DOCTOR OF PHILOSOPHY

Investigating the delivery of antimicrobial proteins and aminoglycoside antibiotics to the airways

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INVESTIGATING THE DELIVERY OF ANTIMICROBIAL PROTEINS AND AMINOGLYCOSIDE ANTIBIOTICS TO THE AIRWAYS

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SUMMARY

Biopharmaceuticals are finding wide applications in the management of diverse disease conditions. Pulmonary delivery of proteins may constitute an effective and efficient non-invasive alternative to parenteral delivery, which is currently the main route of administration of biopharmaceutical drugs. A particular area, in which pulmonary delivery of peptides and proteins may find ready application, is in the local delivery of antimicrobial peptides and proteins to the airway, a measure that could potentially bring about improvements to currently available antipseudomonal therapies.

This thesis has therefore sought to develop inhalable antimicrobial proteins in combination with antibiotics that have particularly good antimicrobial activity against *Pseudomonas aeruginosa* infections in the respiratory tract of people with cystic fibrosis (CF). Through process optimisation, a suitable spray drying method was developed and used for the preparation of active, inhalable dry powder formulations of the antimicrobial protein, lactoferrin, and aminoglycosides (tobramycin and gentamicin). The physicochemical properties, aerosolisation performance and the antibacterial properties of the various spray-dried formulations were assessed. In addition, a relevant *in vitro* cellular model was employed to investigate the potential cytotoxic and pro-inflammatory effects of the various formulations on four bronchial human epithelial cells together with their effectiveness at reducing bacterial colonies when administered on to biofilm co-

cultured on the epithelial cells. It was found that following spray drying the particles obtained were mostly spherical, amorphous and possessed suitable aerosolisation characteristics. The various spray-dried antimicrobial proteins (lactoferrin or apo lactoferrin) and co-spray dried combinations of the proteins and aminoglycosides were found to exhibit bactericidal activity against planktonic and biofilms of *P. aeruginosa*. In general, the spray drying process was found not to significantly affect the antimicrobial activities of the protein. Treatment of the different bronchial epithelial cell lines with the antimicrobial formulations showed that the various formulations were non-toxic and that the co-spray dried combinations significantly reduced established *P. aeruginosa* biofilms on the four bronchial epithelial cells. Overall, the results from this thesis demonstrates that spray drying could potentially be employed to prepare inhalable antimicrobial agents comprised of proteins and antibiotics. These new combinations of proteins and aminoglycosides has promising applications in the management of P. aeruginosa in the airway of cystic fibrosis patients.

Keywords: Antimicrobial Protein, Spray drying, *Pseudomonas aeruginosa*, Cystic fibrosis, Inhalation

For my family

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ABBREVIATIONS

AP	Alkaline Phosphatase
aLf	Apo lactoferrin
ALI	Air-liquid interface
ANOVA	One-way analysis of variance
ASL	Air surface liquid
ATCC	American Type Culture Collection
BCA	Bicinchonic acid
BEAS-2B	Bronchial epithelial cells
BSA	Bovine serum albumin
C38	Corrected IB3-1 cells
CALU-3	Bronchial adenocarcinoma cells
CF	Cystic fibrosis
CFU	Colony forming unit
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
DNA	Deoxyribonucleic acid
DPI	Dry powder inhaler
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS/FBS	Foetal calf serum / Foetal bovine serum
FU	Fluorescence units
GSD	Geometric standard deviation
IB3-1	Bronchial epithelial cells isolated from a CF patient
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IL	Interleukin
IMS	Industrial methylated spirit
kDa	Kilodalton
Lf	Lactoferrin
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MMAD	Mass median aerodynamic diameter
mRNA	Messenger ribonucleic acid
MSLI	Multi-stage liquid impinger
Mw	Molecular weight
NaOH	Sodium hydroxide
NCTC	National Collection of Type Culture
PBS	Phosphate buffered saline
pg	picograms
PI	Propidium iodide
pMDI	Pressurised metered dose inhaler
PPM	Parts per million
SD	Standard deviation
SD Genta	Spray-dried gentamicin
SDaLf	Spray-dried apo lactoferrin
SDaLf + Genta	Co-spray dried apo lactoferrin and gentamicin
SDaLf + Tobi	Co-spray dried apo lactoferrin and tobramycin
SDLf	Spray-dried lactoferrin
SDLf + Genta	Co-spray dried lactoferrin and gentamicin
SDLf + Genta	Co-spray dried apo lactoferrin and tobramycin
SEM	Scanning electron microscopy
TER	Trans-epithelial electrical resistance
T_g	Glass transition temperature

Tmax	Time to reach peak serum concentration
UV	Ultra violet
VMD	Volume median diameter

Chapter One

Introduction

1.1 General introduction

Proteins consist of a series of organic linear polypeptides (from a repertoire of nineteen α-amino acids and one imino acid 'proline') linked together through amide bonds. The amino acid building blocks of polypeptides are naturally occurring and play many vital roles in the body. Interestingly, the amino acid sequences usually vary in arrangement and determine the resultant protein's unique 3-dimensional structure along with its specific function. Interactions of some protein molecules, in what is called the quaternary structure, may lead to the establishment of a larger and well defined structure (Manning *et al.*, 1989). The order of arrangement of amino acids in a polypeptide chain is determined by genes which code for specific polypeptides. A composite analysis of available information in the public domain seems to identify proteins as having the most dynamic and diverse role of any macromolecule in the body, especially since their roles include catalysis of many biochemical reactions, receptor and channel formations, providing intracellular as well as extracellular scaffolding support, and participation in the transport of many molecules either within a cell or between cells.

Today, with the recent advances in biotechnology, peptides and protein therapeutics now represent a promising class of pharmacologically active agents which can be employed in the management or treatment of many ailments (for example, those affecting the immune system) due to their high specificity and potency. Sadly, even though quite a large number of proteins have been found to have some clinical applications, the delivery of these proteins still poses a major challenge. Since they are labile, have a high tendency to adhere to surfaces, are easily metabolised, cleared, and may elicit immunological responses. It is important to mention at this juncture that at present, intramuscular, intravenous and subcutaneous injections remain the method for administering these macromolecules. This is because oral administration which happens to be the most convenient and commonly utilised route for the administration of most

drugs, regardless of their molecular size or structure, is plagued with various challenges when employed in the delivery of proteins and peptides. Some of the challenges for oral delivery range from difficulties in overcoming the enzymatic degradation of proteins to insufficient permeation of the proteins through the gastrointestinal membrane. It has been suggested that the pulmonary route may well be best suited for the delivery of those macromolecules which are poorly absorbed from the gastrointestinal tract (Agu *et al.*, 2001; Patton, 1996; Johnson, 1997).

Notwithstanding polypeptide formulation challenges, proteins and peptides have continued to remain very attractive candidates in drug discovery applications, especially due to their unique functions as active regulators and components of the innate immune system (Leader *et al.*, 2008). Available publication data suggest that there are specialist journals (like current protein and peptide science, the protein journal and protein and peptide letters) which only focuses on publishing breakthrough research in the areas of peptide and protein science. The torrent of information in these journals suggest that concept therapeutic approaches of the future will favour the employment of proteins in the treatment of many ailments (Edwards *et al.*, 1998).

It has been estimated that there are around 20,500 different genes in the human genome (Clamp *et al.*, 2007; www.genome.gov, 2012) and taking into consideration alternative splicing of genes and post-translational modification of proteins (for example, cleavage, phosphorylation, acylation and glycosylation), the number of functionally distinct proteins is likely to be much higher (Pennisi, 2003; Lander *et al.*, 2001; Venter *et al.*, 2001). Viewed from the perspective of harnessing their therapeutic benefit, these proteins represent a tremendous opportunity since they can be employed in alleviating diseases. Currently, approximately 200 protein or peptide based active pharmaceutical agents (API's) from various sources intended for use in the treatment of variety of human diseases and conditions have been approved by the US food and Drug Administration (FDA) (GBI, 2009). So far, much of the approvals have generally resulted

because some peptides and proteins are known to effectively treat a wide variety of specific ailments that are sometimes difficult to manage or are untreatable using other chemical-based agents due to their specific biologic activity. It is important to note that the potential of protein therapeutics seems to be at an early stage. An ever greater number of molecular targets which could be employed in the treatment of various diseases are still been discovered, but even then, formulation technologists have not been able to keep pace with the various discoveries that have been made with regard to the special nature of proteins.

1.1.1 Advantages of employing proteins in therapeutics

The advantages associated with the use of proteins and peptides in clinical therapeutics are vast. The table below summarises some of the many advantages, as well disadvantages, of employing proteins in therapeutics. Interestingly, while most of the advantages outlined are mostly pharmacologic, the disadvantages seem to deal with issues related to formulation design (which can be overcome).

Table 1.1: Advantages and disadvantages of protein therapeutics

Advantages	Disadvantages
High activity	Low oral bioavailability
High specificity	Mostly administered by injection
Reduced unspecific binding to molecular structure other than desired target	Very unstable hence requires careful handling during processing and in use
Minimization of drug-drug interaction	Difficult to deliver across membranes
Decreased accumulation in tissues	Challenging and costly synthesis
Low toxicity	Solubility challenges
Often very potent	Risk of immunogenicity
Biological and chemical diversity	Usually cleared from the body very quickly

Over the last two decades, biotechnological advancements across the techniques of recombinant DNA technology and solid-phase peptide synthesis, which are generally employed in the production of pharmaceutically active proteins on a commercial scale, have been well refined (Agu *et al.*, 2001). These advances have resulted in the following: (1) they effectively address the issue of scarcity, especially for proteins like interferons which are produced naturally in the body in minute quantities; (2) they have improved product safety since this technology effectively eliminates pathogens which may have been present in biological sources (e.g. human immunodeficiency virus (HIV), hepatitis B and C); (3) they also provide a ready alternative to direct extraction from potentially hazardous sources, for example, Follicle-stimulating hormone (FSH) and human chorionic gonadotrophin (hCG) used to be extracted from human urine (Walsh, 2007). In addition, the use of biotechnology has permitted modifications to the molecular structure of these therapeutic proteins. The insertion, deletion or alteration of amino acids in the protein sequence can enhance site-directed interventions and improve activity (Walsh, 2007). It is pertinently obvious that the benefits of recombinant DNA technology, especially in the development of protein drugs, are quite enormous.

Table 1.2: Some therapeutic agents currently licensed for use in the UK, which are prepared through recombinant DNA technology, their therapeutic indications and the various sources from which they have been produced. Information was obtained from the electronic Medicines Compendium (electronic Medicines compendium – eMC).

Product	Therapeutic indication	Source(s)
Human insulin	Treatment of diabetes mellitus	*Saccharomyces cerevisiae, E. coli*
Etanercept	Rheumatoid arthritis, Juvenile idiopathic arthritis, Psoriatic arthritis, Ankylosing spondylitis, Plaque psoriasis	Chinese hamster ovary (CHO) mammalian expression system
Teriparatide	Treatment of osteoporosis	Chinese hamster ovary (CHO) mammalian expression system
Somatropin	Growth hormone deficiency	*Escherichia coli*
Interferon beta-1a	Treatment of relapsing-remitting multiple sclerosis	Chinese hamster ovary Cells (CHO-K1)
Interferon beta-1b	Treatment of relapsing-remitting multiple sclerosis	*Escherichia coli*
Interferon gamma-1b	Reduction of the frequency of serious infections in patients with chronic granulomatous disease (CGD) and severe, malignant osteopetrosis	*Escherichia coli*
Interferon alfa 2b	Chronic hepatitis B, Chronic hepatitis C, Hairy cell leukaemia, Chronic myelogous leukaemia, multiple myeloma, carcinoid tumour	*Escherichia coli*
Dornase alpha (DNase I)	Management of cystic fibrosis	Chinese Hamster Ovary Cell Line CHO A14.16-1 MSB #757
Glucagon	Treatment of severe hypoglycaemia	*Saccharomyces Cerevisiae*
Follitropin alpha	Female infertility Stimulation of spermatogenesis in men	Chinese Hamster Ovary (CHO) cells
Canakinumab	Cryopyrin-associated periodic syndromes Gouty arthritis	mouse hybridoma Sp2/0 cells
Aldesleukin	Treatment of metastatic renal carcinoma	*Escherichia coli*

During the next decade, it has been suggested that as more molecular targets for the treatment of various diseases are identified, hundreds of bio-engineered proteins and peptides would be required in various forms of delivery. This chapter focuses on the discussion of current trends and potential challenges associated with the pulmonary delivery of peptides and proteins, with brief descriptions of the physiology of the lungs, the characteristics of formulations for pulmonary delivery, criteria for selecting pulmonary delivery devices, toxicity and *in vivo* efficacy studies.

1.2 Pulmonary delivery of proteins and peptides

Pulmonary drug delivery has been practiced for several centuries, especially in the widely common practice of 'inhaling narcotics' (Sanders, 2007). More recently, this delivery route has attracted much attention, not for its illegal applications, but for its suitability in delivering proteins and peptides whose characteristic large molecular size, high surface charge, hydrophobicity, lability and rapid ionisation over a wide pH range make their handling, processing into traditional dosage forms (for example, tablets and capsules) and therapeutic drug delivery applications most challenging. Pulmonary delivery of proteins has been suggested to be the most attractive alternative to both the subcutaneous and intravenous routes of protein administration (Selam, 2003), which have been the mainstay for systemic protein delivery. In addition to the desirable formulation characteristics of a pulmonary administered therapeutic agent (such as stability, ease of handling and dose administration), various physiological characteristics of the lungs also makes pulmonary administration an attractive choice in drug targeting and delivery. The large surface area, which, at approximately 140 m^2 is nearly 40 times more than the external body surface area (Agu *et al.*, 2001; Wearley, 1991; Gehr *et al.*, 1978), lower concentrations of metabolic enzymes compared to those of the gastrointestinal tract (Banga, 1995), a very thin epithelial lining (0.1 – 0.2 µm) in the deep lungs permitting relatively rapid equilibration of blood and alveolar fluid proteins

(Patton and Byron, 2007; Patton, 1996), and a highly vesiculated single layer of epithelial cells which permits effective gaseous exchange (Owens *et al.*, 2003; Weibel, 1973) and therefore allows rapid entry of inhaled therapeutics to the systemic circulation.

