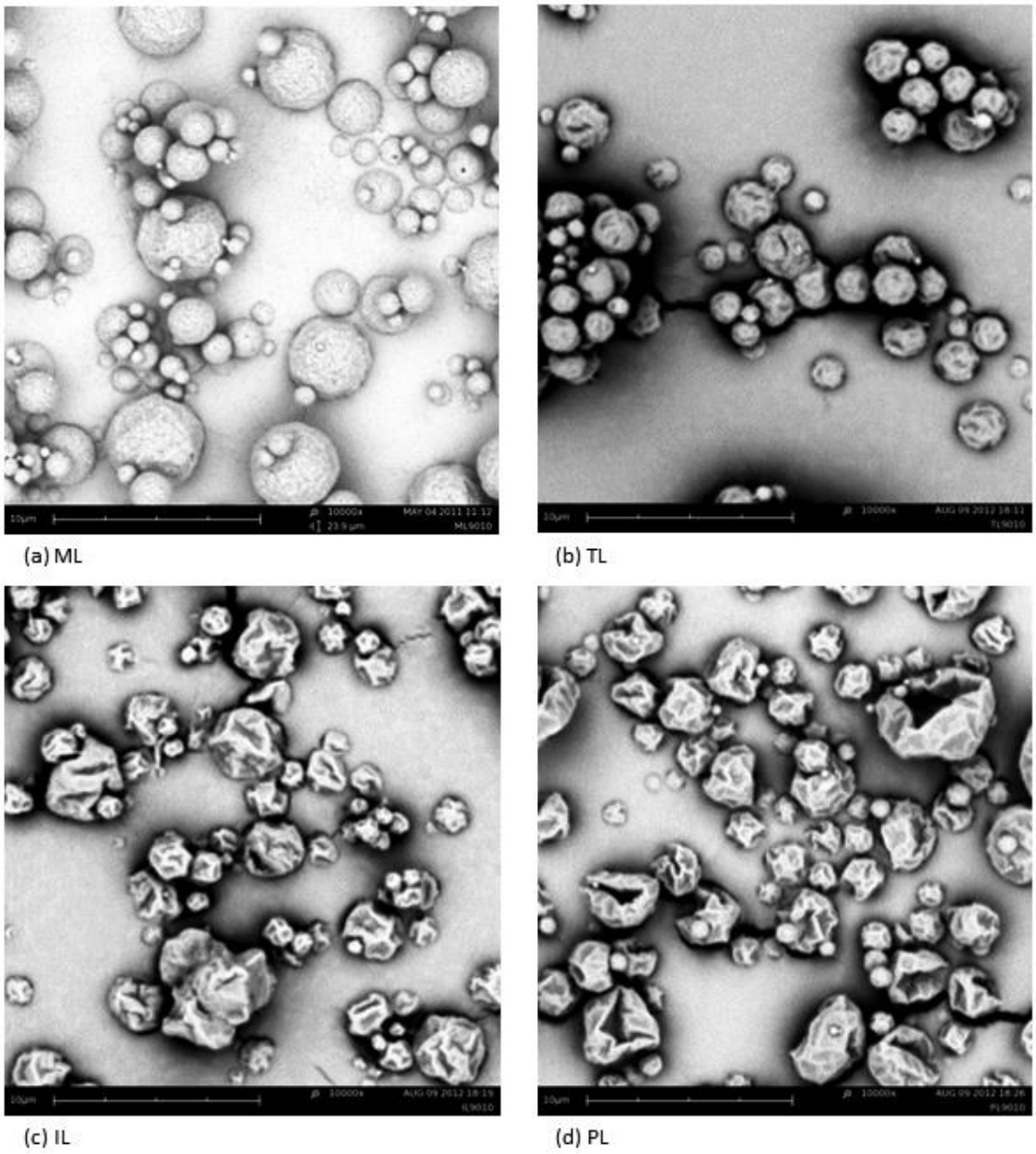

Figure 6. Representative scanning electron micrographs (a) mannitol/leucine (ML), (b) trehalose/leucine (TL), (c) inulin/leucine (IL) and (d) PVP/leucine (PL). [Magnification: 10000x]

5.4. Conclusion

This study set out to compare and contrast the influence of molecular size on the resultant properties including processing yields, solid-state properties and morphology of spray-dried formulations that are relevant to their performance in stabilising biomacromolecules as a pulmonary delivery platform for delivery of potent biomacromolecules. Previously, only formulations containing high protein content have been shown to prevent recrystallisation of spray-dried mannitol. The results in this study have shown that the addition of sodium citrate was highly effective in inhibiting crystallisation of mannitol. However, addition of sodium citrate significantly increased the hygroscopicity of the material, such that it could not be recovered after spray-drying. The addition of leucine dramatically improved yield by assisting particle formation to create a platform combination of excipients with multiple desirable properties suitable for pulmonary delivery that have not been previously reported: leucine as a coating agent for particle formation and efficient spray-drying, sodium citrate as a glass-forming agent for inhibition of mannitol crystallisation, and glycine as a morphological modifier for surface corrugation and flow improvement.

In addition, this study has demonstrated for the first time the potential benefit of higher molecular weight compounds with polymeric properties, as an attractive alternative to small molecule excipients such as polyols and sugars, in providing an amorphous particulate platform suitable for pulmonary delivery. These polymeric systems retained appropriate Tg in spite of the relatively high moisture content after spray-drying. Although these compounds alone were difficult to spray-dry, the addition of leucine assisted particle formation and improved processing yield significantly. Studies to further optimise the combined properties, including aerosolisation delivery,

physical and chemical stability, and to explore the utilities of these formulation platforms, both in vitro and in vivo, with various biomacromolecules are being undertaken.

5.5. Acknowledgements

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CHAPTER SIX

SPRAY-DRIED INFLUENZA VACCINE WITH TREHALOSE AND LEUCINE PRODUCES A HIGHLY AEROSOLISABLE POWDER THAT INDUCES SUPERIOR SYSTEMIC AND MUCOSAL IMMUNITY AFTER PULMONARY ADMINISTRATION

Monash University

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Study initiation, experimental design, laboratory work, data analysis and interpretation, writing up. Formation of hypothesis and conclusion.	80%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution
David AV Morton	Supervision, manuscript revision
Mark Williamson	Histological study
Els N Meeusen	Manuscript revision
Lisa M Kaminskas	Supervision, manuscript revision, rat immunisation study
Michelle P McIntosh	Supervision, manuscript revision

Candidate's Signature [redacted]

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Monash Institute of Pharmaceutical Sciences, Monash University, 381 Royal Parade, Parkville, VIC 3052, Australia

David AV Morton
Mark Williamson
Els N Meeusen
Lisa M Kaminskas
Michelle P McIntosh

[redacted]

6. Spray-dried influenza vaccine with trehalose and leucine produces a highly aerosolisable powder that induces superior systemic and mucosal immunity after pulmonary administration

Tomás Sou[a], David A. V. Morton[a], Mark Williamson[b], Els N. Meeusen[c], Lisa M. Kaminskas[a]* and Michelle P. McIntosh[a]*

[a] *Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia*

[b] *Gribbles Veterinary Pathology, Clayton, Victoria, Australia*

[c] *Dept Microbiology & ARC Centre of Excellence in Structural and Functional Microbial Genomics, Monash University, Clayton, Victoria 3800, Australia*

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*Corresponding authors:

[redacted] (L.M. Kaminskas)

[redacted]

(M.P. McIntosh)

6.0. Abstract

This study aimed to characterise the impact of the incorporation of a model biomacromolecule (influenza antigen, containing 1-10 µg/mg active haemagglutinin) on the solid-state properties of spray-dried powders prepared with trehalose and leucine. The stability of haemagglutinin under the spray drying conditions was also examined. The morphology of the spray-dried powders and the formation of an amorphous glassy matrix were not affected by the incorporation of up to 10 µg/mg haemagglutinin (equivalent to 23.58 µg/mg total protein). However, increasing the proportion of protein in the feed solution reduced the T_g of the formulation and increased water content. Haemagglutinin was stable under the spray-drying conditions employed. Additionally, pulmonary instillation of the reconstituted powder containing 5 µg/ml haemagglutinin to rats induced strong mucosal and systemic immunity compared to subcutaneous injection of the liquid antigen without adverse pulmonary effects. This study demonstrates the utility of this spray-dried carrier system as a promising platform for the pulmonary delivery of influenza vaccine. This delivery system may therefore be applied to the formulation of inhalable dry powder formulations of other biomacromolecules.

6.1. Introduction

Pulmonary immunisation has recently gained increased interest as a means to induce both systemic and mucosal responses while eliminating issues associated with the use of needles in parenteral vaccination. These issues include the requirement for skilled medical personnel to administer the dose, the risk of needle-stick injuries and consequently the potential transmission of blood-borne viruses (Sou et al., 2011a). Dry powder inhalation is an attractive delivery method for pulmonary drug administration due to its many advantages including the non-invasive nature and ease of administration, relatively simple formulation and low cost (Carpenter et al., 1997; Prime et al., 1997; Rave et al., 2004). The feasibility of dry powder influenza vaccines has previously been demonstrated in several studies which have shown that influenza antigen is a suitable candidate for pulmonary immunisation to induce mucosal and systemic immunity (Amorij et al., 2007; Audouy et al., 2011; Saluja et al., 2010). However, the relatively low fine particle fractions (FPF; <5 μm) of ≤38% from the formulations used in these studies suggested that there is room to improve the aerosolisation performance of these dry powder formulations.

In contrast to the inhaled delivery of many small molecule drugs, a well-characterised carrier platform that is readily adaptable to the incorporation of various biomacromolecules as a common standard is lacking. In recent years, studies investigating the possibility of developing a universal dry powder delivery platform that is readily adaptable to pulmonary delivery of various potent biomacromolecules such as vaccine antigens which may only be required in a small loading (i.e., <1% w/w) has been reported (Sou et al., 2013; Sou et al., 2011b). Since the small amount of protein loading may be considered unlikely to impact on the aerosolisation behaviour of the

carrier platform, optimisation of the intrinsic performance of the delivery system is therefore fundamentally important. The active biomacromolecule of interest may then be added to the optimised system to investigate the in vivo activity of the resultant formulation.

For a dry powder carrier platform to be suitable for pulmonary delivery of biomacromolecules, it has to be not only aerosolisable, but preferably capable of stabilising the biomacromolecules at room temperature. According to the glassy dynamics theory, an amorphous glassy solid should provide a highly viscous "vitreous" environment which restricts the molecular mobility of biomacromolecules, thereby stabilising proteins in a dry solid state (Chang and Pikal, 2009; Weers et al., 2007). Any excipient combination used should ideally be a good glass former with high glass transition temperature (Tg) but otherwise inert. A number of research groups are working in the area of developing formulation strategies for the production of a dry powder delivery system containing an amorphous glassy matrix with high aerosolisation performance and sufficiently high Tg (Andya et al., 1999; Costantino et al., 1998; Depreter and Amighi, 2010; Ógáin et al., 2011; Rodríguez Furlán et al., 2011; Sou et al., 2013; Sou et al., 2011b; Tewes et al., 2010).

Sugars are commonly used in the stabilisation of biomacromolecules due to their ability to form hydrogen bonds (Sou et al., 2011a; Vehring, 2008). Trehalose is a non-reducing sugar which has the advantage that it will not undergo Maillard reaction with proteins. Trehalose, with a Tg of 117ºC, tends to form amorphous glasses after spray-drying and has been used to stabilise proteins in several studies (Jin et al., 2010; Maa et al., 1997; Ógáin et al., 2011). However, high concentrations of trehalose (> 50% w/w), without appropriate excipients, may not be ideal for aerosol applications due to the

adhesive nature of the sugar and subsequently the high degree of particle agglomeration and poor aerosolisation (Maa et al., 1997).

Spray-dried trehalose with leucine has previously been demonstrated to produce powders containing an amorphous glassy matrix with high aerosolisation performance (Sou et al., 2013). The present study aimed to further extend our understanding of the use of this multi-component inhalable dry powder carrier platform for the pulmonary delivery of biomacromolecules, using influenza antigen as a model therapeutic antigen. Specifically, this study examined the influence of protein loading on solid-state properties and morphology of particles. An optimised spray-dried vaccine formulation was then selected for further in vivo investigation to probe the stability of the influenza antigen under the spray-drying conditions employed.

6.2. Materials and methods

6.2.1. Materials

D-(+)-Trehalose dihydrate and L-leucine were purchased from Sigma-Aldrich Chemicals (Castle Hill, NSW, Australia). Inactivated influenza virus antigen (A/Solomon Islands/03/2006) containing haemagglutinin (HA) protein content of 3.082 mg/mL (total protein content 7.267 mg/mL) was kindly donated by CSL Limited (Melbourne, Victoria, Australia). Goat anti-rat IgG:HRP and goat anti-rat IgA:HRP for ELISA were purchased from Abcam plc. (Cambridge, UK).

6.2.2. Preparation of spray-dried powders

Aqueous solutions containing the formulations in various compositions were dissolved in 200 mL of Milli-Q water. The prepared formulations were subsequently spray-dried using a Buchi 190 mini spray-dryer with a 0.5 mm two-fluid nozzle, using the following standard operating conditions: airflow rate, 800 L/h; pump setting, 5 (6.67 mL/min); aspirator setting, 20; outlet temperature, 70 °C . The compositions of the formulations are shown in Table 1. The powders were stored in sealed conditions with dessicant before testing.

Table 1. Compositions of the spray-dried formulations and their corresponding glass transition temperatures (T_g) and water contents (%). (Mean ± s.d., n = 3)

Formulation	Trehalose (% w/w)	Leucine (% w/w)	HA (µg/mg)	T_g (C°)	Water content (%)
HA00	90	10	--	61.9	3.23 ± 0.11
HA01	90	10	1	58.7	3.32 ± 0.53
HA05	90	10	5	57.3	3.48 ± 0.16
HA10	90	10	10	52.4	4.60 ± 0.54

Abbreviations HA, haemagglutinin protein influenza antigen.