Hundreds of proteins and peptides are currently undergoing clinical investigation for a wide range of clinical conditions including; cystic fibrosis, cancer, diabetes and multiple sclerosis. Although most of the proteins under investigation are formulated as injections, some of them have specifically been formulated for inhalation, by leveraging the various sites of action which have already been identified; airway surface (e.g. deoxyribonuclease) (Yang *et al.*, 2010), airway cells (e.g. cyclosporin) (Waters *et al.*, 2005) and systemic targets (e.g. insulin) (Huang and Wang, 2006). It is important to note that despite the extensive research which has being carried out in the pulmonary delivery of peptides and proteins, very few therapeutic products have actually made it through clinical trials to full license.

Table 1.3: Some examples of proteins/peptides which are/have been considered for delivery to the airways for the management of various disease conditions. The various protein/peptides listed below are at various stages of clinical trial except for for DNase which has been approved.

Clinical application	Peptide/Protein drug	Clinical status	Sponsors	Ref./Clinical trial registry number
Cystic fibrosis (CF)	Dornase alfa	Approved	Roche	
Respiratory Distress Syndrome in Premature Infant	recombinant human CC10 (rhCC10)	Phase 1 intratracheally (completed in nov, 2011)	Clarassance, Inc. And National Heart, Lung, and Blood Institute (NHLBI)	NCT01473264
Sarcoma Solid Tumors Pulmonary Metastases Lung Metastases Osteosarcomas Ewing Sarcoma	Aerosol IL-2	Phase 1 (currently recruiting march 11 2013)	M.D. Anderson Cancer Center and Shannon Wilkes Foundation	NCT01590069
Asthma	Recombinant human IL-4 receptor	Phase 2 (completed in feb, 2000)	National Institute of Allergy and Infectious Diseases (NIAID) and Immunex Corporation	NCT00017693
Pulmonary Fibrosis	aerosol interferon-gamma	Phase 1 (currently ongoing)	New York University School of Medicine and National Center for Research Resources (NCRR)	NCT00563212
Lung Transplantation Bronchiolitis Obliterans Chronic Rejection of Lung Transplant	liposomal aerosol cyclosporine	Phase 1 (currently recruiting march 11 2013)	University of Maryland	NCT01650545
Chronic Obstructive Pulmonary Disease	Cyclosporine	Phase 1 (completed in sept, 2009)	University of Pittsburgh	NCT00783107

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Even today, as more and more biopharmaceuticals are gaining regulatory approval for general use, pharmaceutical companies and research institutions are still confronted with the challenges relating to inability to efficiently and effectively deliver aerosolised peptides and protein drugs to the airways. This is because there is a need to provide the drug as very small solid or liquid microparticles (usually between 1 and 5 µm) so as to reach the lungs (Carvalho *et al.*, 2011). There is also the need to consider the use of deaggregation mechanisms to improve the delivery of solid microparticles to the airway by inhalation devices. It is also important to avoid *in vivo* biological degradation by proteases that are resident in the lungs, as well as overcome the various clearance mechanisms of the respiratory system (hoiby 2010). Section 1.7 below discusses these challenges in more detail.

1.3 Anatomical features of the human airways

In order to fully understand the therapeutic challenges facing drug delivery via the pulmonary route, it is important to discuss the anatomical organization of the respiratory tract and how this arrangement is structured to provide a very large surface area for efficient exchange of gases (carbon dioxide and oxygen) between the body and its surrounding environment – the main function of the respiratory system. The efficiency of gaseous exchange is highly visible since a healthy adult male inhales approximately 5 – 10 L/min of air at rest, which increases to about 100 L/min during strenuous exercise (Kim and Folinsbee, 1997).

Anatomically, the structures of the respiratory tract can be divided with respect to their position into two; the upper respiratory tract (the nasal cavity, mouth, pharynx and larynx) and the lower respiratory tract (including all airway structures from the trachea to the alveoli) (Suarez and Hickey, 2000). The upper respiratory tract is primarily a conducting passage way for air to reach the lungs. When air first enters through the

nose (which is lined with cilia, mucus membrane and blood capillaries) it is filtered by cilia, moistened by mucus membranes and warmed by blood, then the conditioned air moves on through the pharynx (a common passageway for both air and food) on to the larynx, (which contains the vocal folds). The lower respiratory tract which starts with the trachea at the lower border of the cricoid cartilage (level with the sixth cervical vertebrae C6) and connects the larynx to the bronchial tree. The trachea is made up of fibrous and elastic tissues and is prevented from collapsing due to the presence of about 20 rings of cartilage. The trachea bifurcates into right and left bronchi at the level of the sternal angle. The bronchi enter their corresponding right or left lungs where they subdivide into even smaller branches called bronchiole and at the end of each bronchiole are structures called the alveolar sacs. The alveolar sacs are surrounded by blood capillaries and contain single layer alveoli cells where the gas exchange takes place.

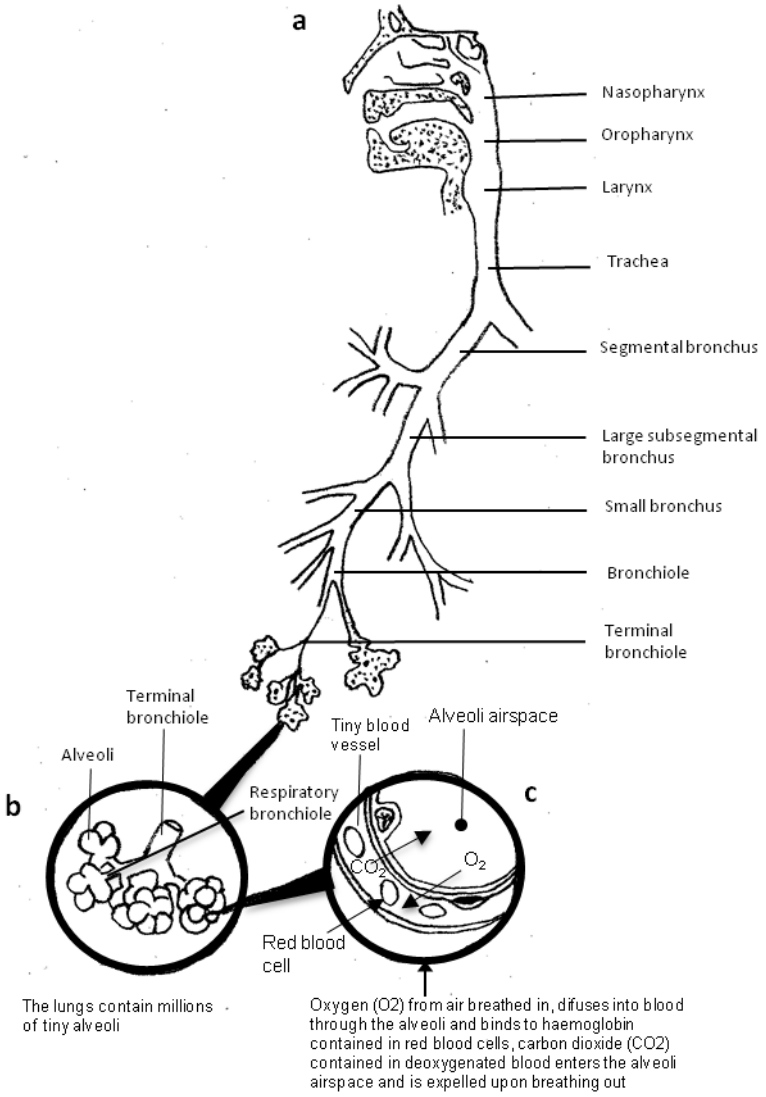

Figure 1 -1: The anatomy of the human airways. The diagram illustrates the locations of the respiratory structures in the body. a) shows both the upper and lower respiratory tract; b) shows an enlarged image of terminal bronchiole and alveoli; c) shows the region where gaseous exchange takes place between the capillaries and the alveoli.

The conducting airways possess mucus producing goblet cells, the mucus produced by the goblet cells, traps small airborne particles and with the aid of the ciliated epithelial cells moves the mucus to the pharynx, where it is swallowed and digested in the gastrointestinal tract. Individuals who suffer from cystic fibrosis where thick sticky mucus

adhere to the linings of their airways and interfere with airflow to the lung surface as well as providing a breeding ground for bacterial infection.

In addition to this anatomical division of the respiratory tract, the respiratory system can be broadly divided into two functional units (Figure 1- 3);

(a) The tracheo-bronchial airways

(b) The bronchial circulation

The tracheo-bronchial airways: The tracheo-bronchial airways enable the efficient distribution of inhaled air to areas where gaseous exchange takes place. Functionally, the structures of the tracheobronchial airway may be further classified into two;

> ***The conducting regions*** – includes all passageways, providing a fairly rigid conduit for the passage of inhaled air to the distal portions of the respiratory tract i.e. the bronchi, bronchioles and alveoli.
>
> ***The respiratory regions*** – these are the actual sites of gas exchange, comprised of all respiratory structures distal to the bronchioles (respiratory bronchioles, alveolar ducts, and alveoli).

Weibel's (1963) tracheobronchial anatomical classification of the human lung (Figure 1-3) – is frequently used in computing pulmonary drug administration – the conducting region consists of the first 16 generations while generation 17 – 23 constitute the transitional and respiratory zones where rapid exchange of gases and solutes takes place.

Figure 1 -2: A schematic of airway branching of the human lung with approximate dimensions, number of branches and total cross sectional area of both the conducting and respiratory zone. (Weibel Dichotomous Lung Geometry) (Weibel, 1965).

Bronchial circulation: The bronchial circulation plays a vital role in the efficient distribution of blood to the airway mucosa especially to the structures of the respiratory region. Mucosal blood supply, which is primarily derived from the bronchial artery, is at a relatively high flow rate (Nørgaard *et al.*, 1999) and is responsible for the absorption, metabolism, redistribution and activation or inactivation of inhaled drugs as well as endogenous substances in the lung environment.

1.3.1 A description of the lung epithelium

The lamina propia (comprised of collagen, elastin and proteoglycan) forms the basic scaffold to which the lung epithelium is attached (Bienkowski and Gotkin, 1995), rings of cartilage – which decreases both in size and frequency as the airways become smaller helps to maintain the rigidity of the luminal space from the trachea to the bronchi. Smooth muscles (also decreasing in size and frequency) surround the airway between the bronchioles and the respiratory bronchioles. The overall progressive reduction in rings of cartilage and smooth muscles, results in a smaller airway wall thickness closer to the alveoli. Interestingly, as the diameter of the respiratory tract decreases, the surface area available for gaseous exchange increases. For example, while the cross sectional area of the trachea is about 2.5 cm^2 (Suarez and Hickey, 2000), the total cross sectional area of alveoli is about 10^4 cm^2 (Okamoto *et al.*, 2002). This means that the alveoli are more than 100 m^2 larger than the small intestine (Okamoto *et al.*, 2002).

1.3.1 Cytology of the airway

The cytology of the airway cells show that the cell population that make up both the nasal and tracheobronchial regions of the mammalian respiratory tract are very diverse (Breeze and Wheeldon, 1977). Generally, cells lining the nasal cavity up to the terminal bronchioles are pseudo stratified ciliated epithelium possessing mostly cilia and mucus secreting cells and primarily function to exclude debris from the airway by moving them up the mucociliary escalator where they may be expectorated or swallowed. Below the bronchioles, the pulmonary epithelium begins to thin, cell height begins to flatten and merge with the alveolar epithelial cells, thus, enabling exchange of gases (Harkema *et al.*, 1991; Gruenert *et al.*, 1995).

1.3.1.1 Airway epithelial cells

1.3.1.1.1 Goblet and serous cells

Goblet and serous cells are primarily secretory cells as they secrete mucin. While goblet cells are located on the surface of the lung epithelium and release vesicles directly into the luminal space, serous cells are present as a collection of cells that form the submucosal glands. Serous cells are normally found below the epithelial cell layer, although they may also be found as single cells within the epithelium surface. Both Goblet and serous cells are responsible for controlling the fluidity of mucus in the airway (Rogers, 1994; Rogers *et al.*, 1993). They are able to do so by expressing high quantities of CFTR (which controls the fluid balance by moderating Cl^- transport across the lung epithelium, which in turn is linked to water content). It is interesting to note that both goblet and serous cells are absent from regions of the lungs where gaseous exchange takes place.

1.3.1.1.2 Ciliated cells

Ciliated cells are usually found between the upper airways and the respiratory bronchioles where they usually make up about 50% of the epithelial cell population of the airways (Harkema *et al.*, 1991). In humans, each ciliated cell usually possess about 200 – 300 cilia that beat approximately 12 – 16 times per second to move mucus towards the larynx (where the mucus is either expectorated or swallowed). Hence, the mucus expelling actions of the ciliated cells make them important components of airway defence.

1.3.1.1.3 Clara and basal cells

Clara cells are commonly found in the bronchiolar epithelium where under normal conditions they have a very low turnover, however when stressed (for example, upon

exposure to NO_2) they can be significantly increased by up to 20 fold (Evans *et al.*, 1978). When clara cells are stimulated, they act as precursors for secretory or ciliated epithelial cells by protruding above neighbouring ciliated cells (Breuer *et al.*, 1990; Inayama *et al.*, 1988). Basal cells on the other hand are attached to the basal membrane, they do not protrude into the lumen of the airway and may sometimes act as precursors for ciliated and/or goblet cells. With increasing airway generations, the number of basal cells decline dramatically with clara cells becoming the major secretory cell type.

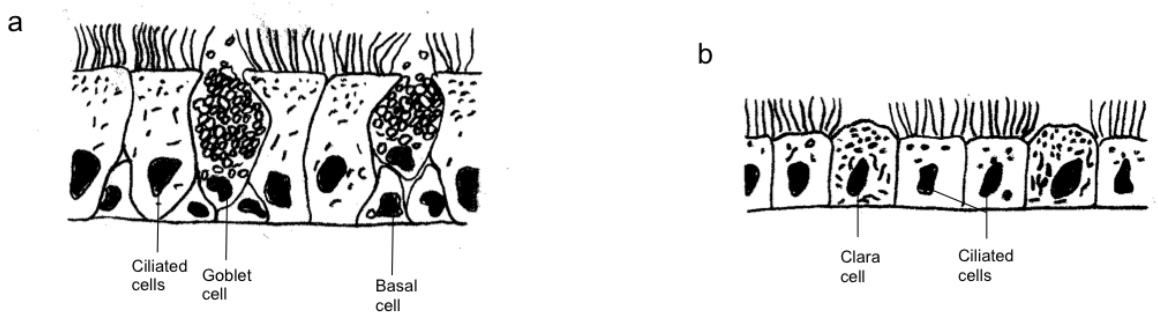

Figure 1 -3: An illustration of the structures of the airway epithelium. a) airway epithelial cells of the trachea and large bronchi where ciliated and goblet cells predominate with numerous basal cells b) airway epithelial cells of the bronchioles where ciliated and clara cells predominate. Goblet and serous cells decrease distally and are absent in the terminal bronchioles. Adapted from Forbes and Ehrhardt (2005).

1.3.1.2 Alveolar epithelial cells

The epithelium of the alveoli is quite thin and populated by two epithelial cell types: the terminally differentiated type I pneumocyte and its progenitor cell, the type II pneumocyte (Yang *et al.*, 2000). Together both cells provide the boundary where gaseous exchange takes place.

1.3.1.2.1 Alveolar type I cells

Alveolar type I cells are terminally differentiated, form the alveolar sacs, have a thickness of around 0.1 µm and typically possess a narrow elongated nuclei with very few other cell synthesis organelles. Alveolar epithelial cells type I cells cover approximately 95% of the total internal alveolar surface area, therefore, primarily functioning as the pathway for gaseous exchange (Johnson *et al.*, 2002).

1.3.1.2.2 Alveolar type II cells

Alveolar type II cells cover between 2-5% of the internal alveolar surface area. Type II cells are generally cuboidal in shape and play no role in gaseous exchange rather they produce, secrete and recycle pulmonary surfactant, pulmonary defence mechanisms and the trans-epithelial transport of ion. Type II cells are the progenitor cells for type I cells and increase their differentiation and turnover rate upon exposure to stressful conditions.

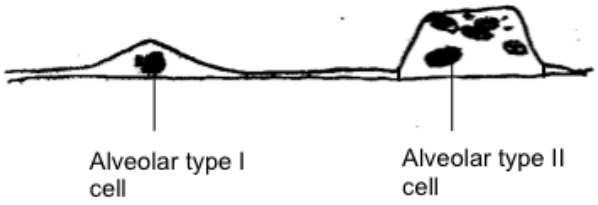

Figure 1 -4: An illustration of the alveolar epithelium showing contrasting alveolar cell morphologies. Alveolar type I cells are extremely thin and accounts for 95% of the epithelial surface, while the type II cell are cuboidal in shape and cover between 2 and 5% of the epithelial surface. . Adapted from Forbes and Ehrhardt (2005).

1.4 Airway defence mechanisms

At rest, the average human being breathes in about 20,000 litres of air every day, inevitably, a myriad of airborne particles including microbes, allergens, dusts and carcinogens are suspended in this inspired air, many of which will cause no harm. However, like all major points of contact with the external environment, the lung has intrinsic protective barriers which function to exclude (physical – e.g. movement of mucus by cilia; and chemical – e.g. pulmonary surfactants, α-defensins, cathelicidin) these undesired airborne particles from the body. (Cohen and Gold, 1975; Newhouse *et al.*, 1976; Richardson and Peatfield, 1981).

1.4.1 Physical defence mechanisms

This defence mechanism, which appears to predominate, includes all the physical barriers that function to prevent the invasion of microbes. Firstly, mucus is produced by the goblet cells of the epithelium and the submucosal gland which functions to prevent the microorganism from adhering to the epithelium as well as expelling any microbe with the outward flow of mucus which is driven by the synchronous beating of the cilia towards the mouth (mucociliary transport or clearance). The beneficial contributions of this defence mechanism is evident in disease conditions like cystic fibrosis, which is characterised by thick mucus secretion or inhibition of ciliary movement (cilia dyskinesia), where the respiratory tract is colonised by bacteria. In addition to expelling microbes, the mucociliary transport also serves to clear insoluble particles depositing in the ciliated region of the airways usually within 24 – 48 hr (Lippmann and Albert, 1969; Svartengren *et al.*, 1991).

1.4.2 Chemical defence mechanism

The airway epithelium secretes several different types of antimicrobial proteins and peptides, which participates in microbial killing. These products are resident in the airway fluid and inducible upon invasion by microbes. The antimicrobial molecules produced can be small cationic molecules like β-defensins, LL-37 and CCL20, or larger proteins like lysozyme, lactoferrin and secretory leukocyte peptidase inhibitor (SLPI) all secreted by serous cells of the submucosal glands (Basbaum *et al.*, 1990; Finkbeiner, 1999)

Other chemical components which contribute to airway defence include:

a) Haematopoietic cells (polymorphonuclear leukocytes, platelets, eosinophils, macrophages) which have origins in the haematopoietic stem cells of the bone marrow and generally make up both the innate (non-specific) and adaptive (specific) immune systems;
b) Lung immunoglobulins (IgA - predominantly in the form of secretory IgA, IgG, IgM and IgE) which are derived form both the vascular compartment (by diffusion through the lung tissue) and local production by cells of the epithelium or lamina propia (Burnett, 1986);
c) Parts of the complement system (C3b, C3bi, C5a) which particularly is involved in the lysis of certain viruses and/or opsonize pathogens (e.g. C3b) thereby facilitating their uptake through the complement receptors on the phagocytic cells (Clarke, 1990; Kroegel and Costabel, 1994);
d) Inflammatory mediators (prostaglandins, thromboxanes, leukotrienes, susbstance P, bradykinin, neurokinin A, platelet activating factor, histamine etc) which are produced by a number of cells in the airway epithelium (Clarke, 1990; Jordana *et al.*, 1994; Raphael and Metcalfe, 1986);

e) Cytokines (interferons, tumour necrosis growth factor, interleukins, chemotaxins) which are produced in response to failure of cell differentiation (Barnes, 1994; Clarke, 1990; Jordana *et al.*, 1994);

f) Protease inhibitors (secretory leukoprotease inhibitor, antichemotrypsin, α_1 – protease inhibitor, α_1 – antichemotrypsin) which are secreted by both the respiratory epithelial cell (SLPI) and produced in the liver (α_1 –protease inhibitor) to inhibit proteases – neutrophil elastase (Clarke, 1990)

Overall, both physical and the chemical airway defence mechanisms operate in a complementary fashion to effectively remove or neutralise particulates with varying physicochemical properties from the respiratory tract. However, for the purpose of the body of work presented in this thesis, our focus will only be on antimicrobial proteins.

1.4.2.1 Role of antimicrobial proteins in the defence against infections

In the airways, several different types of antimicrobial proteins are produced and contribute to airway defence. However, since their contributions to airway defence is primarily dependent upon the concentration produced and the spectrum of activity of the individual antimicrobial protein, three major antimicrobial proteins have been identified to constitute airway defence and they include; lysozyme, lactoferrin and secretory leukoproteinase (SLPI). Interestingly, they are all produced by the serous cells of airway glands (Basbaum *et al.*, 1990)

1.4.2.1.1 Lysozyme

Lysozyme is one of the most predominant antimicrobial factors in the airway surface liquid (ASL). Lysozyme is found at a concentration of approximately 10 µg/ml in airway lavage fluid (Fleming, 1922; Raphael *et al.*, 1989; Thompson *et al.*, 1990) and 1 mg/ml

in sputum (Brogan *et al.*, 1975; Kotlar *et al.*, 1980; Harbitz *et al.*, 1984; Jacquot *et al.*, 1983). Human lysozyme is a 1,4-β-N-acetylmuramidase which selectively cleaves the β(1,4) glycosidic linkage between the C-1 of N-acetylmuramic acid (MurNAc) and the C-4 of N-acetylglucosamine (GlcNAc) (both components of bacterial peptidoglycan) (Jollès and Jollès, 1984), however, since the peptidoglycan layer of Gram-positive bacteria are surface exposed (Fig 1 -4a), lysozyme is able to effectively cause damages to it there by exposing the lipid bilayer of the bacterial cell membrane (Fig 1 -4c). In contrast, since Gram-negative bacteria have an outer layer of lipopolysaccharide (LPS) covering their peptidoglycan layer (Fig 1 -4b), lysozyme is ineffective against Gram-negative organisms (Leive, 1974; Nikaido and Vaara, 1985; Nikaido, 2003). However, Singh *et al.,* (2000) have been able to show that combinations of lysozyme with lactoferrin or lysozyme with secretory leukocyte protease inhibitor (SLPI) or a triple combination of lysozyme, lactoferrin and SLPI showed synergism in killing Escherichia coli (a gram negative bacteria), thereby, inferring that lysozyme does have secondary contributions in preventing the host defence system from invasion by Gram-negative microbes (Martinez and Carroll, 1980).

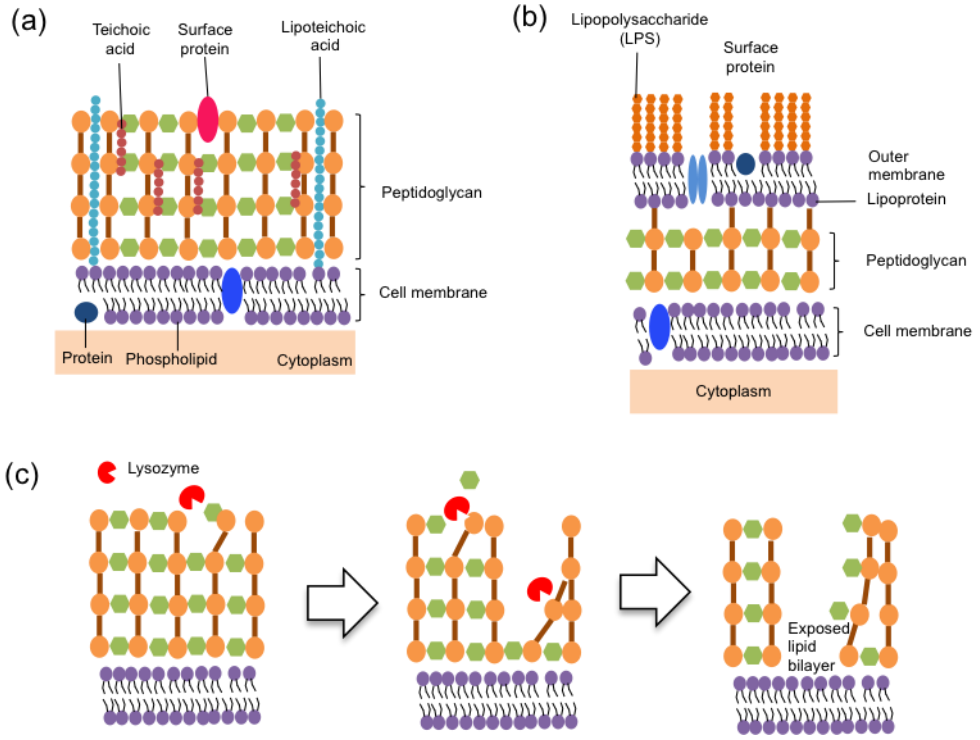

Figure 1 -5: An illustration of bacteria cell membrane showing the various structural components of the bacterial cell wall. N-acetylglucosamine is represented by the olive green hexagon, N-acetylmuramic acid is represented by the orange circle and the cross linked peptide bridges is represented by the dark orange bars. a) Gram-positive bacterial cell wall is predominantly constituted of peptidoglycan and b) Gram-negative bacterial cell wall has a thin inner wall of peptidoglycan covered by an outer lipid membrane that contains proteins and lipopolysaccharide (LPS), c) a schematic showing lysozyme cleaving the β-(1,4) linkages between MurNAc and GlcNAc thereby exposing the underlying cell membrane structures to other antimicrobial agents.

1.4.2.1.2 Lactoferrin

Lactoferrin is an iron binding glycoprotein that is active against a variety of Gram-positive and Gram-negative microbes (Arnold *et al.*, 1980; Flotte *et al.*, 1993). Lactoferrin is relatively abundant at 1 to 10 µg/ml in airway lavage fluid (Raphael *et al.*, 1989; Thompson *et al.*, 1990) and approximately 1 mg/ml in sputum (Brogan *et al.*, 1975; Harbitz *et al.*, 1984; Jacquot *et al.*, 1983). Although lactoferrin is found in

significant quantities in the airways, milk still remains the most abundant source of lactoferrin.