6.2.3. X-ray powder diffraction (XRPD)

Sample powders were sprinkled onto a quartz sample plate smeared with a thin layer of Vaseline at room temperature. The sample was analysed by an X-ray diffractometer (Philips 1140 vertical diffractometer, Philips, Holland) scanning from 2 to 60° in 2θ, with an angular increment of 2°/min. The crystalline status of the powders was assessed qualitatively by examination of the resulting diffraction patterns.

6.2.4. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) measurements were conducted using a TA DSC Q100 (TA Instruments, UK). Samples weighing between 6.8 to 8.0 mg were crimped in Tzero aluminium DSC pans for measurements. Samples were scanned from -10 to 200 ºC at 10 ºC/min. The glass transition temperature (T_g) was defined as the mid-point of the glass transition.

6.2.5. Karl-Fisher titration

The water content of the spray-dried formulations was determined by colorimetric Karl-Fisher titration (907 Titrando, Metrohm, Herisau, Switzerland). Samples were crimper sealed in glass vials after manufacturing and stored in refrigerated conditions until experiment.

6.2.6. Scanning electron microscopy (SEM)

The morphology of particles was visualised under a scanning electron microscope (Phenom™, FEI Company, USA). Powder samples were poured onto double-sided carbon tape mounted on a sample holder for examination under the scanning electron microscopy (SEM). The samples were sputter coated with gold using an electrical potential of 2.0 kV at 25mA for 6 minutes with a sputter coater (K550X, EMITECH). SEM micrographs were captured using the in-built image capturing software.

6.2.7. In vitro powder aerosolisation study

The in vitro powder aerosolisation performance was determined using a modified abbreviated Anderson Cascade Impactor (ACI) system configured to simulate the human respiratory tract (HRT) as described and validated previously (Mitchell et al., 2010). The Monodose inhaler (Miat S.p.A., Milan, Italy) was used as the aerosol dispersion device. Briefly, the abbreviated ACI configuration consisted of, from top to bottom, the throat piece, pre-separator, stage 0, a full metal plate coated with a surfactant (Brij-35), stage F containing a filter paper and elastomer gasket, and the rest of the stages. The cut-off aerodynamic diameter of powders deposited on the filter paper using this configuration at a flow rate of 90 L/min is approximately 4.7 µm according to the manufacturer. Approximately 20 mg of samples were weighed and filled into size 3 HPMC capsules (Capsugel, Peapack, NJ, USA) for the tests which were performed in an air-conditioned laboratory (at 20 ± 3 °C, 40 ± 5% relative humidity). Each capsule was actuated from the inhaler over 6 s for each measurement (n = 3). Fine particle fraction (FPF) was calculated as a percentage of the emitted dose (ED). The ED and amount of powder deposited on the filter paper were determined gravimetrically.

6.2.8. Stability of antigen in spray-dried formulations

The integrity of antigen in the spray-dried formulations was determined using haemagglutination (HA) assay adapted from previously published method (Huang et al., 2004). Briefly, powder formulations were reconstituted with phosphate buffered saline (PBS) of pH 7.4 to achieve an HA antigen concentration of 100 µg/mL. The reconstituted samples (50 µL) were added to the 96-well microplates and serially diluted, followed by addition of 25 µL of 1% chicken red blood cells to each well. The

plates were incubated at room temperature for 30 min. The HA titres of the formulation were compared to the original liquid antigen stock which had been stored in refrigerated condition. All samples were analysed in duplicate. The HA titres of the optimised spray-dried vaccine formulation was measured immediately after production to determine the HA activity after spray-drying. The formulation was subsequently stored at 40 °C and assayed at 3, 6 and 9 weeks after storage to determine the HA activity remained.

6.2.9. Animals

Female Sprague-Dawley rats (6 weeks old) were obtained from the Animal Resources Centre, Perth, Australia. Rats were exposed to a 12 hour light/dark cycle and were given food and water ad libitum. All animal experimentation was approved by the institutional Animal Ethics Committee.

6.2.10. Immunisations

Animals were briefly anesthetised with isoflurane for immunisation and blood sample collection. In this study, we were interested in the stability of the antigen upon exposure to the spray drying conditions rather than the capacity to deliver the powder into the deep lungs (rats are not a suitable species to assess lung deposition (Meeusen et al., 2009)). Therefore, in order to compare the immunogenicity of the dry powder formulation to the liquid antigen after pulmonary delivery, the powder formulation was reconstituted in saline and the liquid formulation was diluted in saline to achieve 5 µg HA in a 100 µL volume. Administering both vaccines as a liquid instillation ensures that the lung exposure to both formulations is equivalent which is important given the

regional distribution of the antigen in the lung dictates the extent of mucosal immunity induced (Holmgren and Czerkinsky, 2005). Animals received an intratracheal instillation of either (i) reconstituted powder vaccine, (ii) liquid vaccine, (iii) reconstituted blank powder (containing no antigen), or (iv) saline or (v) a subcutaneous injection of liquid vaccine. Pulmonary immunisation was performed via liquid instillation of the entire 100 µL dose using a 1 mL syringe attached to a 0.96 mm cannula inserted into the trachea approximately 2 cm past the vocal chords. Instillation of liquid doses was followed by 200 µL of air to expel any remaining liquid. Syringes were weighed before and after immunisations to determine the accurate amount of formulations administered. The animals were divided into 3 separate cohorts to ensure the reproducibility of the immunization results across different batches of rats. Each animal was immunised twice over a 2 week period on day 1 and day 14, and bronchoalveolar lavage (BAL) fluid and lung tissue collected 4 weeks after the initial dose on day 28.

6.2.11. Sample collection

Blood samples (300 µl) were collected via a lateral tail venipuncture immediately prior to the administration of each dose and prior to BAL collection on day 28. Blood samples were allowed to coagulate for 30 mins at 4 °C before centrifugation at 10621 g for 5 min to separate sera.

The bronchiole connecting the caudal lobe was clamped to prevent the infusion of saline into the lobe and BAL fluid was collected from the rest of the lung via the instillation of 3 x 5ml saline into the trachea. The caudal lobe was subsequently removed and placed into 10% formalin for histological analysis after haematoxylin and

eosin (H&E) staining. BAL fluid was centrifuged at 4450 g for 15 min at 4 °C to remove alveolar macrophages and collected pneumocytes. The sera and BAL supernatant were stored at -20 °C until analysis.

6.2.12. Haemagglutination inhibition (HAI) assay

Influenza-specific antibody response was determined using haemagluttination inhibition (HAI) assay (Huang et al., 2004). Briefly, test sera were diluted 1 in 10 with PBS before heat inactivation in a 56 °C water-bath for 30 min to remove non-specific inhibitors of haemagglutination. The treated sera (25 µL) were added to 96-well microplates and serially diluted, followed by addition of 25 µL of four haemagglutination units of influenza HA antigen before incubation at room temperature for 30 min. This incubation was followed by an addition of 25 µL of 1% chicken red blood cells to each well. The plates were incubated at room temperature for another 30 min. The endpoint HAI titre was defined as the reciprocal of the highest serum dilution that completely inhibited haemagglutination of the chicken red blood cells.

6.2.13. ELISA for antibody responses

Influenza HA antigen-specific antibody responses were determined via ELISA using previously published methods (Huang et al., 2004). Briefly, 100 µL of 5 µg/mL HA antigen was added to each well of a 96-well flat bottom microplate (polystyrene, Costar®, NY, USA) and incubated at 4°C overnight. Wells were rinsed in 0.05% Tween-20/PBS (PBST) and blocked using PBST containing 5% skim milk powder (w/v) at 37 °C for 1 hr. The plates were washed prior to the addition of sera and BAL fluid

(100 µL/well, diluted up to 1:2048000) and incubated at 37°C for 1.5 hr. The plates were washed prior to the addition of horseradish peroxidase (HRP) conjugated secondary antibodies and the plates were incubated at 37 °C for 1 hr. Specifically, IgG was captured using goat anti-rat IgG:HRP and IgA was captured using goat anti-rat IgA:HRP. To each well, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB single solution, Invitrogen) was added and incubated for 20 min at room temperature. The reaction was stopped via the addition of 100 µL 1 N HCl and the optical density of each well read at 450 nm (OD450) on a FLUOstar plate reader (FLUOstar OPTIMA, BMG Labtech). The endpoint titres were defined as the highest reciprocal dilution of sera or BAL yielding an OD450 value that was at least 2-fold higher than the background readings obtained at day 0 for sera samples, or at least 2 standard deviations higher than the background readings obtained from the saline group for BAL samples.

6.2.14. Statistical analysis

Statistical analysis was performed using Prism software (Prism 6, GraphPad Software Inc., USA). Antibody responses in BAL at day 28 were compared using one-way ANOVA with Tukey's post-test. Antibody responses in sera at day 0, 14 and 28 were compared using two-way ANOVA with Tukey's post-test. Water contents between the dry powder formulations were compared using one-way ANOVA with Tukey's post-test.

6.3. Results

6.3.1. Amorphous status of the spray-dried formulations

The X-ray powder diffraction (XRPD) profiles of raw trehalose from the manufacturer and the spray-dried formulations are shown in Figure 1. It is clear that the spray-drying process transformed the trehalose which was supplied as crystalline material from the manufacturer into an amorphous material. The result is consistent with a previous study showing that the trehalose/leucine formulation appeared to be fully amorphous (Sou et al., 2013). The leucine peaks at 6° and 20° 2θ were attributable to self-assembled leucine at the surface and were not well-defined with leucine 10% w/w (Sou et al., 2013; Sou et al., 2011b). The XRPD profiles indicate that the range of HA protein content used in the study did not influence the amorphous nature of the spray-dried powders and the formation of leucine coating on particle surfaces as shown by the characteristic 'amorphous halo' in their diffractograms.

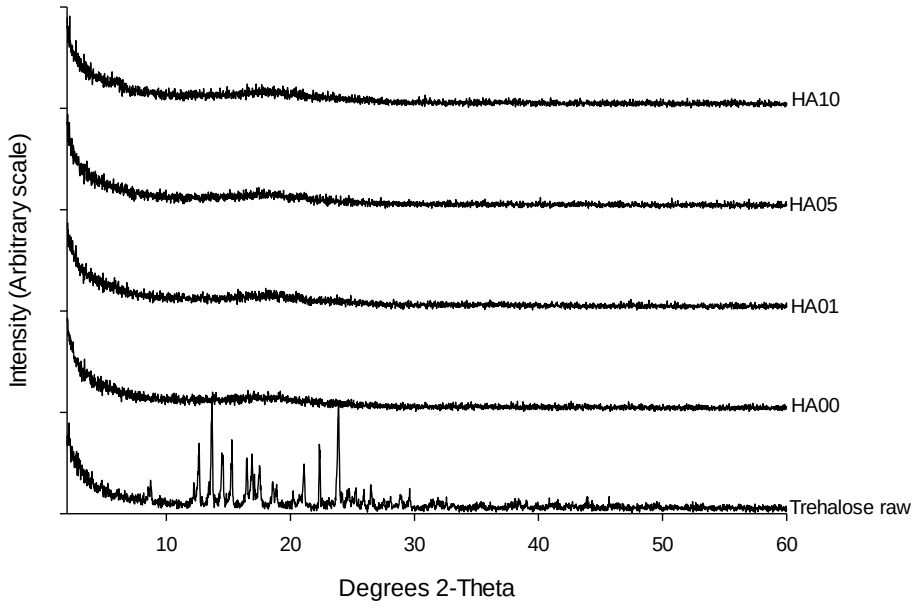

Figure 1. XRPD profiles of the spray-dried formulations and raw trehalose before spray-drying.

6.3.2. Glass transition temperature and water contents

The Tg and water contents of the formulations are included in Table 1. A descending trend of Tg in response to increasing HA protein concentration and water content was observed. The reduction in Tg was particularly prominent when the HA content was higher than 5 µg/mg which corresponds to the greater increase in water content (Figure 2). The result suggests that higher HA protein content reduced Tg due to the higher residual moisture retained in the formulation although it did not interfere with the formation of the amorphous glassy matrix during spray-drying as shown in the XRPD profiles.

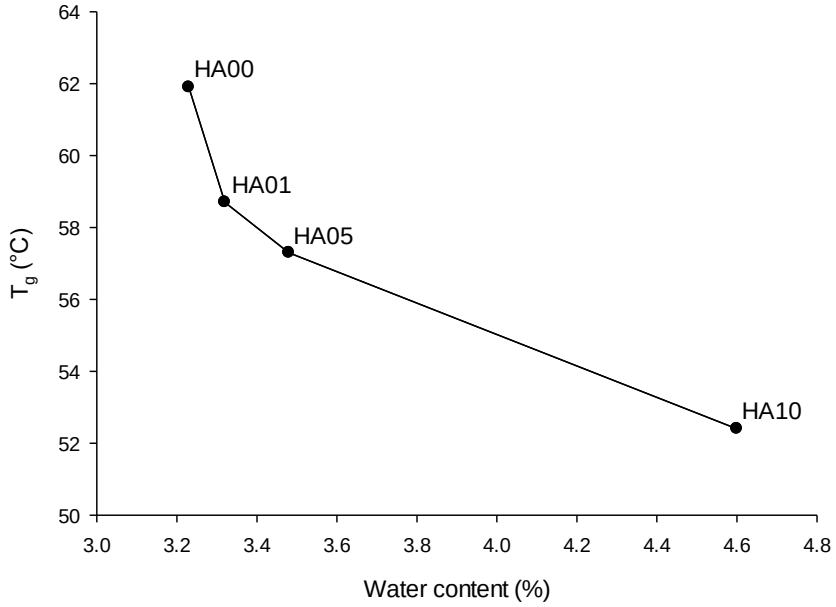

Figure 2. Glass transition temperature (T_g) vs. water content in the formulations.