The antimicrobial activity of lactoferrin has been related to its ability to deprive microorganisms of the essential nutrient of iron (Bullen, 1981; Griffiths, 1975; Norrod and Williams, 1978). However, lactoferrin has also been reported to have direct bacteriocidal activity on both Gram-negative and Gram-positive microorganisms, through interactions with the anionic portion (lipid A) of lipopolysaccharide (Ellison *et al.*, 1988). Lactoferrin is also known to disrupt the negatively charged lipid matrix of the Gram-positive bacterial by electrostatic interactions arising from its positively charged N-terminal region. Lactoferrin is also reported to significantly enhance the activity of secretory IgA (sIgA) (Spik *et al.*, 1978; Stephens *et al.*, 1980; Arnold *et al.*, 1977a; Boesman-Finkelstein and Finkelstein, 1985). All of these mechanisms highlighted above indicate the presence of other mechanisms of antimicrobial actions other than iron sequestration. Lactoferrin also enhances the antibacterial activity of other antimicrobial proteins towards Gram-negative and Gram-positive bacteria by interacting with lipoteichoic acid (present on Gram-positive bacterial cell wall) and lipopolysaccharide (present in Gram-negative bacteria cell membrane) leading to alterations in bacterial membrane permeability barriers which in turn contributes to microbial cell injury and death (Leitch and Willcox, 1999b; Arnold *et al.*, 1982). Lactoferrin has also being found to prevent biofilm formation by *P. aeruginosa* (Singh *et al.*, 2002) and because biofilms play an important role in the progression of lung infections in cystic fibrosis, efficient inhibitors like lactoferrin which prevent biofilm formation hold particular promise as a therapeutic agent.

1..4.2.1.3 Secretory leukocyte proteinase inhibitor (SLPI)

Human secretory leukocyte proteinase inhibitor is a low molecular weight (11.7 kDa), high net negative charge (+11), non glycosylated protein (contains 107 amino acids) that is produced by the serous cells in the submucosal gland of the bronchi and by Clara and goblet cells of the bronchiolar and bronchial lining of the airway epithelium (De Water *et al.*, 1986; Hiemstra *et al.*, 1996), as well as by secretory cells in the genital and lacrimal glands and dermal keratinocytes (Wingens *et al.*, 1998). SLPI provides an antiprotease shield against serine proteases (cathepsin G, trypsin, chymotrypsin but most importantly neutrophil elastase – a pro-inflammatory mediator which predominates in cystic fibrosis) secreted by activated neutrophils during phagocytosis (Williams *et al.*, 2006; Balfour-Lynn, 1999; Hutchison, 1987). SLPI is found at a concentration of 0.1 to 2 µg/ml in airway lavage fluid (Vogelmeier *et al.*, 1991; Kouchi *et al.*, 1993) and 2.5 µg/ ml in nasal secretions (Lee *et al.*, 1993).

SLPI has been shown to have *in vitro* antimicrobial activity against a variety of microorganisms including *Staphylococcus aureus*, *Escherichia coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Mycobacterium tuberculosis, Aspergillus fumigates* and *Candida albicans* (Hiemstra *et al.*, 1996; Gomez *et al.*, 2009; Travis *et al.*, 1999; Wiedow *et al.*, 1998; Fernie-King *et al.*, 2002; Tomee *et al.*, 1997) and HIV (Wahl *et al.*, 1997; Mcneely *et al.*, 1995; Mcneely *et al.*, 1997). Although the biochemical mechanism by which SLPI is able to achieve these antimicrobial and antiviral activities have not been fully elucidated, it is thought that it may function in a similar way as other antimicrobial peptides (Hiemstra *et al.*, 1996; Williams *et al.*, 2006) through its N-terminal domain which may be involved in disruptions to the anionic cell membrane of bacterial cells .

Recombinant SLPI (rSLPI) (identical to the native SLPI) produced by *Escherichia coli* has been considered as a potential therapeutic agent for cystic fibrosis (a condition

characterised by excessive neutrophil elastase activity on the airway epithelium), studies by Gillissen *et al.,* (1993) showed that inhalation of rSLPI resulted in increased gluthathione levels in the epithelial lining fluid, a clear indication that SLPI does augment anti-neutrophil elastase capacity and therefore well suited for therapy of lung diseases characterised by excessive production of serine proteases and oxidants on the respiratory epithelial surface. However, inefficient delivery of rSLPI to affected but poorly ventilated portions of the airway has been identified as a major setback of aerosolised rSLPI, thereby suggesting that rSLPI aerosol therapy may only be beneficial for prophylactic protection against neutrophil derived elastase (Stolk *et al.*, 1995).

1.5 Infections of the airways

If inhaled microbes manage to evade all the host defences and colonise the respiratory tract, microbial infections of the airway result. Some of the organisms encountered include bacteria, viruses and airborne fungi. Since these organisms are deposited in different regions of the respiratory tract, a wide range of respiratory tract infections result and include pharyngitis, common cold, influenza, acute sinusitis, laryngitis, bronchitis, pneumonia, pulmonary tuberculosis, and bronchiolitis. Most of these respiratory tract infections are usually treatable with a course of antibiotic, but for people with respiratory diseases such as cystic fibrosis, repeated infections with opportunistic organisms usually result in death (Moreau-Marquis *et al.*, 2009; Gibson *et al.*, 2003; Lyczak *et al.*, 2000)

1.6 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive lethal disease resulting from mutations in a single gene on the long arm of chromosome 7 that encodes cystic fibrosis

transmembrane conductance regulator (CFTR) protein (Riordan *et al.*, 1989; Kerem *et al.*, 1989; Rommens *et al.*, 1989), a cyclic adenosine monophosphate (cAMP)-regulated Cl^- channel (Dupont *et al.*, 2007). The mutations of which more than 1,500 sequence variation have been identified (Döring *et al.*, 2010), remains the most common life-shortening genetic disorder amongst Caucasians with an estimated median survival age of 41.5 years in the United Kingdom in 2011 (Cystic Fibrosis Trust, 2013).

The incidence of CF varies extensively across the globe, although available data suggests that it is more common among the Caucasian populations (Who, 2013). The Cystic Fibrosis Trust (2013) estimates that there are currently over 8500 people living with CF in the UK (an estimated incidence rate of 1 in every 2500 live births) (Waugh *et al.*, 2012). Clinical presentations of CF vary among individuals and generally include; pancreatic insufficiency, infertility amongst most males (due to congenital bilateral absence of the vas deferens) and females (reduced fertility), bile duct obstruction, high sweat chloride, intestinal obstruction, nasal polyp formation, chronic sinusitis, liver disease, diabetes (Kubesch *et al.*, 1993) progressive lung disease elicited by thick and dehydrated airway mucus frequently infected with *Pseudomonas* and *Staphylococcus*, which usually results in respiratory failure and CF mortality (Kubesch *et al.*, 1993; Collins, 1992; Welsh *et al.*, 1995).

The biology of normal CFTR protein (composed of 1480 amino acids with a molecular weight of 170 kDa) expression and activation is a complex process involving; firstly DNA transcription to mRNA, translations of mRNA to a peptide, glycosylation of the peptide and finally insertion of the protein into the apical membrane (Jenni and Ban, 2003). However, whenever any step of this normal protein expression is impaired, an abnormal *CFTR* protein results, which in turn leads to defective electrolyte transport and defective chloride ion transport in the apical membrane of the epithelial cells of the sweat gland, airway, pancreas, and intestine. In the long term, therapeutics of cystic fibrosis aims to improve/correct the mutant CFTR protein through gene therapy although at the present

moment the therapy is yet to be successful in clinical trials. Interestingly, some exciting CFTR modulation therapies have began to appear; including Ivacaftor (currently licensed for the treatment of patients with G551D CFTR mutation) and Ataluren (phase 3 clinical trials), which seeks to rescue particular mutations which only affect about 5 % of the UK population

As a result of limitations in current CFTR modulation therapies, other therapeutic agents such as hypertonic saline (restoration of airway surface liquid), dornase alfa (mucus alteration), tobramycin (antimicrobial agent) and enzyme supplements also augment CF therapy until a time when a harmless virus, lipososome or adeno-associated virus can successfully deposit a normal CFTR gene into cells of both the lungs and the pancreas in a cost effective way. This thesis will however only focus on managing airway infections caused by *P. aeruginosa* in people with CF.

1.6.1 Pathogenesis of pulmonary infections in cystic fibrosis

The airway of people with CF are particularly susceptible to pulmonary infections due to the loss of innate immune functions, however, the pathogenesis of endobronchial lung infections in cystic fibrosis is still a topic of debate at present, with the exact role played by dysfunctional or absent CFTR in the whole sequence of events still unclear and embroiled in a controversial and incompletely understood mechanisms. The two major competing hypothesis that have been put forward to try and explain early and persistent endobronchial infections in CF include;

The isotonic “low volume hypothesis” which suggests that mutant CFTR causes a decrease in chloride secretion into the lumen and increased sodium absorption, ultimately resulting in reduced flux of water into the lumen (Matsui *et al.*, 1998a), hence, the mucus in the airways becomes dehydrated with the viscous air-surface liquid (ASL)

trapping inhaled bacteria and viruses. The cilia are unable to beat efficiently due to impaired mucociliary clearance consequently resulting in persistent airway infections.

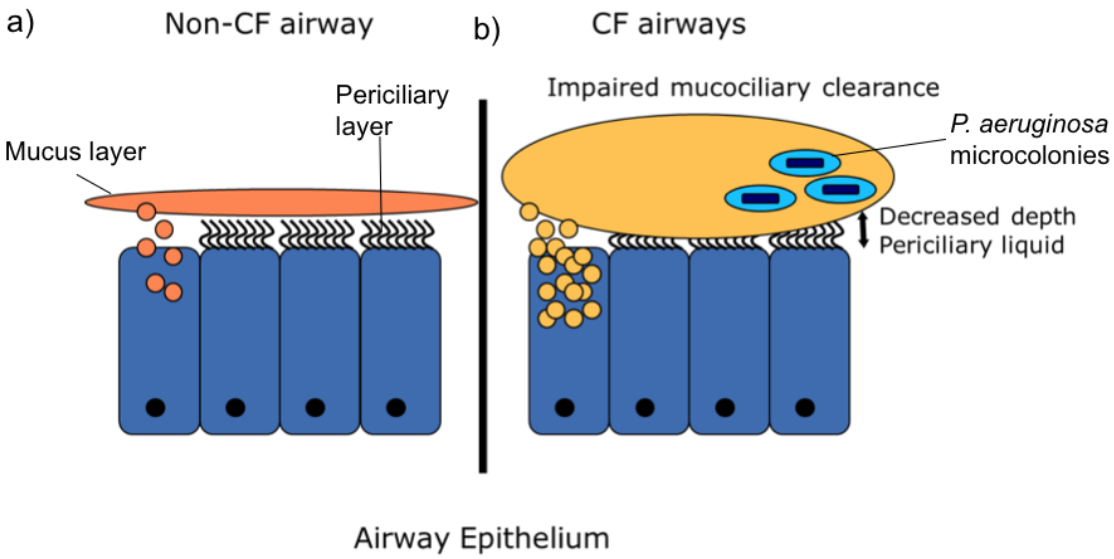

Figure 1 -6: Schematic representation of the mucocilliary escalator in the non-cystic fibrosis and cystic fibrosis airways. a) normal airway epithelia with; low viscosity periciliary layer, normal mucus volume and an efficient mucociliary clearance, b) cystic fibrosis airways where there is hypersecretion of viscous mucus which is plastered down upon the cilia thus inhibiting normal cilia beating. Microbes which are inhaled from the environment penetrate the mucus actively/passively (due to mucus turbulence) and are able to adapt to the local environment. In the case of *P. aeruginosa*, these adaptations usually lead to the evolution of biofilms.

The opposing theory, high salt theory, suggests that various mutation of CFTR protein leads to high levels of sodium chloride (salt) in the airway-surface liquid (Smith *et al.*, 1996) which is unsuitable for the activities of antimicrobial peptides involved in airway defence (for example lysozyme, lactoferrin and beta defensins) (Smith *et al.*, 1996), contrary to this view, *in vivo* experiments by Knowles *et al.,* (1997) have shown that ASL electrolyte levels in CF patients are not different from the electrolyte levels in the airway

of normal patients, also, the diversity and complexity and of the antimicrobial peptides found in ASL makes it difficult to understand how such a situation could be sufficiently compromised in CF to produce the phenotype of this disease (Travis *et al.*, 1999; Travis *et al.*, 2001). In general, the low volume hypothesis is considered the most plausible mechanism for CF airway infections although it does not explain the narrow range of pathogens characteristically encountered in CF.

Notwithstanding, particular mechanisms by which airway infections result, it is clear that accumulations of viscid mucus in the airways makes the lungs of CF patients more vulnerable to colonisation by various micro-organisms such as *Staphylococcus aureus* in infancy, *Haemophilus influenza* in the early years and *P. aeruginosa* in early teens, *Burkholderia cepacia* and mycobacteria in the late teens (Govan and Nelson, 1992). Infections are usually localised in the major and minor airways with localised infections at other non- pulmonary sites or systemic infections being rare. In this study, attention will be focused on *P. aeruginosa* infection (which affects 36% of children and 69.2 % of adults) especially because it is difficult to treat with the currently available antibiotics and hence responsible for many of deaths in CF (Ramsey *et al.*, 1999; Cystic Fibrosis Trust, 2013).

1.6.1.1 Mechanisms of *P. aeruginosa* persistence in the CF airway

In a bid to have a better understanding of how *P. aeruginosa* is able to persist in the CF airways, since colonisation by *P. aeruginosa* is a poor prognostic indicator, several researchers have extensively investigated various adaptation mechanism available to the microbe in the CF airway including, increased binding of *P. aeruginosa* to the airway epithelium and the failure of non-specific immune systems to resolve bacterial infections.

1.6.1.1.1 Adaptions of *P. aeruginosa* in the CF airway

P. aeruginosa is a highly adaptable, aerobic, Gram-negative bacillus that is motile by means of a single polar flagellum. Normally, *P. aeruginosa* is non-pathogenic, becoming pathogenic in individuals with weakened defences (Pitt, 1986). It possesses the ability to develop resistance to multiple antibiotics through a number of broadly specific multidrug efflux systems (Poole, 2001). In addition, hypermutable strains of *P. aeruginosa* which have an increased resistance to antibiotics have also been isolated from the airways of people with CF (Oliver *et al.*, 2000). Initially, *P. aeruginosa* exists as a motile planktonic bacteria (the non-mucoid phenotype) in the airways of patients with CF. As bacterial infections progresses *P. aeruginosa* begins to produce mucoid exopolysaccharide (MEP) or an alginate. Alginate is a strongly anionic polysaccharide composed of linear 1-4 linked β-D-mannuronic acid and its C5 epimer, α-L-guluronic acid (Linker and Jones, 1966). The exopolysaccharide produces highly viscid aqueous solutions and has the capacity to bind water and divalent cations to form flexible gels. Due to the raised Ca^{2+} concentration found in the CF lung, alginate gel formation is induced leading to the formation of bacterial microcolonies. The emergence of mucoid strains usually correlates with a poor patient outlook (Pedersen *et al.*, 1992). This is because the biofilm prevents the phagocytosis of bacteria since it masks the complement on the bacterial surface and the outer membrane of *P. aeruginosa* from antibodies of the host defence (Stiver *et al.*, 1988) as well as from antimicrobial agents. In addition, *P. aeruginosa* also produces an alginate lyase enzyme which functions to cleave MEP and allow for further spread of the bacterial organism to contiguous sites (Boyd and Chakrabarty, 1994). Furthermore, *P. aeruginosa* may also synthesize specific lipid-A moieties containing palmitate and aminoarabinose which provoke increased host inflammatory responses and resistance to antimicrobial peptides (Ernst *et al.*, 1999). The pyocyanin pigment possessed by *P. aeruginosa* has also been found to slow ciliary beat frequency, bind iron and inhibit the growth of other bacteria *(Wilson et al., 1987)*

1.6.1.1.2 Increased binding of *P. aeruginosa* to airway epithelium

There is an increase in asialo-GM1 residues (a class of glycolipids which contain a GalNAcB14Gal sequence and enable *P. aeruginosa* attachment) on CF airway epithelial cells (Saiman and Prince, 1993), which has been found to be responsible for a two fold increase in bacterial binding (Saiman *et al.*, 1992). The possession of adhesins (pilin and flagelin) by *P. aeruginosa* significantly contributes to development of chronic infections by the microbe since these structures contribute to the attachment of the microbe as well as the initiation of an exuberant inflammatory response (Scheid *et al.*, 2001). For example, the binding of pilin to asialo-GM1 activates the pro-inflammatory transcription factor NF-κB and induces production of the neutrophil chemoattractant, IL-8 (Dimango *et al.*, 1998; Dimango *et al.*, 1995). On the otherhand, flagellin just like pilin also promotes *P. aeruginosa* retention in the airways of CF patients by binding to mucin oligosaccharides via flagellar cap protein FliD (Arora *et al.*, 1998; Ramphal *et al.*, 1996) and to epithelial cell oligosaccharides (in which case it stimulates the production of pro-inflammatory cytokines) (Feldman *et al.*, 1998).

1.6.1.1.3 Failure of nonspecific airway defences to kill the bacteria

From birth the human airway is repeatedly challenged by small doses of microbes. In the normal airway, nonspecific airway defences such as defensins, lysozyme, lactoferrin, SLPI and nitric oxide (NO) ordinarily resolve these small microbial challenges. In the airways of CF patients, the non-specific defences are compromised. For example, the major isoform of nitric oxide synthase (NOS) in airway epithelial cells, NOS-2, is reduced in CF airway epithelium (Kelley and Drumm, 1998). This reduction in local NO production (related to abnormal CFTR functioning) In combination with reduced amounts of or inactivated natural antimicrobial proteins and peptides (due to high salt content in the airway-surface liquid as earlier described) probably compromises the

host's ability to handle small bacterial challenges. A direct result of compromised nonspecific defences and reduced bacterial clearance is the recruitment of inflammatory cells and many other systems to fight bacterial invasion (which would otherwise have being resolved by nonspecific host defences) with mostly deleterious outcomes to the airway.

1.6.2 Current treatment strategies of P. aeruginosa in the airway of CF patients

Antibiotic therapy has been the corner stone for improvements to both life expectancy and quality of life of CF patients. Typically, antibiotic therapies are generally geared towards managing early, intermittent infections with particular focus on eradicating the pathogens or at least reducing the bacterial density as well as the inflammatory response that ultimately leads to lung injuries. However, once *P. aeruginosa* establishes itself in the airway of a person with CF, eradication is rarely achieved. Therefore, the most effective and efficient antimicrobial therapies have been those, which are able to supress this bacterial pathogen even though many other pathogens like *Burkholderia cepacia* complex and methicillin-resistant *Staphylococcus aureus* (MRSA) (which are associated to declining lung function) are also present in the airway of people with CF. Recently, clinicians have begun to evaluate eradication protocols for MRSA (www.clinicaltrials.gov identifier NCT01349192). For the purpose of this thesis, mention would only be made about inhaled antibiotics employed for the treatment of *P. aeruginosa* infections in the airways of CF patients.

1.6.2.1 Currently approved aerosol antibiotics in the UK

Commonly employed antibiotics include the aminoglycoside – tobramycin; the polymyxin E – colistin; the monobactam antibiotic – aztreonam; with the fluoroquinolones (ciprofloxacin and levofloxacin) and phosphonic acid-aminoglycoside combinations (fosfomycin-tobramycin combinations) still under clinical trials.

1.6.2.1.1 Nebulised and inhaled tobramycin

TOBI® (Tobramycin 300mg/5 mL nebuliser solution) and TOBI Podhaler (28 mg inhalation powder) both marketed by Norvatis Pharmaceutical UK Limited are currently indicated for the therapeutic management of chronic pulmonary infection resulting from *P. aeruginosa* in CF patients ≥ 6 years of age. The recommended dose for TOBI® in adults and children is 300 mg tobramycin twice daily for 28 days-on followed by 28 days-off therapy while the recommended dose for TOBI Podhaler is 112 mg tobramycin (4 x 28 mg capsules) administered twice daily for 28 days followed by 28 days off therapy. A multicentre, double-blind, placebo-controlled trial published by Ramsey *et al.,* (1999) indicate an average increase in forced expiratory volume in one second (FEV_1) of 10%, 20 weeks after commencement of 300 mg inhaled tobramycin therapy compared with 2% decline in FEV_1 in the patients receiving the placebo.

1.6.2.1.2 Colistimethate sodium

Colistimethate sodium is a polymyxin antibiotic marketed as Promixin (1 million international unts of Colistimethate sodium powder for nebuliser solution) by Profile Pharma Limited for the treatment of *P. aeruginosa* lung infections sensitive to colistimethate sodium in CF patients ≥ 2 years of age. The recommended dose for both children and adult is 1-2 million IU for 8 to 12 hours daily. There has not been a large, randomized controlled study for colistimethate yet, but Jensen *et al.,* (1987) have so far

shown that 90 day treatment with colistin is superior to treatment with a placebo.

1.6.2.1.3 Aztreonam lysine for inhalation (AZLI)

Aztreonam lysine is a monobactam antibiotic which has been used parentrally to treat infections caused by aerobic Gram-negative bacteria. It has however been reformulated as a lysine salt for inhalation. Cayston (75 mg aztreonam which forms aztreonam lysine *in situ*) is marketed by Gilead sciences Limited for suppressive therapy of chronic *P. aeruginosa* infections in CF patients' ≥ 6 years of age. The recommended dose for children and adults is 75 mg every 8 hours for 28 days followed by 28 days off therapy. A double blind, randomized, placebo-controlled phase 2 study carried out by Retsch-Bogart *et al.,* (2008) showed that 75 and 225 mg of aztreonam lysine administered 12 hourly for 14 days using eFlow electronic nebuliser (Pari Innovative Manufacturers, Inc., Midlothian, VA) significantly reduced bacterial density in sputum with the 75 mg dose being associated with fewer adverse events.

1.6.2.1.4 Aerosol antibiotics in clinical development

The continued improvement of the available therapies for managing *P. aeruginosa* infection as well as the fear that overuse of the same antimicrobials overtime may place selective pressure on CF microbiome has seen clinical explorations into the reformulation of some other already effective antibiotics for inhalation purposes including;

Table 1.4: Some antipseudomonal antibiotics, which are currently at various stages of clinical development.

Antibiotic class	Antibiotic formulation	Status	Sponsors	Ref./Clinical trial identifier
Fluoroquinolones	Inhaled ciprofloxacin	Phase 2 clinical trial completed	Bayer Healthcare AG, Germany	NCT00645788
	liposomal ciprofloxacin	Phase 3 clinical trials about to begin	Aradigm corporation, USA	NCT01515007
	Nebulised levofloxacin	Phase 3 clinical trials is currently ongoing	Mpex Pharmaceuticals USA	NCT01180634
Aminoglycoside	Liposomal amikacin	Phase 3 clinical studies about to begin	Insmed Incorporated, USA	NCT01315691
Phosphonic acid	Nebulised fosfomycin-tobramycin combinations	Phase 2 clinical trial completed	Gilead Sciences, USA	NCT00794586

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1.6.3 Antibiotic resistance

Unfortunately, one of the recurring problems with the currently available antimicrobial therapies employed in managing infections in CF patients is antibiotic resistance. Although many of the relevant antibiotics (aminoglycosides, β-lactams, fluroquinolones polymixin) employed for the treatment of *P. aeruginosa* infections in CF remain active against majority of respiratory isolates in the UK and Ireland (www.bsacurv.org/mrsweb/respiratory), resistance is still notable especially among patients with cystic fibrosis. An overview of some factors responsible for *P. aeruginosa* resistance have been previously reviewed by Lambert, (2002), Costerton *et al.,*(1999) and Gilbert *et al.,* (1997) and they include; restricted penetration of antimicrobial agents into a biofilm, decreased growth rate of microbes in a biofilm and possible expression of resistance genes.

Table 1.5: Resistance among *P. aeruginosa* isolates in the United Kingdom and Ireland, 2008 – 2011. Data was obtained from the resistance surveillance website of the British Society for Antimicrobial Chemotherapy

Antimicrobials	Year	No. of isolates tested	Breakpoints		Susceptible N (%)	Intermediate N (%)	Resistant N (%)
			S ≤	R >			
Ciprofloxacin	2008 - 2009	238	0.5	1	184 (82.1%)	15 (6.7%)	25 (11.2%)
	2009 - 2010	206	0.5	1	168 (81.6%)	9 (4.4%)	29 (14.1%)
	2010 - 2011	224	0.5	1	186 (78.2%)	20 (8.4%)	32 (13.4%)
Gentamicin	2008 - 2009	238	4	4	230 (96.6%)	0 (0%)	8 (3.4%)
	2009 - 2010	206	4	4	193 (93.7%)	0 (0%)	13 (6.3%)
	2010 - 2011	224	4	4	215 (96.0%)	0 (0%)	9 (4.0%)
Colistin	2010 - 2011	224	4	4	224(100%)	0 (0%)	0 (0%)
Imipenem	2008 - 2009	238	4	8	197 (82.8)	20 (8.4)	21 (8.8%)
	2009 - 2010	206	4	8	168 (81.6%)	23 (11.2%)	15 (7.3%)
	2010 - 2011	224	4	8	184 (82%)	19 (8.5%)	21 (9.4%)

1.6.3.1 Restricted penetration of antimicrobial agents into a biofilm

When *P. aeruginosa* forms biofilms, the exopolysaccharide matrix serves as a shield for the microbe by restricting the diffusion of an antibiotic through it (Nichols *et al.*, 1988), unfortunately, all the major classes of antibiotic used to treat *P. aeruginosa* need to cross these biofilm to reach their target. For example, the mechanism of action of the aminoglycosides (tobramycin, gentamicin and amikacin) involves inhibition of protein synthesis through its binding to the 30S ribosomal subunit, the fluoroquinolones (ciprofloxacin, levofloxacin) inhibit DNA gyrase (an isozyme of topoisomerase found only in bacteria) through its binding to the subunit A of the enzyme, the β-lactams (aztreonam, ceftazidime, imipenem, meropenem and piperacillin) inhibit bacterial cell wall synthesis through its inhibition of peptidoglycan assembling transpeptidases and the polymyxins (colistin) which disrupt the outer cell membrane of Gram-negative bacteria by binding to lipopolysaccharides and phospholipids. Therefore the inability of the antibiotic agent to accumulate within the organism either due to restrictions presented by the outer membrane or the biofilms in which the bacteria exists, ensures the survival of the organism in a hostile environment

1.6.3.2 Mutations and resistance

All the antimicrobial agents currently being used to manage *P. aeruginosa* infections are all prone to compromise by mutational resistance. For example, mutations to topoisomerase II (gyrA) and topoisomerase IV (parC) in *P. aeruginosa* confer fluoroquinolone resistance (Jalal and Wretlind, 1998), depression of chromosomal AmpC β-lactamase confers cephalosporin and penicillin resistance (Livermore, 1995), up-regulation of MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM compromises the fluoroquinolones, penicilins, cephalosporins and to some extent, meropenem (with only up-regulation of MexEF-OprN causes some reduced susceptibility to imipenem),

reduced aminoglycoside transport results in aminoglycoside resistance and changes to the membrane architecture of *P. aeruginosa* resulting in polymixin resistance.