6.3.3. Particle morphology and in vitro powder aerosolisation

The SEM images of the spray-dried formulations are shown in Figure 3. The co-spray-dried trehalose/leucine carrier platform alone (HA00) formed distinctive particles as previously reported (Sou et al., 2013). Incorporating the HA protein did not significantly affect formation of the spray-dried particles as shown by the consistent particle size and morphology across the HA concentration range in the SEM images. It should be noted that precipitation of leucine from the feeding solution for the HA10 formulation was observed during the spray-drying process. It is proposed that the higher protein solution composition in the feeding solution contributed to the precipitation of leucine. Consequently, there was relatively less leucine available in the solution to influence particle surface formation. This may also have contributed to the higher water content in the HA10 formulation with less leucine available for surface protection. The HA05 formulation appeared to contain the most appropriate amount of protein loading without compromising the favourable physical properties of the spray-dried particles. This vaccine formulation was subsequently selected for in vitro powder aerosolisation study and demonstrated a high FPF of 49.3 ± 2.6 %.

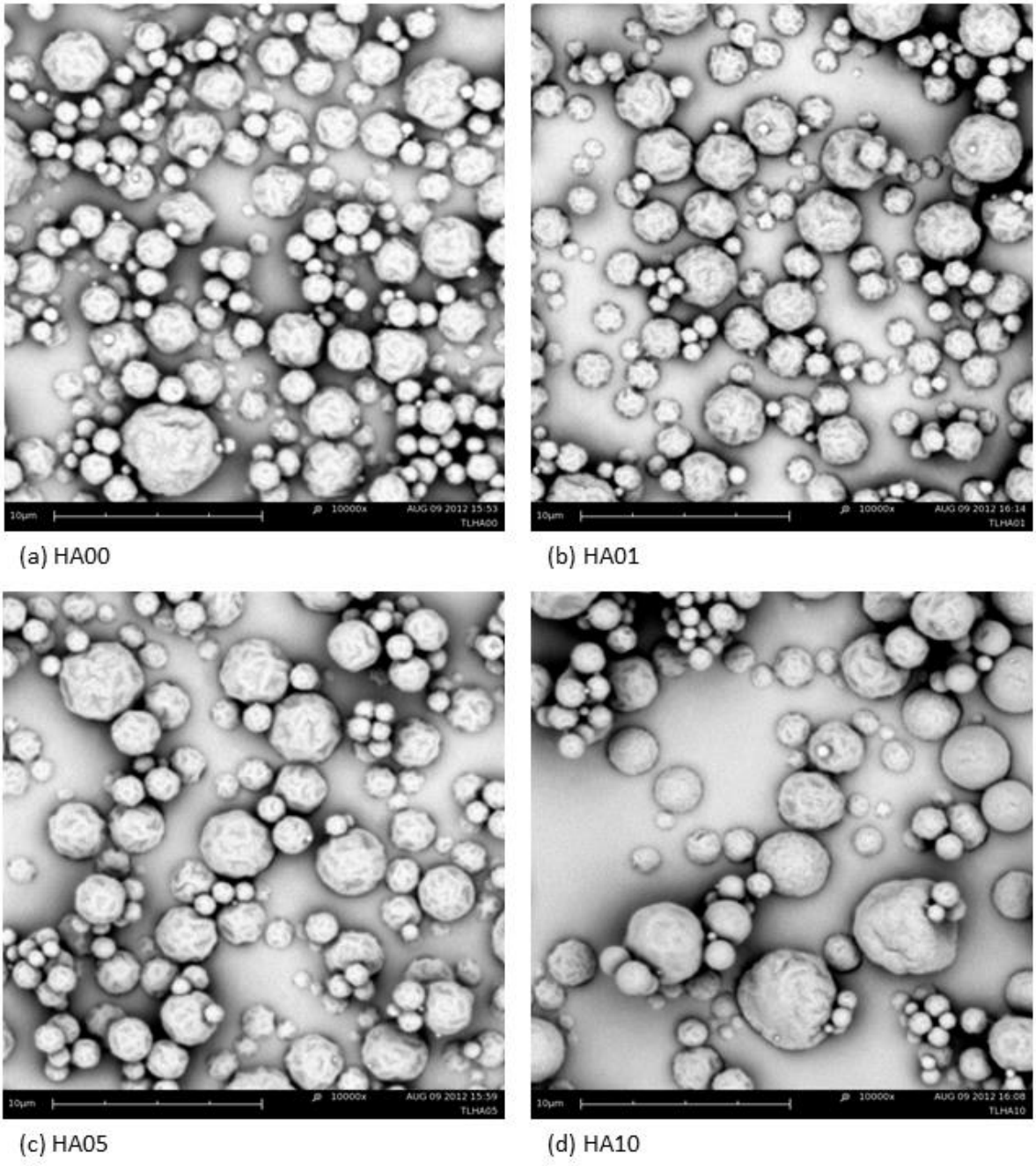

Figure 3. Representative scanning electron micrographs of spray-dried trehalose/leucine formulations containing no HA antigen (HA00), 1 µg HA/mg (HA01), 5 µg HA/mg (HA05) and 10 µg HA/mg (HA10).

6.3.4. Stability of antigen in the spray-dried formulations

To evaluate stability of the powder vaccine (HA05) at an accelerated condition, the HA titres of the reconstituted powder vaccine stored at 40 °C were compared to the HA titres of the liquid antigen stored in refrigerated conditions. The result shows (data not shown) that the integrity of the HA antigen was not affected by the spray-drying conditions used in the study as demonstrated by the equivalent HA titres of the dry powder formulation when compared to the liquid antigen before spray-drying. In addition, the reconstituted powder vaccine also showed HA titre comparable to the liquid antigen stored in refrigerated conditions after being stored at 40 °C for 2 months.

6.3.5. Systemic immunity

In order to compare the ability of the spray-dried powder vaccine to induce immunity after pulmonary administration to the liquid vaccine after pulmonary and subcutaneous administration, rats were immunised with either the spray-dried powder vaccine or the liquid vaccine reconstituted to the same HA concentration. Comparative analysis of the sera HAI, IgG and IgA titres induced from each formulation are shown in Figure 4 and 5. The influenza antigen-specific antibody responses in serum as demonstrated by the HAI titres were higher from rats immunised via the pulmonary route compared to the SC route for both the reconstituted powder and liquid vaccines (Figure 4). Sera IgG and IgA against the specific influenza antigen were also significantly higher after pulmonary immunisation compared to subcutaneous immunisation (Figure 5). The integrity of the influenza antigen, therefore, did not appear to have been compromised by the spray-drying process as demonstrated by the

comparable immunity induced by the spray-dried powder and liquid vaccines. Interestingly, the reconstituted powder vaccine appeared to induce immunity more efficiently as demonstrated by the higher antibody titres compared to the liquid vaccine via both the pulmonary and subcutaneous routes after the first immunisation at day 14.

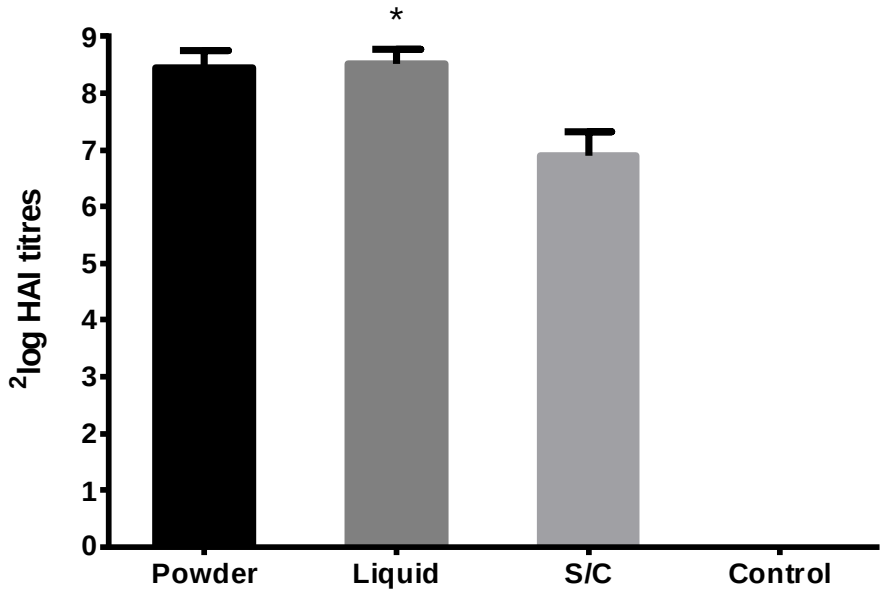

Figure 4. Haemagglutination inhibition (HAI) titres of sera at day 28 (mean ± S.E.M.; n = 4 for the S/C group, n = 5 for all other groups). Comparative analysis of HAI titres: (*) liquid vs. S/C, $p < 0.05$.

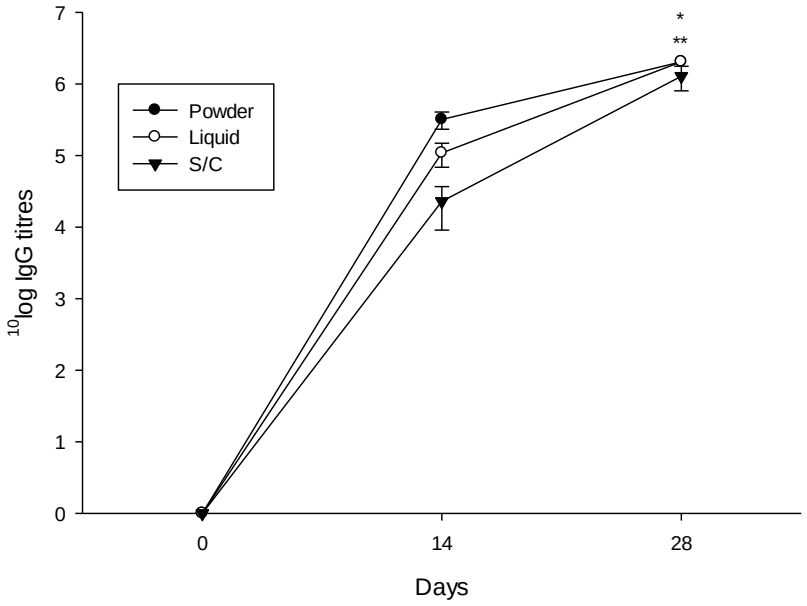

Figure 5a. Serum IgG (mean ± S.E.M.; n = 4 for the S/C group, n = 5 for all other groups). Comparative analysis of specific mucosal IgG responses against inactivated influenza virus antigen (A/Solomon Islands/03/2006) in sera of rats: (*) powder vs. S/C at day 28; (**) liquid vs. S/C at day 28; $p < 0.05$.

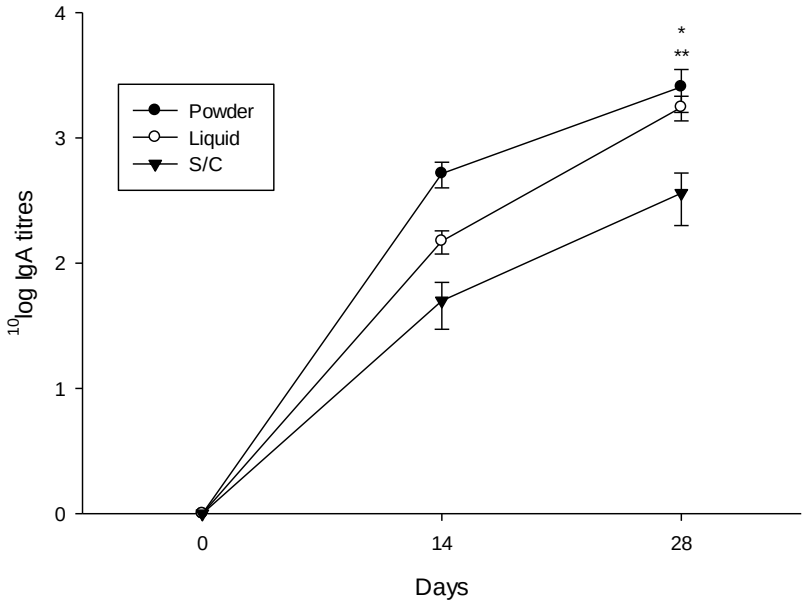

Figure 5b. Serum IgA (mean ± S.E.M.; n = 4 for the S/C group, n = 5 for all other groups). Comparative analysis of specific mucosal IgA responses against inactivated influenza virus antigen (A/Solomon Islands/03/2006) in sera of rats: (*) powder vs. S/C at day 28; (**) liquid vs. S/C at day 28; $p < 0.05$.

6.3.6. Mucosal immunity

To evaluate the mucosal immunity induced from the vaccines via different routes of administration, antibody titres were determined in BAL collected from the animals at day 28. The mucosal IgA and IgG titres from BAL at day 28 are shown in Figure 6. The results indicate that pulmonary immunisation induced stronger mucosal immunity compared to subcutaneous immunisation, as demonstrated by the higher IgA and IgG titres. In particular, both the reconstituted powder and liquid vaccines induced higher IgA titres in BAL after pulmonary administration.

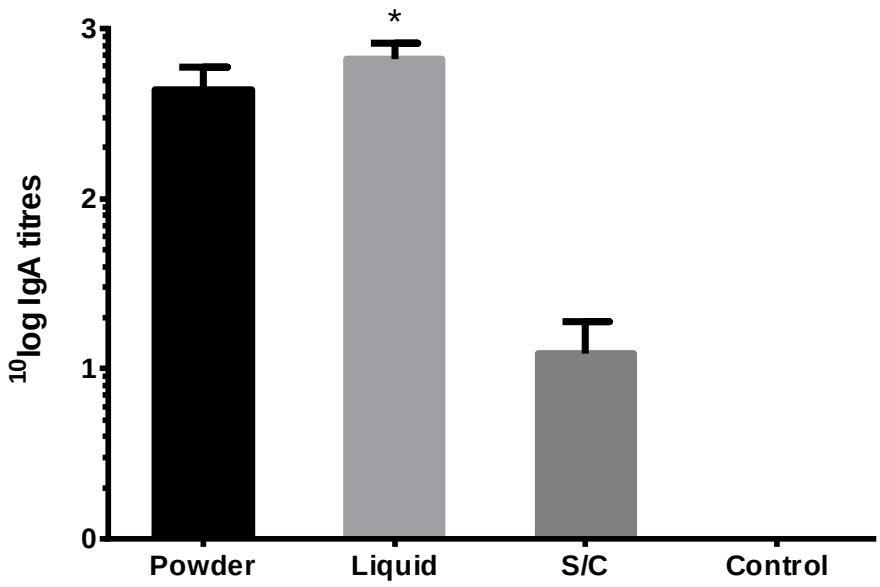

Figure 6a. Mucosal IgA titres in bronchial-alveolar lavage (BAL) fluid at day 28 (mean ± S.E.M.; n = 4 for the S/C group, n = 5 for all other groups). Comparative analysis of specific mucosal IgA responses against inactivated influenza virus antigen (A/Solomon Islands/03/2006) in BAL fluid at day 28: (*) liquid vs. S/C, $p < 0.05$.

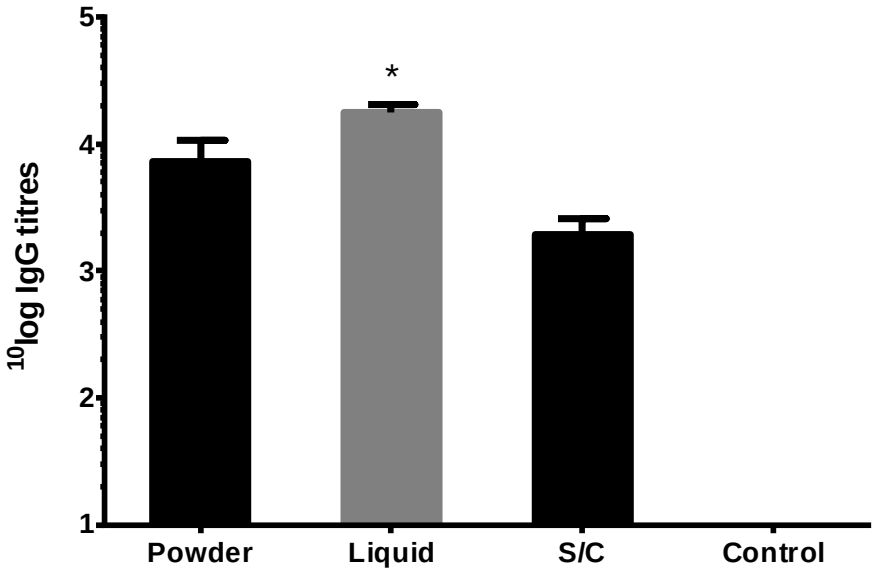

Figure 6b. Mucosal IgG titres in bronchial-alveolar lavage (BAL) fluid at day 28 (mean ± S.E.M.; n = 4 for the S/C group, n = 5 for all other groups). Comparative analysis of specific IgG responses against inactivated influenza virus antigen (A/Solomon Islands/03/2006) in BAL fluid at day 28: (*) liquid vs. S/C, $p < 0.01$.

6.3.7. Histological examination of lung parenchyma

To determine any potential impact on the lung parenchyma after pulmonary immunisation of the spray-dried formulation, the caudate lobes were collected from each rat and examined for histological evidence of tissue damage. The H&E images of the lungs from the various treatment groups are shown in Figure 7. There was no evidence of pulmonary inflammation from any of the formulations administered. The spray-dried powder formulation platform did not cause any acute toxicity to the lung parenchyma as demonstrated by the absence of tissue damage, oedema or inflammatory cell infiltration.

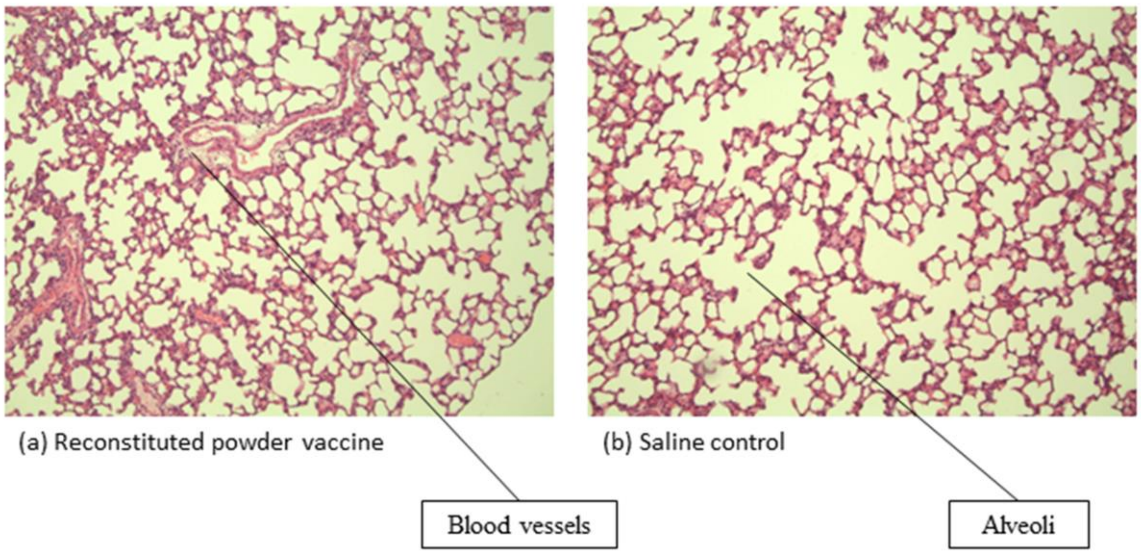

Figure 7. H&E sections of the caudate lobe of the lungs collected from the treatment groups: (a) reconstituted powder vaccine; (b) saline control. [x 100 HE]

6.4. Discussion

Most vaccines are formulated as liquid formulations which typically require storage in refrigerated conditions. Dry powder formulations that can circumvent the need for cold-chain storage would be beneficial, especially in developing countries where access to refrigeration and reliable electricity supply is an issue (Carstens, 2009; Geeraedts et al., 2010; Sou et al., 2011a). Spray-drying as a methodology to generate respirable dry powders has gained increased interest due to its simplicity, adaptability, cost-effectiveness and scalability (Fourie et al., 2008). Particles can be engineered to contain various ingredients (e.g., different actives and excipients) by adjusting the content of the feed solution to manipulate the properties of the dry powder formulation.

Most antigens are labile biomacromolecules, including proteins and peptides, with complex tertiary and quaternary structures. The maintenance of these macro structures in their native state is essential for the activity of the biomacromolecules.

Spray-drying generates heat stress during the drying process and mechanical stress during the atomisation process that can compromise the integrity and viability of the antigen (Andya et al., 1999; Namaldi et al., 2006; Tzannis and Prestrelski, 1999a, b). Furthermore, dehydration stress caused by the removal of water molecules from the surface of the antigen may also destabilise the protein (Tzannis and Prestrelski, 1999a, b). Ideally, the temperature used in the drying process should be high enough to allow efficient drying but not too high such that the viability and integrity of the antigen is compromised. While the mechanical stress achieved during the atomisation process cannot be avoided, tests should be performed to ensure sufficient biological activity is maintained. In the present study, the manufacturing process was developed to minimise impact on the integrity of the HA antigen by using a relatively low outlet temperature of 70°C. The results indicate that the spray-drying conditions used in the present study did not compromise the bio-activity of the HA antigen as demonstrated by the comparable HA titres and immunogenicity of the spray-dried vaccine to the liquid antigen before spray-drying.

In order to preserve the biological characteristics of biomacromolecules in a dried solid-state, the use of sugars and polyols as stabilising excipients has been studied extensively (Sou et al., 2011a; Vehring, 2008). For instance, mannitol has been widely reviewed as a stabiliser for dried protein pharmaceuticals (Bakaltcheva et al., 2007; Kibbe, 2000; Schüle et al., 2007; Schule et al., 2008; Torrado, 2002). However, previous studies have demonstrated the reliance upon comparably large amounts of 50% to 90% w/w of protein present for the formation of amorphous mannitol glassy matrix (Andya et al., 1999; Costantino et al., 1998; Hulse et al., 2008; Maa et al., 1997; Schule et al., 2008; White et al., 2005). These systems may not be ideal for more potent active proteins such as vaccine antigens for which only a small amount of the active protein

(e.g. <1% w/w protein loading) is typically required in the formulation. The potential utility of co-spray-dried trehalose/leucine formulation as a universal dry powder delivery platform for pulmonary delivery of biomacromolecules has been previously reported. This approach retained the amorphous nature of the powders as a standalone carrier platform with a high fine particle fraction of >65% (Sou et al., 2013). In the present study, the HA protein content contained in the formulations ranged from 0.1% to 1% w/w, which corresponds to a total protein content of 0.24% to 2.36% w/w. The results show that this formulation platform was able to incorporate the range of protein concentrations studied without compromising the formation of the amorphous glassy matrix.

While various influenza antigens have previously been incorporated into dry powder formulations, the impact of HA concentrations on the thermal properties and therefore the optimal concentration of influenza antigen to be used in dry powder formulations have not been thoroughly investigated (Amorij et al., 2007; Audouy et al., 2011; Coucke et al., 2009; de Jonge et al., 2007). Previous studies have shown that the concentrations of various proteins had little influence on the Tg values of lactose after spray-drying (Haque and Roos, 2004; Shrestha et al., 2007). It is likely that the HA protein in our formulation was more hydrophilic and therefore retained more water molecules within the particles which in turn reduced the Tg of the formulation. This could potentially impact negatively upon the storage stability of the formulation. The result indicates that due consideration should be given to the concentration of HA protein being incorporated into the carrier platform for delivery of the required dose without excessively suppressing the Tg and therefore compromising the physical stability of the resultant formulation. The HA05 formulation appeared to be the most suitable vaccine and remained amorphous with an appropriate level of HA protein and

water content that maintained a reasonably high Tg. This was sufficient to maintain the activity of the HA antigen without refrigeration for an extended period of time up to 2 months.

Pulmonary dry powder influenza vaccines have been previously produced by spray-drying and spray-freeze-drying using inulin as a stabilising agent (Amorij et al., 2007; Audouy et al., 2011; Saluja et al., 2010). The results of those studies demonstrated that pulmonary administration is a promising route of delivery which induces systemic immunity that is comparable to parenteral immunisation in mice (Amorij et al., 2007; Saluja et al., 2010). Intranasal dry powder influenza vaccine containing trehalose as an excipient has also been studied in a rat model (Huang et al., 2004). However, the powder vaccine failed to induce systemic immunity comparable to parenteral immunisation even in the presence of chitosan as adjuvant. Interestingly, the antigen loading used in that study was 20 times higher than the dose used in the current study (Huang et al., 2004). In contrast, the results in the present study showed that pulmonary administration of spray-dried influenza vaccine using trehalose and leucine as excipients can induce strong systemic immunity comparable to parenteral immunisation in a rat model even in the absence of specialised adjuvant. In addition, the reconstituted powder vaccine appeared to induce immunity more rapidly as demonstrated by the higher antibody titres compared to the liquid vaccine via both the pulmonary and subcutaneous routes at day 14. While the exact mechanism is subject to further investigation, it is possible that the exposure of trehalose and/or leucine to the pulmonary mucosa may have stimulated the antigen presenting cells to process antigen more efficiently and therefore enhanced the immune response against the antigen.

Secretory IgA specifically transported into mucosal secretions plays an important role in mucosal defence against pathogens via various mechanisms such as

inhibiting bacterial adhesion and absorption, neutralising virus and bacterial toxins and inhibiting the pro-inflammatory effects of other immunoglobulins (Holmgren and Czerkinsky, 2005; Pulliam et al., 2007). Mucosal immunity at the pulmonary mucosa therefore provides more immediate and direct protection, especially against respiratory pathogens such as influenza and tuberculosis. Previous studies have demonstrated stronger mucosal immunity induced after pulmonary immunisation of influenza vaccines stabilised with inulin when compared to the intramuscular route (Amorij et al., 2007; Saluja et al., 2010). However, the fine particle fractions (FPF) of those dry powder formulations were less than 38%. In contrast, the vaccine formulation employed in the present study demonstrated a substantially higher FPF of about 50%. Furthermore, in contrast to the previously reported intranasal dry powder influenza vaccine containing trehalose with chitosan as adjuvant, the trehalose-based vaccine formulation used in the current study induced higher mucosal IgA titres when compared to the parenteral route (Huang et al., 2004). In particular, this high immune response was achieved with a lower antigen loading even in the absence of any mucosal adjuvant. With the proven aerosolisation performance and high immunogenicity demonstrated, the spray-dried delivery platform in the present study appears to be an optimal delivery system for pulmonary immunisation of influenza vaccine.