Overall, data from the European Antimicrobial Resistance Surveillance Network (EARS-NET) indicate that there is growing Europe-wide increase in antimicrobial resistance of Gram-negative pathogens (including *P. aeruginosa*) to third generation cephalosporins, fluoroquinolones and aminoglycosides (Ecdc, 2012). It is an unfortunate situation that aminoglycosides which are the mainstay antibiotic component of most inhaled/aerosolised antipseudomonal therapies are gradually becoming less effective in combating *P. aeruginosa* infections in CF patients (Ecdc, 2012). While aminoglycoside toxicity (nephrotoxicity and ototoxicity) may be resolved by increasing dosing intervals (Turnidge, 2003), resistance of clinical isolates of *P. aeruginosa* are more difficult to manage.

This thesis focuses on developing an inhalable antipseudomonal therapy (combination of aminoglycosides and antimicrobial protein) with the view of increasing the susceptibility of *P. aeruginosa* in the airways of CF patients. It is premised on the understanding that one of the major causes of reduced susceptibility of *P. aeruginosa* to antimicrobial agents is the formation of biofilms (free-living, independent planktonic bacteria attaching to surfaces and becoming sessile) which retard the diffusion antimicrobial agents or in which bacterial cells are non-growing (hence not very susceptible to many antimicrobial agents). A study by Singh *et al.,* (2002) reported that depriving *P. aeruginosa* of iron, enhanced twitching motility and limited biofilm formation and development, an observation which suggests that an important feature of *P. aeruginosa*, antibiotic resistance, could be curtailed by combining an antimicrobial protein (lactoferrin) with other antibiotics.

1.7 Pulmonary delivery of antimicrobial peptides and proteins

Inhaled medications have been available for many years and are widely accepted as the most effective first line therapy for local airway disorders: chronic obstructive pulmonary disorder, asthma and infections in people with cystic fibrosis. Improvements in the understanding of inhalational science have now been employed to deliver peptide and protein based drugs as earlier mentioned (table 1 -3). Although, antimicrobial proteins have a lot of perceived benefits, there is so far no clinically available inhaled antimicrobial protein or peptide formulation. This is despite the fact that there is still a limited knowledge on the optimal lung deposition site for maximum therapeutic benefits of antibiotics. For example, we now know that chronic lung infection with *P. aeruginosa* in patients with CF resides in the airway lumen (beginning from the bronchioles and gradually proceeding to the larger airways) with limited invasion of the lung parenchyma (Baltimore *et al.*, 1989; Potts *et al.*, 1995). It is thought that the optimal site of deposition for inhaled antimicrobial therapy should therefore involve a uniform distribution on the conducting airways, however, this is not always feasible as the viscous mucus in the airway (bronchi and bronchioles) may prevent the deposition of inhaled antimicrobial particles distal to the airway obstruction thereby limiting the therapeutic effectiveness of the aerosolized antimicrobial agent (Alderson *et al.*, 1974; Ilowite *et al.*, 1987; Anderson *et al.*, 1989). In order to gain insight into some of the challenges such an antimicrobial protein formulation may encounter, factors affecting the therapeutic delivery of proteins and peptides are briefly reviewed.

1.7.1 Factors affecting the effectiveness of aerosolised protein and peptide medications.

1.7.1.1 Drug-associated factors

1.7.1.1.1 Protein intergrity

Many proteins are structurally unstable in solution, as a result they may undergo conformational changes during purification, formulation and storage with resultant physical damage (for example, protein aggregation) arising from exposures to stresses such as high temperatures, mechanical shear stress, extremes of pH or exposure to organic solvents (Wang, 2005; Ohtake *et al.*, 2011) that affect their unique three-dimensional structure (a key requirement for protein activity). In addition to physical degradation, therapeutic proteins are also subjected to degradation by lung peptidases and proteases, the extent of which is dependent on the unique protein characteristics.

1.7.1.1.2 Macromolecular chemistry

Knowledge of the concise chemistry of a protein/peptide to be employed in therapy could aid understanding and prediction of the therapeutic behaviour of the protein when administered to the respiratory tract. For example, understanding the hydrophilicity or hydrophobicity of administered proteins could serve in predicting how they may traverse across the airway epithelial membrane. It is known that most proteins are hydrophilic in nature thereby creating some difficulty in getting them across the lung epithelium (Patton and Byron, 2007), therefore strategies which increase hydrophobicity would be expected to improve their therapeutic effectiveness, if the aim is to deliver the proteins and peptides for systemic delivery. In other cases where local delivery is desired (especially in the delivery of antimicrobial proteins and peptides), strategies that enhance the hydrophilicity of the protein or peptide drug should be embarked upon.

Small hydrophobic chemical moieties are also known to be absorbed by simple passive diffusion throughout the lungs, while large hydrophilic moieties are thought to be absorbed via tight junctions or specific transporters (Patton, 1996). Unfortunately, the exact mechanism(s) by which protein drugs are absorbed through the lung/airways epithelium have not yet been fully understood. There still remains a considerable number of investigations that need to be carried out to fully ascertain the mechanism(s) by which protein drugs are absorbed.

1.7.1.1.3 Mucociliary and macrophage clearance

An important defence barrier factor which proteins need to overcome when they are administered to the respiratory tract is the mucociliary escalator, as it acts as a clearance mechanism for particles deposited in bronchi and bronchioles. As previously stated, the cilia drives the particles trapped in the mucus using coordinated ciliary beating to move mucus from the lower airways to the pharynx, where they can be either swallowed and degraded in the gastrointestinal tract, or ejected (Yu and Chien, 1997; Hoiby *et al.*, 2010). On the other hand, even if the protein formulation is able to avoid mucociliary clearance, phagocytic cells like macrophages may degrade the protein particles. This is the major clearance mechanism of the lungs at the alveolar level and occurs when alveolar macrophages migrate to the particles and phagocytose them via chemotaxis involving opsonisation. The whole process of macrophage uptake is believed to be completed within 6–12 h after initial deposition of particles in the alveoli. Once internalised in the macrophages, the particles are either disintegrated (e.g. by enzymes in lysosomes) or accumulated in the lymphatic system, which drains the airways and alveoli. However, a minor fraction of the particle- carrying macrophages may eventually migrate to the ciliated airways, where they are removed by mucociliary clearance (Zhang *et al.*, 2011).

1.7.1.1.4 Clearance by lung proteases

Lung proteases specifically present a significant challenge to aerosolized/inhaled protein formulations. It has been suggested that addition of a protease inhibitor to the formulation may offer a way to protect the protein and consequently increase its local concentration in the lung (Siekmeier and Scheuch, 2009). However, the efficacy of this strategy will typically rely on what enzyme the protease inhibitor inhibits, for example while SLPI - inhibits serine proteases, α_1 antitrypsin – inhibits neutrophil elastase (Greene and Mcelvaney, 2009). Some other protease inhibitor which have been investigated include nafamostat mesilate and bacitracin.

1.7.1.2 Formulation-associated factors

1.7.1.2.1 Aerosol particle size and site deposition

The role of particle/droplet size in the formulation of aerosols intended for pulmonary application is of utmost importance, as particle size directly influences the mechanism of deposition (Weda *et al.*, 2002; Usmani *et al.*, 2003; Weda *et al.*, 2004; Srichana *et al.*, 2007; Glover *et al.*, 2008).

Generally, it is thought that particles with aerodynamic diameter greater than 5 µm mainly deposit in the upper airways by the process of inertial impaction while particles with aerodynamic diameter between 1 and 5 µm mainly deposit in the bronchial tree and the alveoli by the process of sedimentation as a result of progressively decreasing air velocity. On the otherhand, particles within the size range of 1 and 3 µm are particularly suited for alveolar deposition while even smaller particles (i.e particles having an aerodynamic diameter of below 0.5 µm) usually undergo Brownian motion and in some instances are exhaled during the expiration process.

For effective drug delivery of antimicrobial protein to the respiratory tract, the recommended aerodynamic particle size has been suggested to be between 1 – 5 µm (Hinds, 1982; Gonda, 1992). Particle sizes within these ranges are quite susceptible to deposition in the conducting regions where infections usually occur.

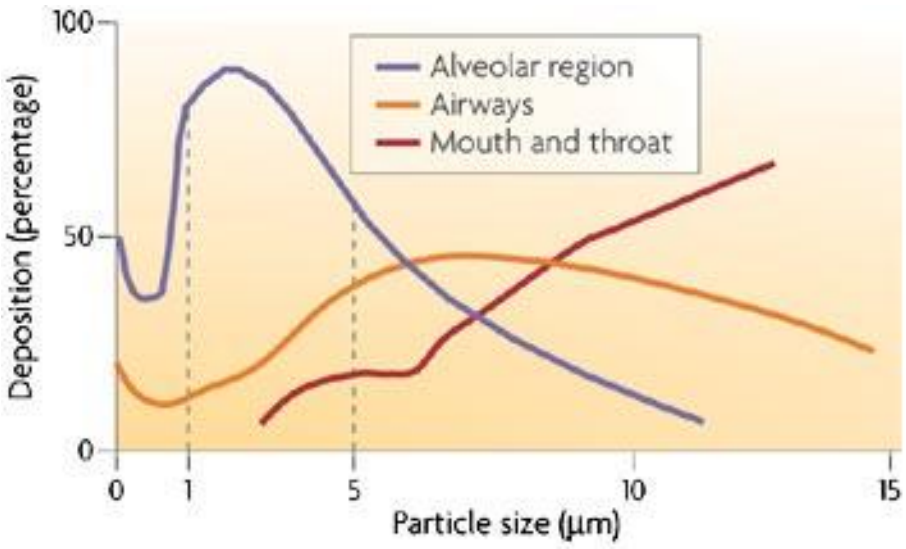


Figure 1 -7: Effect of particle size on the deposition of aerosol particles in the human respiratory tract following a slow inhalation and a 5-second breath hold. For optimal deposition in the alveolar region, aerosolised protein formulations must be within a particle size range of between 1 and 3 µm. For maximum percent deposition in the airways especially for local delivery, the particle size of the formulation should be between 5 and 7 µm. Adapted from Patton and Byron (2007).

1.7.1.2.2 Aerosol mass density and aerodynamic diameter

Since most aerosolised particles are not perfect spheres, the particle sizes of anisometric inhaled particles are commonly described in terms of their equivalent aerodynamic diameters (diameter of a unit density sphere having the same terminal settling velocity as the particle under laminar flow conditions). Summarily, aerodynamic diameter defines particle size, shape and density of inhaled particles. So far, therapeutic

dry powders for inhalation have been engineered to achieve a typical mass density (ρ) of approximately 1 ±0.5g/cm^3 and a mean geometric diameter of <5 µm (Edwards *et al.*, 1997). Although, some researchers have generated large porous microparticles having a low mass density of <0.4 g/cm^3 and aerodynamic diameter between 1 – 5 µm with actual geometric diameter of >5 – 10 µm for use in advanced delivery systems (Edwards *et al.*, 1997; Lee *et al.*, 2007; Kwon *et al.*, 2007; Edwards *et al.*, 1998).

1.7.1.3 Device-associated factors

The selection of an appropriate aerosol device that best suits the protein or peptide formulation is essential, since the role an aerosol device plays in the delivery of any protein or peptide formulation cannot be underestimated. For example, formulations presenting as solution will be well suited for nebulisers, while dry powders are generally delivered using DPI or pMDI devices.

1.7.1.3.1 Devices for liquid protein formulations

The device employed to generate aerosols from liquid protein formulations has a key role to play in ensuring a high degree of protein stability. Nebulisers are the device of choice to generate aerosol droplets; Briefly, a nebuliser can be best described as an aerosol device that atomises a drug containing solution into a mist of small fine droplets, either through the use of compressed gas (as in air jet nebulisers) or through piezo-electric crystal transducer (as seen in ultrasonic nebuliser). Unfortunately, nebulisers have some serious drawback when used to aerosolise protein solutions or suspensions. These drawbacks will be discussed under the two types of nebulisers on the market.

1.7.1.3.1.1 Operational principles of a jet nebuliser

A portable compressor delivers a jet of high velocity air stream through a nozzle which causes liquid to be drawn up from the reservoir due to the drop in partial pressure created at the orifice. An aerosol droplet is generated when the liquid formulation establishes contact with the air stream. Subsequently, larger droplets impact upon an appropriately placed baffle and are refluxed, i.e. returned to the reservoir, while sufficiently small aerosol droplets remain in the air stream and exit from the inhalation port of the nebuliser. Over the course of nebulisation, over 99% of the liquid protein formulation would be expected to be refluxed; this undoubtedly leads to repeated stress by the multiple exposures of the protein to the air-water surface, an experience that may essentially denature the protein formulation. For example, an investigation by Niven and Brain (1994) shows that there is an irreversible time-dependent loss of lactate dehydrogenase activity following jet nebulisation. This may have occurred due to physical disruptions to the protein structure resulting from shear stress and surface effects, which are dependent on the volume of fluid and rate of aspiration. However, Cipolla and co-workers (1994a) were able to maintain the activity and structural integrity of recombinant human deoxyribonuclease (i.e. the first protein approved for inhalational therapy amongst patients with cystic fibrosis) when a 2.5 mL of either 1 or 4 mg/mL aqueous protein solution was nebulised with the aid of four different variations of jet nebulisers. Interestingly, the activity of between 20 – 28% of the administered rhDNase dose having an aerosol droplet size of approximately 1 – 6 µm remained unchanged following jet nebulisation. Unfortunately, at the moment jet nebulisers are not being used to aerosolise recombinant proteins.