Since the spray-dried carrier platform constitutes the majority of the vaccine formulation, it is important that the excipients are safe and non-toxic to the lung parenchyma. In contrast to treatments for chronic conditions where ongoing administration of therapies is required, acute damage to local tissues is particularly relevant since vaccines are usually given as a once-off treatment with booster immunisations as required. Specific histological examination of lung parenchyma after pulmonary administration of trehalose and leucine has not been previously reported. In

the present study, these excipients in the vaccine formulation did not cause detectable acute toxicity to the lung parenchyma. The results indicate that the spray-dried carrier platform was able to deliver the influenza antigen for induction of immunity without causing acute adverse effects on the epithelium.

6.5. Conclusion

The present study has demonstrated the potential of our spray-dried carrier platform as an approach to produce stable and inhalable dry powders of influenza vaccine. The spray-drying conditions used were gentle enough to allow incorporation of the influenza antigen without compromising its haemagglutination activity and immunogenicity. The results indicated the amount of protein incorporated might impact on the water content and Tg of the dry powder formulation. However, the low protein loadings did not appear to have a substantial effect on the formation of amorphous matrix and morphology of the spray-dried particles. In addition, the formulation remained aerosolisable and was shown to induce superior mucosal immunity with at least comparable systemic immunity after pulmonary delivery when compared to subcutaneous immunisation. The excipients in the spray-dried formulations did not cause any detectable acute toxicity to lung parenchyma. With proven aerosolisation performance and high immunogenicity, this spray-dried formulation appears to be a promising platform for pulmonary delivery of influenza vaccine. The highly aerosolisable dry powder delivery platform may also be employed for pulmonary delivery of other biomacromolecules. Studies to further explore the potential utility of this delivery system with other biomacromolecules are currently being undertaken.

6.6. Acknowledgements

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CHAPTER SEVEN

CONCLUSIONS AND FUTURE PERSPECTIVES

7. Conclusions and future perspectives

7.1. General conclusions

The introduction to this thesis provides a review of the literature on pulmonary immunisation and on respective spray-drying technology. Previously, pulmonary immunisation has been demonstrated to be an effective means for vaccination. In comparison to parenteral vaccination, the attractive properties of pulmonary vaccines include the superior local mucosal immunity and systemic immunity, absence of complications associated with injectable delivery, and ease of administration. While superior local immunity is particularly beneficial for protection against infections originating from the respiratory tract, such as influenza and tuberculosis (TB), the connection of various mucosal sites through a compartmentalised mucosal immune system presents the opportunity to immunise against diseases originating from a remote mucosal site via the pulmonary route.

In particular, dry powder vaccine formulations are argued to provide a real opportunity for improved antigen stability compared with conventional liquid formulations, and may enhance the immunity induced after vaccination. By using appropriate excipients and manufacturing process, the formulation may be engineered to be readily aerosolisable and capable of stabilising the antigen. The improved stability may negate the necessity of cold-chain storage that is a typical requirement of most liquid vaccine formulations. The properties of such dry powder formulations may also be optimised (e.g., chemical stability, physical stability, delivery efficiency) by using the appropriate processing conditions. In addition, needleless vaccines could remove the need of medically trained personnel for administration, and thus the complications

resulting from the risk of needle-stick injury and the subsequent transmission of blood-borne viruses, and could be particularly appealing to patients with needle-phobia.

Spray-drying has received increased attention for the production of dry powder vaccine formulations owing to its relative simplicity, cost-effectiveness and scalability. Spray-drying also allows the engineering of multi-component particles which cannot be readily achieved with other manufacturing processes. Furthermore, the incorporation of suitable stabilising excipients during spray-drying can help to preserve the integrity of antigenic macromolecules and improve the stability of dry powder vaccine formulations. It is proposed that the spray-dried formulations may be engineered to provide the most desirable properties by adjusting the processing parameters. With improved understanding about the particle formation mechanisms and distribution of compounds within the drying droplets, the process could potentially be optimised to enhance properties of the resultant formulations. To date, the optimal formulations with the most ideal properties (i.e., robust stability, reproducible aerosolisation, moisture protection, particle separation, high processing yield) remained to be defined.

To capitalise on inhalable spray-dried vaccine formulations, it is necessary to resolve issues in the development process, such as the most suitable combination of parameters during the spray-drying process and the development of a dry powder composition carrier platform that is capable of stabilising the antigen in a dry solid state using toxicologically acceptable excipients. Many excipients have also been considered to improve the aerosolisation properties and performance of inhalable dry powder formulations. Leucine has been consistently demonstrated under selected conditions to improve aerosolisation and performance of dry powder inhaler formulations. This dispersibility enhancing property appears to be specific to leucine, as other amino acids do not confer equivalent improvements in aerosolisation. While effects produced from

the use a single amino acid on the performance of a dry powder formulation has been extensively-studied, the specific interaction effects resulting from the combined use of multiple amino acids is not well understood. Such interactions may allow or prevent multiple powder functionalities being achieved. Consequently, in the thesis introduction, the hypothesis was proposed that a novel dry powder delivery system for pulmonary vaccine delivery can be developed via the combined use of appropriate excipients and optimisation of spray-drying process.

The first aim to test this hypothesis was to determine interactions effects of adjusting amino acid excipient compositions on various proportions of a mannitol-based spray-dried powder. Using a design of experiment (DoE) approach, the results, in Chapter 2, indicated that interactions between combinations of excipients used in spray-dried formulations can be identified hence leading to a deeper study of such combination effects and greater understanding of the study design space. The results from the study show that the use of glycine and/or alanine, though structurally related to leucine, can provide detrimental effects on particles properties both during and after spray-drying with mannitol. However, the combination of leucine with glycine and/or alanine provides particles with the inherited benefits of leucine on particle separation and aerosolisation. This work further indicates that there is potential to investigate broader ranges of concentrations of combined amino acids that may lead to additional benefits, such as particle morphology control or stable glass formation. It is worth noting that the results from the DoE analysis revealed a high degree of complexity with the lack of linearity of the effects achieved from the combination use of these amino acids across the concentration range within the study design space. This information should be considered in future study design when investigating the optimal concentration and effect of these potential performance enhancing excipients.

The second aim to test this hypothesis was to look further at the effect of amino acid excipients on morphology and more specific solid-state properties of multi-component spray-dried powder formulations for pulmonary delivery of biotherapeutics. The study described in Chapter 3 has provided some important new insights into particle engineering using amino acid and sugar excipients, namely mannitol and trehalose, for the development of dry powder carrier platforms for pulmonary delivery of potent biomacromolecules. The results in the study are consistent with the notion that leucine is able to facilitate particle formation as an encapsulating agent and therefore allows the formation and production of multi-component particles while maintaining appropriate particle size and dispersibility for pulmonary delivery that would otherwise not be possible. The study looked further at the resulting leucine precipitate, and the results have demonstrated that a partially ordered leucine, resulting from self-assembly on the particle surface, is important for the amino acid to function effectively as an encapsulating agent, for optimal particle formation and might also play a role in inhibiting crystallisation of other components within the formulation. This conflicted with earlier studies, giving a new and enhanced insight into both the quantity and quality of the layers. While earlier publications had indicated around 40% w/w leucine was required, the results from this study suggest that, with suitable particle size, dispersibility and solid-state properties, the trehalose/leucine combination with leucine 10–20% w/w appears to have potential for development into a universal carrier platform for pulmonary delivery of potent biomacromolecules. These combinations deserve further investigation, especially with respect to moisture sensitivity and a thorough investigation of glass transitions. Higher concentration of leucine (i.e., >20% w/w) were shown to lead to significant changes in particle morphology, but these may not be necessary to achieve the desired outcomes. With the particle engineering strategies

utilised in this study, other baseline material such as alternative sugar molecules and polymers may also be investigated.

The third aim to test this hypothesis was to identify the effect of bio-compatible polymers on the formation of an amorphous matrix in a multi-component spray-dried particulate platform for pulmonary delivery of biotherapeutics. While sugars such as mannitol and trehalose have been widely studied, polymeric excipients have received less attention in this context. The results presented in Chapter 4 revealed some interesting behaviour of several polymer-based formulations after co-spray-drying with leucine. While polymers are typically more difficult to spray-dry, the addition of leucine as a particle formation agent was shown to be a promising approach to substantially improve yield. The results from X-ray powder diffraction (XRPD) studies suggested the formation of the same self-assembled leucine shell coating the surface of these amorphous particles as previously reported. These polymer-based formulations formed particles with high surface corrugations that were likely to assist dispersion. In addition, these polymers effectively inhibited crystallisation of the model sugar after co-spray-drying. However, these amorphous mannitol-containing particles were highly hygroscopic and the recovery of particles from the spray-dryer was unacceptably low in the absence of leucine. Scanning electron micrographs (SEM) revealed the different morphologies of these particles depending on the polymer incorporated and the formation of extensive solid bridges between the PVP-based particles.

As an extension of this work, the fourth aim was to identify the effect of molecular weights of excipients ranging from polyol, disaccharide, polysaccharide and synthetic-polymer on solid-state properties of a multi-component spray-dried particulate platform for pulmonary delivery of biotherapeutics. The study in Chapter 5 compared and contrasted the influence of molecular size on the resultant particle properties

including processing yields, solid-state properties and morphology of spray-dried formulations that are relevant to their performance in stabilising biomacromolecules as a particulate platform for pulmonary delivery of potent biotherapeutics. Previously, only formulations containing high protein content have been shown to prevent recrystallisation of spray-dried mannitol. The results in this study have shown that the addition of sodium citrate was highly effective in inhibiting crystallisation of mannitol. However, addition of sodium citrate significantly increased the hygroscopicity of the material, such that it could not be recovered after spray-drying. The addition of leucine dramatically improved yield by assisting particle formation to create a platform combination of excipients with multiple desirable properties suitable for pulmonary delivery that have not been previously reported: leucine as a coating agent for particle formation and efficient spray-drying, sodium citrate as a glass-forming agent for inhibition of mannitol crystallisation, and glycine as a morphological modifier for surface corrugation and flow improvement. In addition, this study has demonstrated for the first time the potential benefit of higher molecular weight compounds with polymeric properties, as an attractive alternative to small molecule excipients such as polyols and sugars, in providing an amorphous particulate platform suitable for pulmonary delivery. These polymeric systems retained appropriate glass transition temperature (T_g) in spite of the relatively high moisture content after spray-drying. Although these compounds alone were difficult to spray-dry, the addition of leucine assisted particle formation and improved processing yield significantly.

The fifth aim to test the main hypothesis was to determine the impact of incorporating a model antigen on physical and aerosolisation properties of a prototype spray-dried particulate platform and the in vivo immunogenicity of the subsequent spray-dried vaccine after pulmonary administration. The results reported in Chapter 6

provided in vivo proof-of-concept for the work reported in Chapters 2 to 5 and characterised the impact of inclusion of a model vaccine antigen, influenza haemagglutinin, on dry powder properties. The study demonstrated the potential of a selected prototype spray-dried carrier platform derived from the earlier studies as an approach to produce stable and inhalable dry powders of influenza vaccine. The spray-drying conditions used were shown to be gentle enough to allow incorporation of the influenza antigen without compromising its haemagglutination activity and immunogenicity. The results indicated the amount of protein incorporated impacted on the water content and T_g of the dry powder formulation. However, the low protein loadings did not appear to have a substantial effect on the formation of amorphous matrix and morphology of the spray-dried particles. In addition, the formulation remained aerosolisable and was shown to induce superior mucosal immunity with at least comparable systemic immunity after pulmonary delivery when compared to subcutaneous immunisation. The excipients in the spray-dried formulations did not cause any detectable acute toxicity to lung parenchyma. With proven aerosolisation performance and high immunogenicity, this spray-dried formulation appears to be a promising platform for pulmonary delivery of influenza vaccine. The highly aerosolisable particulate platform may also be employed for pulmonary delivery of other potent biotherapeutics.

The work in this thesis set out to improve the scientific knowledge and understanding towards the development of dry powder antigen formulations for pulmonary vaccine delivery. While the potential of pulmonary immunisation has been demonstrated for several decades, the use of this route for vaccine delivery has not been widely employed. Recent advancement in respiratory drug delivery using dry powder aerosols presents novel opportunities in formulation development. In particular, the

improved understanding in rational particle design and engineering via spray-drying enables unprecedented formulation strategies and designs. With most vaccine antigens being potent biomacromolecules required in small amount in the formulation, it became apparent that the characteristics of the resultant dry powder formulation would be dominated by the behaviour of the excipients. Subsequently, the necessity of improving the understanding and optimising the properties of a generic particulate platform delivery system, in conjunction with a suitable process that is capable of incorporating biomacromolecules for pulmonary delivery, became a logical priority. Hence, a major proportion of the work presented in this thesis, Chapters 2 to 5, has been dedicated to the development of such a particulate delivery system.

While the study reported in Chapter 2 investigated the interaction effects of various amino acids on a model sugar, mannitol, a formulation strategy, using an appropriate amount of leucine as particle formation agent while varying the composition of the other excipients, has been developed and validated in the subsequent chapters as a promising approach. This formulation strategy has been proven to produce highly aerosolisable powders consistently while allowing the adjustment of other properties (e.g., amorphicity and T_g) by facilitating the formation of multi-component spray-dried particles containing various excipients that might otherwise not be suitable for spray-drying. This particle formation effect of leucine was particularly prominent in Chapters 4 and 5 as demonstrated by the improvement in spray-drying yields of the polymer-based formulations. While non-reducing sugars (e.g., mannitol and trehalose) have been common candidates for stabilisation of dried protein pharmaceuticals, these small molecule compounds are more likely to experience crystallisation failure upon storage. In comparison, some higher molecular weight compounds such as polymers are typically characterised with a wide range of molecular weights and a resultant low

tendency of crystallisation. Several model polymers (i.e., inulin, dextran, PVP) were therefore investigated to determine their ability to form an amorphous matrix, either alone or in combination with a model sugar, and the thermal properties of these formulations in relation to change in molecular sizes after spray-drying, in Chapters 4 and 5, respectively. Though there is much room for further optimisation, these polymer-based systems showed desirable properties (i.e., high spray-drying yield, amorphicity, high T_g, aerosolisation) that warrant further investigation.

To this end, the most well-characterised dry powder delivery system in this work has been the trehalose/leucine combination. To validate the dry powder delivery system and manufacturing process is capable of incorporating an antigen for vaccine delivery, in Chapter 6, a model antigen, haemagglutinin, has been incorporated into the particulate platform to produce an influenza vaccine. While in vivo powder aerosolisation in a large animal model was beyond the scope of this work, the results from in vitro aerosolisation study and subsequent immunisation of the reconstituted vaccine powder via the pulmonary route in a rodent model have supported the trehalose/leucine formulation as a promising particulate platform for pulmonary delivery of vaccine. While the work in this thesis set out to develop a dry powder platform system for pulmonary vaccine delivery, the studies and formulation approaches have been designed to cater for the respiratory delivery of, not only antigens, but the family of biomacromolecules as a therapeutic category. Though beyond the scope of this work, with the promising results, the potential use of the various formulation platforms reported herein for pulmonary delivery of other biotherapeutics deserve much further investigation. For instance, towards completion of this programme, the outcomes have recently been employed in the home institution as the foundation in designing a novel inhaled formulation for systemic delivery of a peptide, oxytocin, via

pulmonary administration and is currently forming a potential product with significant opportunity to save lives at childbirth.

7.2. Future perspectives

This work has highlighted a number of areas that require further research, in order for the broader aims to be addressed. Firstly, a low-cost, easy-to-use, single-dose disposable device for aerosol pulmonary vaccine delivery of such a spray-dried powder is not widely available yet. The testing and optimisation of device technology to achieve the highest delivery efficiency to complement the formulations will be highly beneficial. The optimised design of device may not only be used for pulmonary immunisation, but also more broadly for systemic delivery of other biotherapeutics. Such a device could have major implications for developing countries, where affordable treatment options are of particular importance. For populations in the poverty, inhaled therapy for various conditions remained a luxurious treatment options that is largely underutilised, compared to more conventional treatments such as oral dosage forms. The device would allow opportunities for the provision of not only low-cost once-off interventions such as vaccines, but also the possibility of breaking down the cost of chronic therapies into more affordable treatment sessions to the populations for whom the full course of such therapies could be prohibitively expensive.

Secondly, options of adjuvants suitable for pulmonary immunisation remain limited. The lung parenchyma and thin epithelial linings of the alveolar region, while providing greater permeability for biomacromolecules compared to other mucosal membranes (e.g., oral, buccal and nasal), are also more susceptible to damages to exogenous compounds. Common mucosal adjuvants such as cholera toxin and heat-

labile enterotoxin from *Escherichia coli* are not suitable for pulmonary delivery due to toxicity concerns. Novel classes of mucosal adjuvants which are compatible to the lung parenchyma for pulmonary immunisation are yet to be explored. New discovery of such adjuvants might not only enhance the immunogenicity of vaccines, thus reducing the dose of antigens required, but also expand the options of antigens, which are not usually sufficiently immunogenic alone for pulmonary vaccination, that could be delivered via the pulmonary route.

Thirdly, the dissolution rate of dry powder formulations upon contact with respiratory mucosal membranes after delivery has not been thoroughly investigated. The change in dissolution properties of inhaled particles may influence absorption profiles and subsequently the pharmacological responses. However, the fate of dry powder formulation after inhalation within the respiratory tract remains largely unclear. Improved understanding of particle dissolution after inhalation will provide insight in future formulation strategy such as the use of different excipients and drying process to optimise dissolution properties and, ultimately, the efficacy of the formulation. This field is particularly relevant for compounds with low solubility for which the pharmacokinetic responses after inhalation are most likely influenced by their dissolution profiles within the respiratory tract. The dissolution properties may also be studied in conjunction with regional deposition profiles of the formulation after inhalation to investigate the influence of regional concentration of the formulation in situ within the respiratory tract on the subsequent pharmacokinetic profiles.

The optimisation of spray-drying process and spray-dryer design catering for the production of dry powder formulation for potent biotherapeutics could also be valuable. For instance, spray-dryers with higher drying efficiency will allow the use of lower drying temperature, and thus minimise stability issues when processing heat sensitive

biotherapeutics. The different designs in atomising mechanisms (e.g., two-fluid nozzles and piezoelectric vibrating mesh), and thus the different mechanical stress during the atomising process, will have different implication on stability of the antigen. In addition, nanoparticle technology may be utilised to improve the design of dry powder formulations with more sophisticated particle engineering. Since rational particle design and engineering for dry powder formulations using spray-drying is a relatively young discipline. With continuing advancement in the understanding of particle formation process during spray-drying, and thus the improved control over the properties of the resultant formulations, optimisation of the spray-drying process is likely to be an area to receive much further interest.

Furthermore, the generation and characteristics of immune responses after delivery of antigen to different regions of the respiratory tract, and therefore the optimal site for antigen delivery and the underlying antigen presentation mechanisms remains largely unclear. The recent advancement of drug delivery technology targeting the lymphatic system may be further exploited to enhance the induction of immunity in the lymph nodes. Such vaccine delivery strategy may potentially induce immunity more efficiency by allowing more direct access of antigen to the immune system. Recently, the uptake of nanoparticles by macrophage has received much interest. Nanoparticles with suitable properties may be preferentially taken up by macrophages. Carefully designed and engineered nanoparticles may be used to promote uptake by these antigen-presenting cells in the immune systems to promote induction of immune response. The systemic delivery of other potent biotherapeutics using a similar inhalable dry powder delivery platform via the pulmonary route may also be investigated.

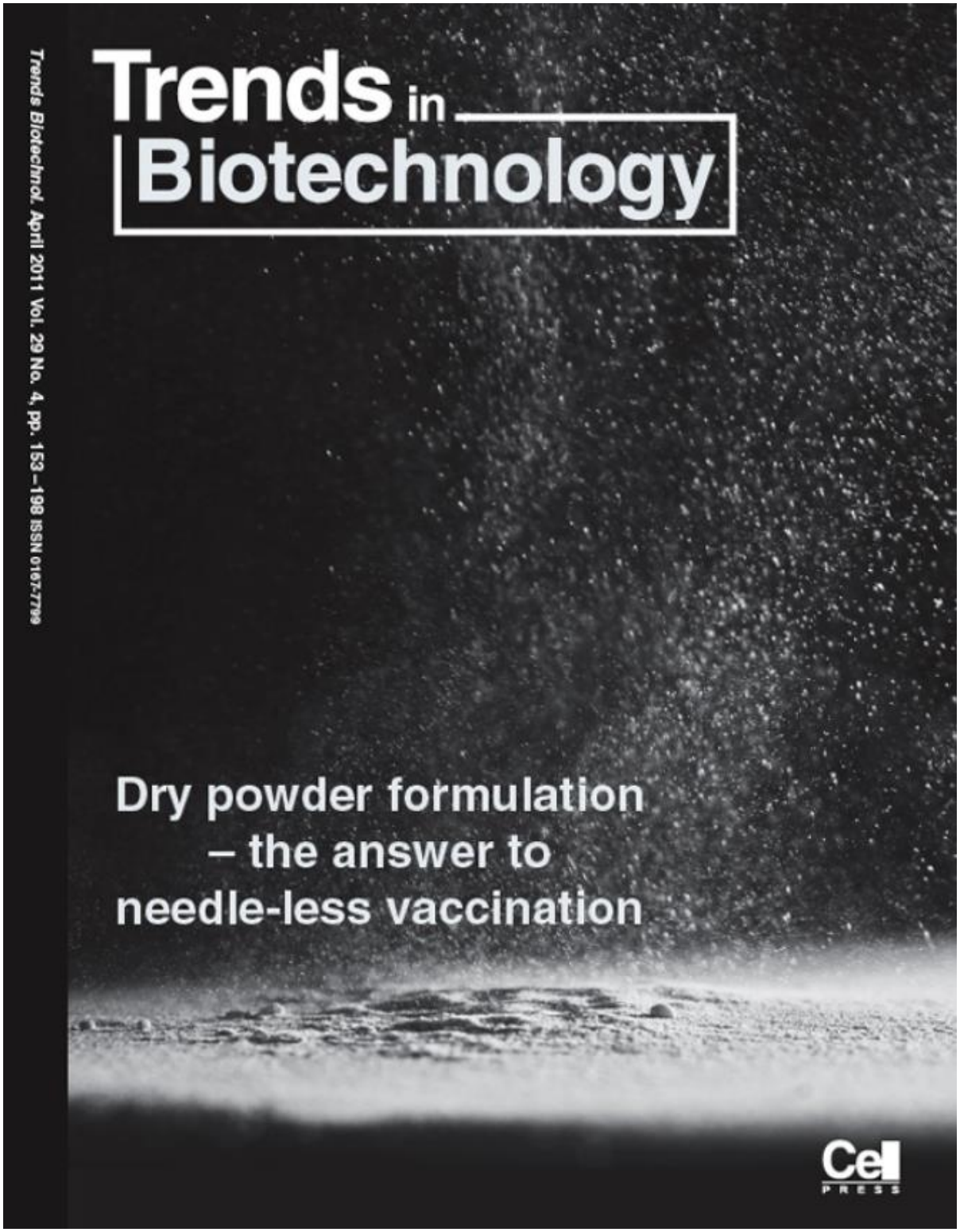
CHAPTER EIGHT

APPENDICES

8. Appendices

8.1. Appendix I

Sou, T., Meeusen, E.N., de Veer, M., Morton, D.A.V., Kaminskas, L.M., McIntosh, M.P., 2011. New developments in dry powder pulmonary vaccine delivery. Trends in Biotechnology 29, 191-198. (Featured Journal Cover)


Trends in Biotechnology
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Dry powder formulation – the answer to needle-less vaccination
Cell PRESS

Review

New developments in dry powder pulmonary vaccine delivery

Tomás Sou[1], Els N. Meeusen[2], Michael de Veer[2], David A.V. Morton[1], Lisa M. Kaminskas[1] and Michelle P. McIntosh[1]

[1] Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria 3052, Australia
[2] Biotechnology Research Laboratories, School of Biomedical Sciences, Monash University, Clayton, Victoria 3800, Australia

Pulmonary immunization has gained increased recognition as a means of triggering both a mucosal and systemic immune response without the use of needles. The appropriate formulation of antigens in a dry, solid state can result in improved stability, thereby removing cold-chain storage complications associated with conventional liquid-based vaccines. The particulate nature of dry powder vaccines could also induce a better immune response. This review describes our current understanding of pulmonary immunization, including possible barriers facing the development of pulmonary vaccines, and discusses recent advances in spray-drying technologies applicable to the production of dry powder formulations for pulmonary vaccine delivery.

A need for alternative routes of vaccination

Immunization is an important public health strategy, and vaccination of susceptible populations is regarded by the World Health Organization (WHO) as the most effective way to reduce disease and death from infectious diseases [1]. Although vaccines for several diseases are widely available in many developed countries, access and uptake of immunization programs need improvement, especially in developing countries. The WHO estimates that at least 2 million children die from vaccine-preventable diseases each year, and millions more suffer from disability and illness because they have not been appropriately immunized [1]. In 2009, it was estimated that more than 23 million children worldwide, mostly in India and Nigeria, did not receive three doses of diphtheria–tetanus–pertussis (DTP) vaccine during their first year of life [2], and at least 49 countries, mostly in Africa, were not able to achieve 80% coverage of infants with measles-containing vaccines (MCV) [3]. The rapid mobilization of mass immunization campaigns internationally in response to the H1N1 swine influenza pandemic in 2009 reiterates the importance of vaccination in both developing and developed countries [World Health Organization (2009) Pandemic (H1N1) 2009. (http://www.who.int/csr/disease/swineflu/en/index.html)].

Most currently available vaccines are administered via a parenteral route as either intramuscular (i.m.) or subcutaneous (s.c.) injections. Parenteral administration is typically required because most antigens, such as proteins and polysaccharides, are macromolecules and are unable to penetrate into the systemic circulation via other less-invasive routes, such as oral and transdermal. However, parenteral vaccination has several drawbacks, which are particularly problematic in developing nations, including the requirement for skilled medical personnel for administration, the risk of needle-stick injuries, and consequently the transmission of blood-borne viruses. Furthermore, the majority of vaccine formulations available are dependent on the effective management of uninterrupted refrigerator storage conditions to maintain vaccine integrity. This cold chain requirement poses significant practical difficulties in the effective implementation of immunization campaigns in third world countries where a reliable supply of electricity and cold-chain storage is often lacking. Therefore, alternative formulations and routes of administration of vaccines are being explored to circumvent these issues [4]. Pulmonary vaccination utilizes the unique physiology of the respiratory system for immunization and confers several advantages over parenteral routes. Nevertheless, there are still no commercially available pulmonary vaccines on the market. Recent advances in drug delivery and formulation technologies provide unprecedented opportunities for potential growth in this field. This review describes our current understanding of pulmonary immunization and discusses dry powder formulations for pulmonary vaccine delivery.

Pulmonary vaccination to induce mucosal and systemic immunity

Mucosal immunization is the local delivery of antigen to a mucosal site to induce specific immunity against an invading pathogen. Given that many pathogens invade the host via mucosal membranes, non-invasive delivery of antigens to mucosal membranes provides more local and direct protection at the site of infection [5] and has the capacity to remove many of the complications associated with parenteral vaccinations. Furthermore, it has been suggested that this approach is more effective at inducing mucosal immunity (i.e. secretory IgA), without compromising the induction of systemic immunity [5,6]. For instance, influenza vaccines have been shown in several animal models to induce significantly higher mucosal antibody titres when delivered directly to the respiratory tract, compared with s.c. or i.m. injection [7–10]. In addition, the existence of an interconnected, compartmentalized mucosal immune

Corresponding author: Meeusen, E.N. [redacted]

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system might provide novel opportunities for more sophisticated immunization approaches such as multiple or remote mucosal site immunization via a more readily accessible mucosal membrane [5].