1.7.1.3.1.2 Operational principles of the ultrasonic nebuliser

The ultrasonic nebuliser is another suitable device which can be employed for the nebulisation of a liquid protein formulation. Basically, there two types of ultrasonic

nebuliser; (1) those which employ the use of vibrating screens and (2) those which use high frequency sound waves to cause the generation of aerosol droplets. However, in the latter, the transfer of the high energies generated as well as the potential build-up of heat energy in the protein formulation may well lead to the thermal degradation of the protein (Cipolla *et al.*, 1994b; Phipps and Gonda, 1990; Taylor and Hoare, 1993). For example, recombinant consensus α-interferon (rConIFN) and lactate dehydrogenase have been reported to denature during nebulisation while employing commercially available ultrasonic nebulisers (Niven *et al.*, 1995; Ip *et al.*, 1995). Despite this, there are some advantages of ultrasonic nebulisers over jet nebulisers including; production of higher mass output, reduction in duration of nebulisation and it actually produces less noise in operation (Mccallion and Taylor, 1999). Additionally, the denaturation of proteins during ultrasonic nebulisation may be device dependent. Flament *et al.,* (1999) reported that upon aerosolisation of an α_1 protease inhibitor using an SAM LS ultrasonic nebuliser (Systeme AssistanceMedicale, Le Ledat, France), the anti-elastase activity was not reduced.

Most recently, Boehringer Ingelheim have designed, tested and secured approval for Respimat®, a device which is able to generate small aerosol droplets by ultrasonic nebulisation of very small volumes of liquid formulations (15 µL) in one atomisation cycle lasting 1.2 seconds (Dalby *et al.*, 2011). Although, the design features of Respimat® allows for circumvention of problems associated with degradation of the protein formulation upon aerosolisation, it will potentially have limited application for the delivery of antimicrobial peptides and proteins since it will necessitate administration of multiple doses and the use of high concentrations of liquid protein formulations hence making such a therapeutic applications relatively expensive.

An alternative device that may be employed to improve the delivery efficiency and reproducibility of nebulised proteins and peptides is the AERx™ device developed by Aradigm Corporation. The device combines precise aerosol particle size and a control of

the patient's inhalation to deliver a higher amount of drug to the lung with minimal loss of the drug in the mouth and throat. In comparison to Respimat®, the AERx™ is able to deliver a higher volume of drug (50 µL) in about 1 second and also incorporates temperature controllers which minimise effects of ambient air conditions on the generation of small respirable droplets thereby optimising pulmonary deposition (Deshpande *et al.*, 2002; Gonda *et al.*, 1998; Schuster *et al.*, 1997).

a) **b)**

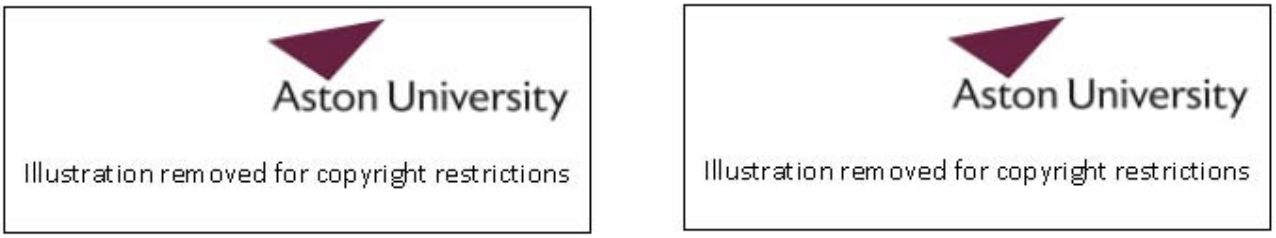


Figure 1 -8: New generation of nebulisers. (a) AERx Essence® device by Aradigm Coporation (b) Respimat device by Boehringer Ingelheim. Currently AERx is being used in phase 2 clinical trials to deliver liposomal ciprofloxacin for the treatment of infections in cystic fibrosis and Non-CF bronchiectasis patients and also for delivery of inhaled insulin. Respimat device is currently used in delivering a combination of ipratropium bromide and albuterol sulphate as Combivent respimat inhalation spray in the United States and tiotropium bromide monohydrate as Spiriva Respimat in the United Kingdom

1.7.1.3.2 Devices for dry powder protein formulations

Traditionally, small volume nebulisers (SVN) like Respimat and AERx are best suited for the pulmonary delivery of very potent proteins and peptides but in some special cases like the local delivery of antimicrobial peptides and proteins requiring the delivery of

much larger quantities of the therapeutic peptide, dry powder inhalers (DPI) are much more efficient in delivering high concentrations of protein or peptide agents to the airway. In general, DPI devices are categorised as into two namely; multidose dry powder inhalers (mDPI: where the drug is stored as a bulk and metered inside the device prior to inhalation) and single-unit dose dry powder inhalers (DPI: where the drug is pre-metered into individual storage units). In addition, these two devices can be further sub-divided into two categories based on the inspiratory efforts required for the dispersion of the drug-powder formulation; passive devices (i.e. patient provides the energy required for dispersion) and active systems (i.e. an external source provides the energy required for dispersion) (Clark and Shire, 2000).

1.7.1.3.2.1 Unit dose dry powder inhaler devices

These groups of dry powder inhaler devices constitute the first generation DPIs and are the most commonly used devices for the aerosolisation of protein powders. In terms of device design, the unit dose dry powder inhalers have various forms of presentations, ranging from the early designs in which drug formulations are stored as single doses in hard gelatine capsules (e.g. the Spinhaler and the Rotahaler) to more recent complex variant in which individual doses are premetered into blisters, disks, tubes and dimples by the manufacturer. Overall, the blistered devices offer superior multidose convenience for patients and have become the device of choice for aerosolisation of dry powder formulations of proteins (Clark and Shire, 2000). However, among the many disadvantages of single dose devices is the requirement for dexterity to load each capsule means that a patient undergoing an asthma attack may find it difficult to use for immediate relief. Also, the contents of the capsules maybe vulnerable to extremes of temperature and humidity.

1.7.1.3.2.2 Multidose dry powder inhaler devices

The multidose inhaler constitute the second generation of DPIs and represents an attempt to improve upon the DPI technology. It features a powder reservoir from which individual doses are metered. Unfortunately, these groups of DPI devices present some major challenges in terms of the delivery efficiency of protein powders. This is generally due to destabilization of the formulation, which is brought about by the tendency of the amorphous excipients employed as solid stabiliser to crystallise thereby leading to an increase in the moisture content of the powder and ultimately plasticized powder formulation. However, even though there is the possibility of developing protein formulations which are quite stable at ambient humidities, the instabilities commonly associated with amorphous solid will still severely limit the use of multidose dry powder inhaler devices for the delivery of protein powders.

1.7.1.3.2.3 Passive and active dry powder inhaler devices

Dry powder inhalers devices can be divided into two; passive and active devices (sometimes considered as third generation DPI devices). While passive devices rely on the patient's inspiration flow to provide the energy required for dispersion, active devices rely on an external energy source to power dispersion. Currently, the majority of DPI devices in the market are patient-driven (passive) devices with relatively few active DPI devices. The very first approved active DPI device was the Exubera® device (manufactured by Pfizer) which has since been discontinued due the high cost of the inhaler and poor patronage by physicians and patients (Davila *et al.*, 1990). The device relied on patient-generated compressed air as energy source to deliver insulin to the lung epithelium (White *et al.*, 2005). Other active DPI devices include; Aspirair® (Vectura) – a multidose device employing individual foil blisters to deliver systemic products to the deep lungs.

From a pulmonary delivery perspective, the choice between employing either a patient-driven or a powered system is purely based on design attributes such as; delivery efficiency, stability and reproducibility of the device and patient based factors like; ease of use, compliance and cost of the device. Ideally, a device featuring unit dose packaging with some form of powered aerosol generation and an optimum control of inhalation flow rates may be deemed as a gold standard. However, since we live in an imperfect world, where all these attributes may well not be present in any one particular device design, some form of trade off may well be appropriate. It could also be the case that for some proteins and peptides, where cost and therapeutic effectiveness are less important, less efficient devices may be acceptable. For local delivery of therapeutic moieties like antimicrobial peptides, it is understandable that passive devices are sufficient while active devices may be better suited for systemic delivery of therapeutic moieties through the airways due to efficient and effective deep lung deposition.

1.7.1.3.3 Pressurised metered dose inhaler devices for protein delivery

Pressurised metered dose inhaler (pMDI) devices were first introduced in 1955 and at the time revolutionised the delivery of therapeutic agents to the airway. Since then, the pMDI devices have proven to be a convenient delivery system for targeting bronchodilators and corticosteroids to the airways of patients with asthma (Leach, 1998; Pauwels *et al.*, 1997). The device is usually small, inexpensive and convenient to carry around and use providing reproducible dosing, hence they are usually considered the "gold standard" in many ways for the delivery of therapeutic agents to the airways. However, they have not been the preferred device of choice for the delivery of protein and peptide formulations. In a pMDI device, the drug is suspended or dissolved in liquefied gas such as hydrofluoroalkane propellants (HFA-227 and HFA-134a). pMDI devices are also not suitable for the delivery of antimicrobial peptides and protein as the metering valves can only deliver between 10 – 30 µL per actuation hence the quantities

of antimicrobial agents required to manage airway infections will require multiple actuations (an inconvenience to the patient) or very high concentration of the protein (making the therapy very expensive).

1.7.1.4 Some other considerations

Furthermore, the choice of excipient, interactions between the formulation and the device (e.g. adsorption), method and conditions of storage, target of drug activity (local or systemic), the nature of the aerosols to be delivered (droplets or particles) are some of the considerations which would inform the choice of any one aerosol delivery device. It is clearly understood that since efficient delivery of aerosols to the desired site of action is pertinent, it is of paramount importance to ensure that proteins/peptides for inhalation are of requisite aerodynamic size and possessing sufficient pharmacologic activity, since if it is otherwise, the inhaler/nebuliser device may only deliver a small percentage of the inhaled dose thereby making the cost of therapy prohibitive.

Investigations are currently ongoing in order to determine the best way of circumventing formulation and operational challenges in the pulmonary delivery of peptides and proteins for example Li *et al.,* (2010) have considered the use of sodium carboxymethylcellulose (NaCMC) to protect the activity of an active enzyme, alkaline phosphatase during processing (spray drying) and have found that 94.2 ±8.5% of the protein remained active when co-spray dried with NaCMC compared to 73.7 ±7.5% when spray-dried alone.

1.8 Aims and objectives

The main aim of this study was to explore the benefit of co-formulating an antimicrobial protein and an aminoglycoside in order to increase the susceptibility of *P. aeruginosa* to antimicrobial agents. The specific aims were therefore:

- To optimize a method of spray drying using alkaline phosphatase as a model protein in order to produce a functionally active micronized formulation suitable for inhalation
- To determine the physicochemical and aerosolisation characteristics of a spray-dried antimicrobial protein
- To determine the efficacy of a spray-dried antimicrobial protein against planktonic cultures and biofilms of *P. aeruginosa*
- To develop and characterise co-spray dried combinations of an antimicrobial protein and an antibiotic, as well as evaluate their efficacy against planktonic cultures and biofilms of *P. aeruginosa*
- To determine the cytotoxicity and proinflammatory properties of various spray-dried antimicrobial agents against submerged cultured human bronchial epithelial cells *in vitro*
- To develop an *in vitro* static co-culture model of *P. aeruginosa* biofilm on human bronchial epithelial cell growing at air-liquid interface

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Chemicals

The materials used and their sources are listed in Table 2-1

Table 2.1: Materials sources

Material*	**Source**
Sodium carboxymethylcelluose Diethanolamine Phosphatase alkaline from bovine intestinal mucosa Bromophenol blue indicator reagent Coomassie Brilliant blue R Sodium dodecyl sulphate Magnesium Chloride D-(+)-Glucose Luria broth Sodium acetate Sodium dihydrogen phosphate Ethylenediaminetetraacetic acid Kodak GBX developer Kodak GBX fixer Formaldehyde solution	SIGMA-ALDRICH (Poole, UK)
Spectrosol Ferric Nitrate (Standard solution for atomic spectroscopy)	VWR (Leicestershire, UK)

Fetal bovine serum (FBS) Dulbecco's modified eagle medium: Nutrient mixture 12 – Ham (DMEM/F12) growth medium Phosphate buffer saline (1x, PBS) L-glutamine Penicillin/streptomycin Trypsin-ethylenediamineteraacetic acid	PAA Laboratories Ltd (Somerset, UK)
1-Step™ PNPP Pierce Silver stain kit BCA protein assay reagent	Thermo Scientific (Rockford, IL USA)
CellTiter-Blue™	Promega (Wisconsin, USA)
Human IL-8 ELISA development kit	Peprotech EC Ltd (London, UK)
Casamino acids	Becton Dickson (NJ, USA)
Analytical grade, Absolute Ethanol	Fisher Scientific. (Loughborough, UK)
98% 4-Nitrophenol Glycine	Acros organics. (New Jersey, USA)
Mini-PROTEAN®TGX™ Precast gels	Bio-Rad laboratories (Hercules USA)
PageRuler™ Plus, prestained protein ladder	Fermentas (York, UK)
LIVE/DEAD kit	Invitrogen Molecular Probes, Eugene, OR, USA
Lactoferrin	DMV Fonterra, Netherlands
Gentamicin Tobramycin	Fagron, (Newcastle upon Tyne, UK)

Primary antibodies (IG12, 4C6 and 4C3)	Kindly provided by Dr Andrew Devitt of Aston University
Mueller-Hinton Agar Antibiotic susceptibility disk	Oxoid LTD (Basingstoke, UK)
Immobilon® Western Enhanced Chemiluminescent substrate (ECL)	Millipore (Watford, UK)

*All chemicals are of analytical grade

Distilled water was generated in-house using Fison's Fi-streAm Water Still.