Pulmonary vaccination confers several advantages over parenteral and other mucosal routes of immunization. The higher mucosal antibody titre induced within the respiratory tract provides more immediate protection at the invasion site against pathogens entering via the lungs, such as influenza and tuberculosis (TB). In addition, the large surface area, extensive vascularization and thin epithelium in the alveolar lung tissue [11] might facilitate efficient systemic delivery of antigens, thereby reducing the dose required to induce protective immunity. Portable devices and ease of administration without the need of trained medical personnel would allow rapid and efficient immunization of mass populations during pandemics. Strategies to utilize the pulmonary system as an alternative approach for immunization have been investigated on numerous occasions over the past 50 years [12–15]. Recently, inhaled antigens of human papillomavirus (HPV) and pneumococcal polysaccharides have been shown to induce specific antibodies safely in patients and healthy volunteers [16–18].

Barriers towards development of pulmonary vaccines

As discussed in the previous section, several antigens have induced immunity after pulmonary administration, especially for antigens of respiratory infections [7,8,19]. However, despite the demonstrated efficacy, there are no commercially available pulmonary vaccines on the market. The major barrier in the development of pulmonary vaccines is the development of appropriate formulations for these antigen candidates. The thin epithelium that lines the respiratory tract provides good permeability to macromolecules; however, as a thin barrier, it can be vulnerable to external disturbances. Conventional formulations for parenteral vaccines might not be suitable for respiratory delivery because the safety profile of these formulations have not been established for pulmonary use. In particular, little is known with respect to adjuvants that can be used safely in the lung. The use of permeability enhancers such as surfactants and bile salts which bind to and open lipid membranes, might improve penetration and uptake of the antigen delivered, but could cause irreversible damage to the epithelium and even short-term loss of integrity of the pulmonary epithelium can have negative implications [20]. Long-term toxicity studies are required to identify chronic toxicity of novel molecules delivered to the lung. The recent success of ISCOMATRIX™, a saponin-based adjuvant [21–24], and chitosan, a non-toxic biodegradable polymer [25], both for pulmonary delivery, appears to be promising. Muramyl dipeptide (MDP) and trehalose dibehenate (TDB) have also been screened in *in vitro* studies for potential use in pulmonary tuberculosis vaccines [26]. The optimal region in the respiratory tract for vaccine delivery remains to be defined. Recent studies in mice suggest that access to the lower airways is required to produce a strong immune response [9,22]. However, there are distinct differences in the branching structures [27] and respiratory immune systems of humans and rodents [28]. It remains to be determined which structures within the complex human lung will elicit optimal immune responses.

Requirements for induction of a pulmonary immune response through vaccination

The structure of the lung is an important aspect in determining the type and strength of the immune response that will be induced after antigen delivery [29]. In humans and large animals, the lung parenchyma is supported by a dense network of conducting airways, consisting of the trachea separating into two major bronchi and branching out over several generations of progressively smaller bronchioles (Figure 1). The terminal bronchioles empty into the alveolar ducts and alveoli that constitute the lung parenchyma [30,31]. Rodent models have a restricted branching pattern and other important differences in lung structure that need to be taken into account when interpreting experimental results [32]. The lining of the conducting airways is a true mucosal surface, consisting of ciliated epithelial cells interspersed with mucus producing cells, and support the local production of antibodies in the underlying lamina propria. The first barrier when targeting this region of the pulmonary system is the dense mucus layer that facilitates trapping and continuous removal of particulate material through upward ciliary motion. The alveolar lining, however, consists of thin epithelial cells designed to allow maximum diffusion of inhaled gases into the underlying pulmonary circulation. The alveolar surface is coated with a thin aqueous film containing surfactants and is therefore ideal for absorption of drug molecules for systemic delivery. However, exclusive targeting of a vaccine to the alveolar regions might reduce the induction of local respiratory immunity by inducing a predominantly systemic immune response.

The induction of an immune response through vaccination requires the delivery of both pathogen-specific antigen(s) and an innate stimulus ('danger' signal), generally provided by an adjuvant. Most successful respiratory immunization studies have used vaccine preparations that have inherent adjuvant and/or epithelial cell targeting activity, including viral extracts or virus-like particles [16,33,34]; but incorporation of appropriate innate stimulators are required for antigens that have no inherent adjuvant properties [35]. Innate immune receptors that sense the presence of pathogen- or tissue-derived 'danger' signals are present on epithelial cells and alveolar macrophages, which are the first points of contact for inhaled vaccines (Figure 1). Pulmonary dendritic cells (DCs), once activated by innate stimuli, can transport antigens via the draining lymphatics to the local lymph nodes where they instruct naive T and B cells [36]. It is the effective priming and mobilization of antigen specific T and B cells that ultimately gives rise to immunity. Recently T- and B-cell priming was shown to also occur in inducible bronchus-associated lymphoid tissue (iBALT) within the lung [36,37]. The iBALT structure requires DCs for induction and maintenance, thus suggesting that bronchial DCs are attractive targets for vaccination [28]. It might be possible to target antigen presenting cells (APCs) within the lung by altering the composition and size of antigen particles to

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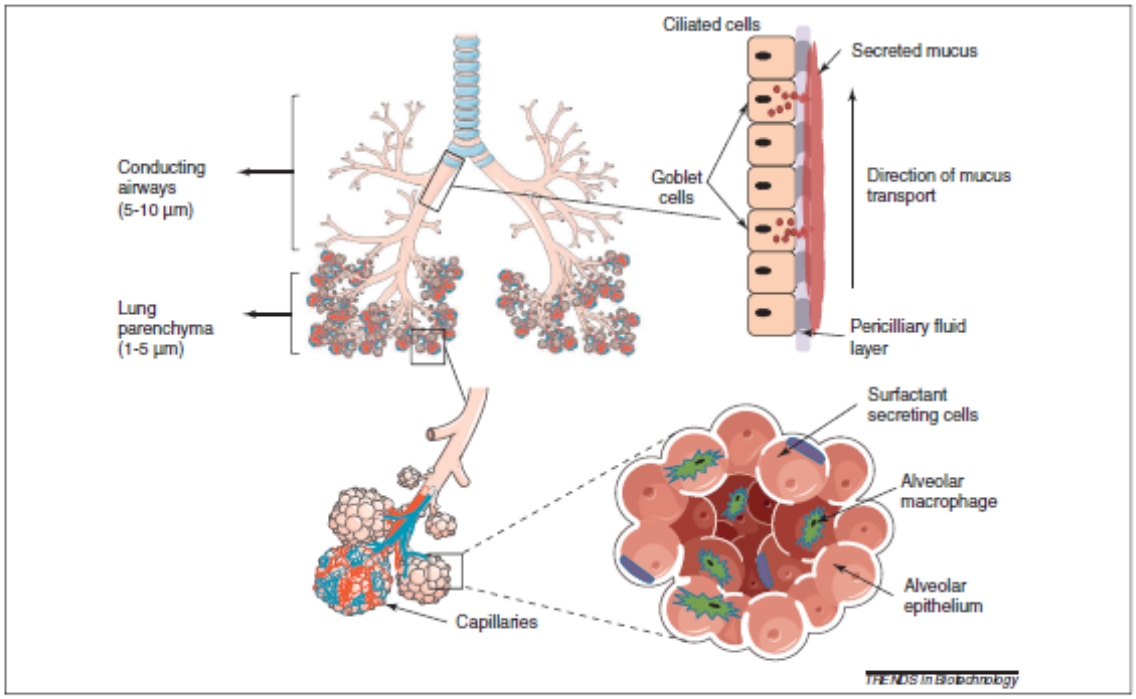

Figure 1. Diagram of the lung and particle-size requirements based on intended deposition region in the respiratory tract. Images on the right are magnified, cartoon views of tissue structures of the conducting airways (i.e. trachea, bronchi and bronchioles) and alveoli in the parenchyma region. The mucosal tissue of the conducting airways consists of ciliated epithelium and mucus producing goblet cells which remove inhaled antigens through upwards mucociliary clearance, with the help of secretary IgA produced by local plasma cells. Blind-ended alveolar sacs are lined by a specialized, thin-walled epithelium to aid gas exchange with the underlying capillaries, interspersed with surfactant producing epithelial cells. Immunosuppressive alveolar macrophages and serum-derived antibodies provide a final line of protection against invading pathogens. A cellular and humoral immune response can be generated after interaction with innate immune receptors present on epithelial cells and tissue-resident dendritic cells.

enhance phagocytosis [38] because it has been shown that antigens in particulate form are more immunogenic [33]. Optimization of particle size and incorporation of appropriate innate stimuli to enhance immunity are likely to be important factors in developing effective pulmonary vaccine strategies, as discussed below.

Dry powder pulmonary vaccine formulation

Advantages

Dry powder inhalers (DPIs) are available as simple, cheap, highly compact and disposable devices in a single use format, which is ideal for administration and distribution of vaccines (Table 1). Most antigens are macromolecules, such as polysaccharides, proteins and peptides, and are generally at a greater risk of chemical and physical degradation in liquid formulations [39,40]. Delivery of macromolecules in the form of dry powder aerosols to the pulmonary system has been widely suggested as a promising non-parenteral drug delivery route that provides improved stability when compared with conventional liquid formulations [20,39,41]. Stability of proteins can be further increased by formulating them in a dry, solid state with additives such as trehalose, sucrose and inulin [39,42,43]. The recent reports on dry powder insulin and measles vaccine formulations demonstrate stability without refrigeration [44–49].

The feasibility of dry powder aerosol vaccines has been demonstrated in several studies (Table 2). Dry powder influenza subunit vaccines prepared by spray-freeze-drying were shown to induce superior systemic and mucosal humoral and cell-mediated immune responses in mice after pulmonary delivery when compared with liquid vaccines administered via either the pulmonary or the i.m. route [8,50]. The effectiveness of aerosol delivery of spray-dried

Table 1. Qualitative comparison of different pulmonary vaccine delivery systems

	Nebulizers	Metered-dose inhaler (MDI)	Dry powder inhaler (DPI)
Delivery efficiency	+	–	+
Ease of use	–	–	+
Portability	–	+	+
Power supply	–	+	+
Stability of antigen	–	–	+
Cost	–	+	+

Key: (+), favorable; (–), unfavorable.

Table 2. Current status of representative dry powder vaccines

Pathogen	Dry powder methodology	Species used	Route	Outcome	Refs
Anthrax toxin	Freeze-drying	Rabbit	i.n.	Increased survival rate upon challenge	[72]
Bacille Calmette-Guerin (BCG)	Spray-drying	Guinea pig	p.	Dry powder vaccine was able to produce comparable tuberculin reaction compared with s.c. or i.d. routes and maintain viability after 9 months in refrigeration	[51]
Diphtheria toxoid + tetanus toxoid + pertussis toxin + *Haemophilus influenza*	Spray-drying	Guinea pig	s.c.	The tetravalent vaccines were strongly immunogenic in guinea pigs and high titres of persistent antibodies were induced	[73]
Hepatitis B virus (HBV)	Spray-drying	Guinea pig	p.	Dry powder vaccines induced protective systemic immunity and superior local mucosal immunity	[74]
Influenza	Spray-freeze-drying	Rat	i.n.	Dry powder vaccines induced much better IgA response with improved stability	[34]
Influenza	Spray-freeze-drying	Mouse	p.	Dry powder vaccine induced superior systemic humoral, cell-mediated and mucosal immune responses, as compared with the i.m. route	[8]
Influenza	Freeze-drying	Rat	i.n.	Dry powder vaccine induced good immunity and improved stability compared with liquid formulations	[10]
Influenza	Spray-drying and spray-freeze-drying	Mouse	p.	Dry powder vaccines induced significantly higher IgG titres and remained stable for at least 3 years at 20 °C	[50]
Measles	Spray-drying	Macaque	p.	Specific immunity was induced after dry powder vaccination although levels induced were lower than injection and nebulization	[52]
Tuberculosis	Spray-drying	Guinea pig	p.	Dry powder aerosol delivery of the antigens was able to reduce the extent of granuloma and necrosis growth in lung and spleen upon challenge	[75]
Yersinia pestis	Spray-freeze-drying	Mouse	i.d./i.m./i.n.	Dry powder vaccines induced good seroconversion from all routes	[76]

Abbreviations: i.d.: intradermal; i.m.: intramuscular; i.n.: intranasal; p.: pulmonary; s.c.: subcutaneous.

nanoparticles of Bacille Calmette-Guérin (BCG) in inducing immunity against *Mycobacterium tuberculosis* has been shown recently in guinea pigs [51], and specific immune responses have been demonstrated in macaques after dry powder inhalation of a measles vaccine [52]. In addition, it has been suggested that dry particulate antigens, as opposed to solubilized antigens, have more efficient access to APCs, leading to a more powerful immune response [33,53,54]. The development of inhalable dry powder vaccines therefore appears to be a promising new approach to pulmonary vaccination (Box 1).

Spray-drying for dry powder vaccine production

Spray-drying has gained interest owing to its relative simplicity, cost-effectiveness and scalability [19]. Spray-drying is a process in which the compound of interest is first prepared in a liquid form, typically a solution, which is then atomized and sprayed into a drying chamber where droplets are dried by heated air. The process can be optimized and has been used in the production of numerous stable protein-containing powders for inhalation [39,45,55] (Box 2). Because most antigens are proteins or peptides, spray-drying is an ideal methodology for the production of dry powder pulmonary vaccines that can readily be scaled up for commercial production.

Box 1. Ideal properties of dry powder formulations for pulmonary vaccine delivery

Particle size: Depending on the target delivery region, particles should exhibit desirable particle size distribution ideally with aerodynamic diameters of 5–10 μm for airways and 1–5 μm for deep lung delivery.

Aerosolization efficiency: The dry powders should aerosolize well upon inhalation with low surface energy and inter-particle cohesion forces and high fine particle fraction for maximal delivery efficiency.

Device: The dry powder inhaler should be simple to administer, without the need for trained personnel, and available as a single-dose, low-cost device for affordable production and efficient distribution in mass immunization programs.

Immunogenicity: The dry powder formulation can ideally be engineered with compounds that selectively stimulate innate immunity to improve the induction of appropriate immune responses.

Manufacturing: The manufacturing process of the dry powders should be readily scalable at low cost without compromising the integrity of the vaccines for mass production.

Stability: The formulation should be able to stabilize the antigen in a dry solid state without refrigeration for a reasonable shelf-life (i.e. 0.5–2 years).

Safety: Excipients and adjuvants used in the formulations need to be safe and biocompatible with lung tissue so to prevent irritation and irreversible damage to the lungs.

Box 2. Examples of spray-dried proteins and peptides in the literature

Insulin
Insulin has been spray-dried into inhalable dry powder formulations with various excipients, including leucine, glycine and lipids, to produce either a powder with high glass transition temperature (T_g) or particles coated with lipids for improved protein stability [45,49].

Influenza vaccine
The spray-dried influenza vaccine with inulin as a stabilizer was demonstrated to have good inhalation characteristics with a fine particle fraction (<5 μm) of 37%. The formulation has been shown to induce significantly higher antibody titres than i.m. injection and liquid aerosol and remain biochemically and physically stable after 3 years at 20 °C [50].

Bovine serum albumin (BSA)
BSA was spray-dried with different concentrations of polyvinyl alcohol (PVA) to produce various controlled-release formulations. The release profiles of the protein were shown to be altered with the use of PVA [68].

Recombinant human growth hormone
Recombinant human growth hormone was spray-dried, with polysorbate 20, Zn^{2+}, and lactose as stabilizing agents. A formulation with good aerosol performance (fine particle fraction = 39%) and protein stability (2.37% protein aggregation) has been successfully developed [69].

Immunoglobulin
Immunoglobulin has been spray-dried with various stabilizing agents including mannitol, sorbitol, trehalose, sucrose, glycine and isoleucine. The studies specifically discussed the effect of glassy stabilization and water replacement properties of sugars on aggregation of the protein and questioned the dominant role of glassy matrix immobilization in protein stabilization [39,42].

Lysozyme
Lysozyme was spray-dried with mannitol and empty liposome dispersions in an aqueous solution prepared by direct mixing of these components. The study successfully demonstrated lysozyme-loaded liposomal powders can be produced by spray-drying without preloading the liposomes with lysozyme before spray-drying [70].

Unfractionated heparin
Unfractionated heparin was spray-dried with L-leucine to reduce cohesiveness of the particles produced with the protein alone. The formulation produced small spherical particles (1–5 μm) that are suitable for inhalation with reduced tendency to form aggregates [55].

Serine alkaline protease
The protease enzyme was spray-dried with maltodextrin, sucrose and PEG 4000 as stabilizing agents. The additives were shown to improve stability of the enzyme at room temperature, with maltodextrin demonstrating the most significant effect in reducing the loss of initial proteolytic activity to 18%, compared to 38% with the spray-dried enzyme alone after incubation at 20 °C for 70 days [71].

There are many parameters that can be adjusted in the spray-drying process, such as the drying air temperature and humidity, the rate and fluid dynamics of the air flow, the atomization process (e.g. droplet size, spray rate, spray mechanism) and the composition of ingredients and excipients in the feeding solution [42,56–59]. Each of these parameters can be adjusted to fine-tune the characteristics of the powder produced until the desired properties for delivery are achieved (Table 3). The fluid dynamics of the air flow and the atomization process determine the droplet size, which in turn affects the size of the formed particles. The temperature and humidity of the drying air affects the drying rate which is a critical factor in the movements of compounds within the drying droplet and therefore distribution of compounds within the resulting particles. The composition of the feeding solution determines the composition of the resulting particles and can be customized to produce particles with multiple active ingredients or excipients. The ability to engineer particles with optimized characteristics for lung delivery will greatly improve the likelihood of the successful development of inhalable vaccine formulations.

Particle size of spray-dried particles

Particle size influences the site of deposition of particles delivered via inhalation, with particles larger than 10 μm in diameter primarily deposited in the airways, and particles under 3 μm in diameter deposited most effectively in the alveolar region (Figure 1). Smaller particles (i.e. <1 μm in diameter) are arguably less suitable for alveolar delivery because they might be exhaled. Additionally, it is well-known that inter-particle cohesive forces become more dominant with decreasing particle size [60], which in turn reduces flow and aerosolization properties of the powder bulk. However, there is evidence to suggest that nanoparticles could be taken up more effectively into the lymphatic system [61,62], which acts as communication channels for immune effector cells to allow efficient immune response. Therefore, the control of particle size is of utmost importance in the development of dry powder vaccine formulations.

Spray-dried particles are often near-spherical, and their size can be appropriately described by their geometric equivalent diameter [59,63]. However, the geometric diameter alone does not provide a complete reflection of the behaviour of the particles because it does not take into account the influence of the envelope density and other morphological features on the aerosolization performance of the particles. In respiratory delivery, a more important metric is the aerodynamic diameter, which is the diameter of a unit-density sphere that has the same settling velocity as the measured particle [59]. Particles with the same aerodynamic diameter are likely to deposit at a similar region in the respiratory tract. The aerodynamic diameter of spray-dried particles is primarily determined by the feed solution concentration and the initial droplet size, the latter determined by the atomizer performance [59]. Therefore, the size distribution of the spray-dried particles can, in principle, be controlled by carefully manipulating these parameters in the drying process to achieve the desired particle size for the vaccine formulation (Table 3).

Distribution of compounds in spray-dried particles

The ability to incorporate various compounds into a single-particle engineering process is a powerful advantage. The composition, and thus the physicochemical properties of spray-dried powders, can be controlled by adjusting the composition of the feed solution (Table 3). However, the architecture and morphology of the resulting powder is not only determined by the ingredients in the feed solution but also the distribution of compounds within the particles. This is a function of several complex phenomena, such as the preferential adsorption of certain components at the liquid/gas interface, the diffusional flux of compounds

Table 3. Examples of dry powder formulations and engineered particles

Key properties studied	Manufacturing process	Key process parameters	Comments	Refs
Stability of antigen	SFD	Trehalose as a stabilizer	The influenza dry powder formulation with trehalose retained bioactivity longer than the corresponding liquid formulation	[34]
Morphology, particle size, aerodynamic properties	SD and SFD	Effect of the two different drying processes	SD produced smaller particles and narrower particle size distribution, whereas SFD produced larger particles with higher porosity	[50]
Aerosolization performance	SD	L-Leucine as an aerosolization enhancing agent	L-Leucine-containing formulation improved fine particle fraction more than fourfold	[55]
Stability of active protein	SD	Drying temperature and stabilizing additives	Glucose was found to best stabilize the enzyme at 1% (w/v) during spray-drying whereas maltodextrin demonstrated the best stabilizing effect at 0.5% (w/v) during storage time	[56]
Morphology, particle size, fine particle fraction, SD yield	SD	Concentration of ethanol as co-solvent and leucine as additive in feed solution; effect of initial formulation viscosity	Leucine-containing powders demonstrated irregular morphology; SD yield and particle size decreased, whereas tapped density and respirable fraction increased with increasing viscosity and ethanol concentration	[57]
Controlled release	SD	PVA as a release modifier	Controlled-release formulation containing PVA demonstrated a ~25-fold increase in bioavailability of the active drug after lung administration	[68]
Stability and aerosol performance	SD	Effect of polysorbate 20; Zn^{2+} in the formulation	Fine particle fraction decreased with increasing polysorbate 20 concentrations; a 2:1 molar ratio of Zn^{2+} improved conformational stability of the protein	[69]
Antigen encapsulation for targeting	SD	PLGA as an encapsulating agent	Microparticles of PLGA encapsulated antigen was hypothesized to interact with APCs to induce cell-mediated immunity	[75]
Morphology, aerosolization efficiency	SD	Concentration of active compound in feed solution	Corrugated particles produced from reduced feed concentration of the active compound demonstrated a higher percentage of fine particles rather than smooth particles	[77]
Aerodynamic behavior and PK parameters	SD	Effect of vehicles in feed solution	The type of solvents used in the feed solution significantly altered the absorption profile and lung deposition of the antibiotic particles	[78]

Abbreviations: PK, pharmacokinetic; PLGA, poly(lactic-co-glycolic acid); PVA, poly(vinyl alcohol); SD, spray-drying; SFD, spray-freeze-drying.

owing to mass transfer rates and concentration gradients produced during evaporation in the drying process [59].

The rate of evaporation of the solution droplet is another factor that influences the distribution of compounds and is determined by the balance of the energy delivered to the droplet surface, the energy required to vaporize the solution, and the mass transfer of vapour from the droplet surface. The small size of the solution droplet rapidly decreases during the evaporation process. Where the diffusional motion of the solute is fast compared with the radial velocity of the receding droplet, surface enrichment of the components is small, thus compounds are evenly distributed in the droplet during evaporation and particle density is likely to be close to the true density [59]. Conversely, where the droplet surface moves faster than the components in the solution, a high concentration of the compound on the droplet surface leads to the formation of a shell. The final morphology of the particle (e.g. spherical vs. collapsed) depends on the nature of the active pharmaceutical ingredients and additives as well as the properties of the shell formed [59]. The resulting morphology of the particles will determine the aerosolization properties and thus performance of the formulation. For instance, porous particles have lower densities and aerodynamic diameters, whereas wrinkled particles provide a corrugated surface that improves dispersibility. Detailed mechanisms of particle formation are beyond the scope of this review, but are discussed elsewhere [59].

Stabilization of spray-dried biological macromolecules

The stabilization of proteins and peptides are of particular interest because they are the main class of antigenic biomacromolecules used in vaccines. Proteins and peptides are labile macromolecules with complex tertiary and quaternary structures. The maintenance of these macrostructures in the native form is essential for activity. However, these macrostructures are easily damaged by various stresses encountered during spray-drying, such as thermal stress during drying and mechanical stress during atomization. Furthermore, dehydration stress caused by the removal of water molecules from the surface can also destabilize protein molecules [39,58,64,65]. Due consideration must be given to these factors to ensure parameters are optimized and any loss of activity is minimized during production.

The inclusion of excipients to stabilize macromolecules in dry powder formulations is another important consideration for vaccine formulations. Inclusion of glass-forming agents such as saccharides, polyols and organic acids in

formulations of solid-state spray-dried proteins has been demonstrated to enhance stability of proteins [49,59,64–66]. These excipients stabilize macromolecules via two main mechanisms. Firstly, the glass-forming ability of these excipients stabilize the protein by trapping it in a rigid, amorphous glass matrix with high glass transition temperature (T_g) that preserves the structure. Secondly, these excipients can form hydrogen bonds with proteins in the dry solid state to replace the hydrogen bonds that water forms with proteins in solution to stabilize the structure [20,59,64]. Common stabilizing excipients include sucrose, trehalose, mannitol and inulin [39,43,50,59,66]. Spray-drying allows incorporation of these stabilizing agents in a single-step process into dry powder vaccine formulations.

Conclusions and future perspectives

Pulmonary delivery has been demonstrated as an effective means for vaccination. In comparison to parenteral vaccination, the attractive properties of pulmonary vaccines include the superior local mucosal immunity and systemic immunity, absence of complications associated with injectable delivery, and ease of administration. Although superior local immunity is particularly beneficial for protection against infections originating from the respiratory tract (e.g. influenza and TB), the connection of various mucosal sites through a compartmentalized mucosal immune system presents the opportunity to immunize against diseases originating from a remote mucosal site via the pulmonary route.

Dry powder vaccine formulations provide a real opportunity for improved antigen stability compared with conventional liquid formulations, and could enhance the immunity induced after vaccination. Spray-drying has received increased attention for the production of dry powder vaccine formulations owing to its relative simplicity, cost-effectiveness and scalability. Spray-drying also allows the engineering of particles which cannot be readily achieved with other manufacturing processes. Furthermore, the incorporation of suitable stabilizing excipients during spray-drying can help to preserve the integrity of antigenic macromolecules and improve the stability of dry powder vaccine formulations. In addition, nanotechnology might be utilized to improve the design of dry powder formulations with more sophisticated particle engineering. Dry powder measles and TB vaccines are expected to enter clinical trials in India and South Africa, respectively, within 2 years [67] (http://www.grandchallenges.org/ImproveVaccines/Challenges/NeedleFreeDelivery/Pages/respirablepowder.aspx; http://www.medicineinneed.org/artscience.html).

To capitalize on inhalable spray-dried vaccine formulations, it is necessary to resolve issues surrounding the development process; for example, the most suitable combination of parameters during the spray-drying process or the development of a dry powder carrier platform that is capable of stabilizing the antigen in a dry solid state using toxicologically acceptable excipients. Future research should be directed towards understanding the generation and characteristics of immune responses after delivery of antigen to different regions of the respiratory tract, and therefore the optimal site for antigen delivery and the underlying antigen presentation mechanisms. The recent advances in drug delivery technology targeting the lymphatic system might be further exploited to enhance the induction of immunity in the lymph nodes.

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