2.1.2 General consumables and Equipment

The general consumables used and their sources are listed in Table 2-2

Table 2.2: General consumables

Consumables	**Source**
Variable volume Micro pipettes	Appleton woods (Birmingham, UK)
BD Falcon Transwell Inserts (polycarbonate with 0.4 µm pore size,) and companion plates	VWR (Leicestershire, UK)
Nucleopore track-etched Membranes (13 mm diameter and 25 mm diameter) with pore size 0.2 µm	Whatman Int Ltd (Kent, UK)
X-ray film	Amersham Biosciences (Buckinghamshire, UK)
Nunc Maxisorp ELISA plates Microscope slides	Fisher Scientific (Loughborough, UK)

Coverslips Filters for the biofilm assay	
Petri dishes	Sarstedt Ltd (Leicester, UK)

Table 2.3: Equipments

Equipment	**Source**
Multiskan Spectrum - UV/Vis Microplate Spectrophotometer	Thermo Scientific (Basingstoke, UK)
G:BOX	Syngene (Cambridge, UK)
Mini Spray Dryer	Büchi Laboratoriums-Technik AG, Flawil, Switzerland
Small scale crimper and propellant filler	Pamasol Willi Mäder AG, Pfäffikon, Switzerland
Sympatec HELOS H1867 coupled to a RODOS dispersing unit and a VIBRI vibratory feeder used for particle sizing	Helium-Neon Laser Optical System, Sympatec Inc. (Clausthal-Zellerfeld, Germany)
Pyris 1 Thermogravimetric analyzer AAnalyst 100	Perkin Elmer (Seer Green, UK)
Vertical gel electrophoresis tanks and western blotting system	Mini-PROTEAN®TGX™ tetra system Bio-Rad laboratories (Hercules, USA)
Confocal laser scanning microscope	Leica TCS SP5 II Leica Microsystems (Milton Keynes, UK)
UV light box	UVP Inc (Cambridge, UK)
TE22 mini transfer tank	Hoefer Scientific (Newcastle-under-lyme, UK)

2.1.3 Bacterial strains

Pseudomonas aeruginosa strain PAO1 and *Escherichia coli* NCTC 10418 (control strain) were identified and donated by Prof. Peter Lambert of Aston University. All bacterial strains were routinely grown on Mueller-Hinton agar (MHA) and liquid cultures were prepared in either 5mL of casamino acid media (*P. aeruginosa*) or 5mL of Luria Bertani media (*E. coli*)

2.2 Methods

2.2.1 Alkaline phosphatase enzyme assay

2.2.1.1 Assay validation

An assay procedure that ensures conformity to both Good Laboratory and current Good Manufacturing Practice standards, while maintaining a high degree of reliability and reproducibility in the various results obtained, was utilised for all investigations.

2.2.1.1.1 Preparation of 4-nitrophenol standards

Stock standard, p-nitrophenol, 1 mmol/litre

139.1 mg of p-nitrophenol was dissolved in a 70 mL of deionised water, upon complete dissolution the final volume was made up to 100 mL with distilled water. Then the solution was diluted in a volumetric flask at 25 ± 1 °C to 1 litre. Then the entire solution was mixed thoroughly to produce a final concentration of 1 mmol/litre solution of 4-nitrophenol.

p-nitrophenol calibration

A standard calibration graph of p-nitrophenol (p-NP) in diethanolamine-HCl (1mM, pH 9.8 at 25°C) was prepared by measuring the absorbance at 405 nm (Jenway 6305 spectrophotometer) of serial dilutions ranging from 10µM to 50 µM of p-NP diethanolamine-HCl solution.

A calibration graph of absorbance against concentration (µM) was plotted and a linear regression fitted using Microsoft Office Excel (2007). The equation of the line followed the equation below;

$$y = mx + c \qquad \text{(Equation 2.1)}$$

where (*y*) is the absorbance value of the product formed, (*m*) is the slope of the line of best fit, (*x*) is the known concentration of the enzyme and (*c*) is the intercept on the y-axis

2.2.1.1.2 Calculating alkaline phosphatase activity in the various spray-dried powders

The alkaline phosphatase activity of the of the various spray-dried powders were determined by adding 10µL portions of 100 µg/mL concentrations of the powders into 1mL of p-NPP/Diethanolamine solution (1-Step™PNP) and allowed to react for 5 mins. The reaction was quenched by the addition of 500µL of 2 N NaOH and the absorbance values of the various solutions determined at a wavelength of 405 nm using a Jenway 6305 spectrophotometer (Essex, UK).

Principle of the enzyme activity assay.

The equation for the reaction for the method described above in section 2.2.1.1.2 is shown in Figure 2 -1.

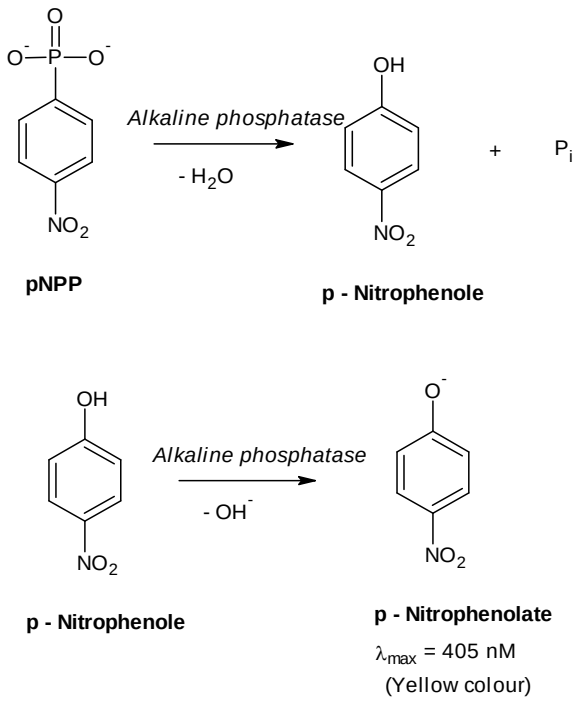


Figure 2 -1: Equation of reaction of alkaline phosphatase reacting with para-nitrophenol phosphate, producing the yellow product para-nitrophenolate

2.2.1.1.3 Evaluating the specificity of the assay

The specificity of the chosen assay method for the enzymatic activity of alkaline phosphatase was evaluated to confirm that the assay procedures were duly suited for the analytical procedure. This was done by spiking known concentrations of standard stock solutions of alkaline phosphatase with different amounts of sodium carboxymethylcellulose prior to measurement of alkaline phosphatase activity as described above. Potential interferences of the sodium carboxymethyl cellulose with the absorbance values of the activity of the enzymes were assessed by comparison with the enzyme activity results from unspiked standard solutions.

2.2.1.1.4 Limit of detection and quantitation of alkaline phosphatase

The limit of detection (LOD) and limit of quantitation (LOQ) were determined for all the triplicate assessments of the alkaline phosphatase standard stock solutions using equations 2.2 and 2.3 (ICH, 1996).

$$LOD = \frac{3.3\sigma}{S} \qquad \text{(Equation 2.2)}$$

$$LOQ = \frac{10\sigma}{S} \qquad \text{(Equation 2.3)}$$

where; σ = standard deviation of the response

S = Slope of the calibration curve

The slope S was estimated from a linear plot of the calibration curve while σ was determined from the standard deviation of the y-intercepts of the regression lines.

The standard deviation of the response (σ) was calculated from the standard deviation of the y-intercepts on the various regression lines for all the triplicate assessments of the enzyme standard stock solutions.

2.2.1.1.5 Linearity of the calibration plot of absorbance against standard concentrations of alkaline phosphatase

A calibration curve of absorbance against concentration was plotted and for each set of calibration standards studied (20 - 100 *μ*g/mL), the equation of the line as well as the

coefficient of determination (r^2) was determined. A blank of (the 1 Step PNPP solution) was used in calibrating the instrument.

2.2.1.1.6 Accuracy of the calibration plot of absorbance against standard concentrations of alkaline phosphatase

Accuracy is defined as the degree of closeness between the “true” value and the observed value. It was determined by analysing the absorbance values of freshly made standard solutions of alkaline phosphatase at concentrations between 20 - 200 µg/mL. Each concentration was analysed in triplicate. For every concentration, each absorbance value obtained was then compared to the expected value (determined from the equation of the line) and the percent difference calculated to determine the intra-day accuracy. This procedure was repeated on 3 different days to establish the inter-day accuracy.

2.2.1.1.7 Precision of the calibration plot of absorbance against standard concentrations of alkaline phosphatase

Precision, defined as the degree of closeness among multiple independent observations was assessed from the percent relative standard deviation around the mean of all observations. Both inter- and intra-day precision was evaluated by determining the coefficient of variation for each set of calibration standards at the following concentrations 20, 40, 60, 80, 100µg/mL.

2.2.2 Preparation of powder formulations

2.2.2.1 Alkaline phosphatase and alkaline phosphatase NaCMC combinations

Aqueous formulations containing alkaline phosphatase alone and in combinations (0.4 % w/v enzyme with 1.6 % w/v sodium carboxymethylcellulose) with NaCMC were prepared with a total powder mass of 2% w/v. Modified formulations containing two ethanol concentrations (10 and 30%) were also prepared. The viscosity of the formulations, % enzyme activity and pH were measured prior to spray drying. Viscosity was measured (in triplicate) using a Brookfield Engineering Laboratories (Stoughton, MA) Viscometer (Programmable DV-II) using spindle #2 at a shear rate of 12rpm. The spindle was rotated in the medium for 1 minute prior to taking a reading.

Table 2.4: Formulation design for the investigation of the influence of co-solvent on co-spray dried carboxymethylcellulose (NaCMC) and alkaline phosphatase (AP)

Formulation	Feedstock solvent	Formulation ID	Viscosity (cP)	pH	% enzyme activity
Excipient-free AP	water	SD-AP	3.1 ± 0.8	9.7 ±0.1	100.1 ±0.7
AP/NaCMC (2:8)	water	SD-N-AP	33.4 ±0.4	9.5 ±0.1	102.3 ±2.0
AP/NaCMC (2:8)	Water-ethanol (9:1)	SD-N-AP 1	36.7 ±1.2	8.7 ±0.1	99.6 ±1.2
AP/NaCMC (2:8)	Water-ethanol (7:3)	SD-N-AP 2	40.1 ±0.8	8.5 ±0.1	97.2 ±0.8

2.2.2.2 Spray drying

All the various powder formulations employed in this investigation were obtained by spray drying using a Büchi 290 Mini Spray Dryer (Büchi Laboratoriums-Technik AG, Flawil, Switzerland) fitted with a standard 0.7mm two fluid nozzle. The spray drying

process was repeated in triplicates for each of the powders. The yields of the spray-dried powders were quantified as a percentage of anticipated yields. The activity of the various spray-dried powders were measured in triplicates using validated assay methods described in previous sections and expressed as a percentage of nominal dose.

2.2.2.2.1 Preparation of spray-dried alkaline phosphatase

Alkaline phosphatase was spray-dried as a 2 % w/v aqueous solution. The spray drying parameters utilised in the micronisation of the enzyme were: Inlet temperature, 180 ^{0}C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator setting, 85 % (34 m^3/hr); to produce an outlet temperature of 99 – 101 ^{0}C.

2.2.2.2.2 Preparation of spray-dried alkaline Phosphatase – Sodium carboxymethylcellulose combinations

2 % w/v NaCMC:alkaline phosphatase (80:20) mixture was co-spray dried from water-only and a water:ethanol (90:10 and 70:30) suspension feedstock using the following spray drying parameters; Inlet temperature, 180 ^{0}C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator setting, 85 % (34 m^3/hr); to produce varying outlet temperatures (Table 2-12)

2.2.2.2.3 Preparation of spray-dried lactoferrin (SDLf)

Lactoferrin was spray-dried as a 2 % (w/v) aqueous suspension. The spray drying parameters utilised include; inlet temperature – 180 °C, aspirator setting – 95 %, pump setting – 3.2 mL/min, to produce an outlet temperature of 99 – 101 °C.

2.2.2.2.4 Preparation of apo Lactoferrin (aLf)

An iron deplete form of lactoferrin (aLf) was prepared from partially iron saturated lactoferrin as previously described by Newsome et al., (2007). Briefly, the partially iron saturated lactoferrin was resuspended at 1% (w/v) in deionzed water, transferred to dialysis membrane and dialyzed for 24h at 4 °C against 20 mmol/L sodium acetate, 20mmol/L sodium dihydrogen phosphate and 40mmol/L EDTA (pH 3.5). The protein was then dialyzed against 900 mL of deionzed water with at least 2 changes and used immediately or quick frozen at -80 °C. Iron removal was confirmed by atomic absorption spectroscopic measurements.

2.2.2.2.5 Preparation of spray-dried apo lactoferrin (SDaLf)

Following dialysis, the resulting protein suspension was spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature ranging from 100 – 102 °C. This procedure was repeated two more times to produce a total of three batches of spray-dried apo lactoferrin powders.

2.2.2.2.6 Preparation of co-spray dried lactoferrin with sodium carboxymethylcellulose (SDLf + NaCMC)

A 2 % w/v suspension of lactoferrin and sodium carboxymethylcellulose was prepared by mixing 1 g of lactoferrin and 1 g of sodium carboxymethylcellulose and making the mixture up to a final volume of 100 mL of deionised water. The resulting slurry was then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator rate, 95 %. These setting resulted in an

outlet temperature ranging from 97 – 99 °C. This procedure was repeated two more times to produce a total of three batches of co-spray dried lactoferrin with sodium carboxymethylcellulose dry powders.

2.2.2.2.7 Preparation of co-spray dried apo lactoferrin with sodium carboxymethylcellulose (SDaLf-CMC)

1g of lactoferrin was made up to a final volume of 100 mL with deionized water (1 % w/v) then transferred to dialysis membrane and dialyzed for 24h at 4 °C against 20 mmol/L sodium acetate, 20mmol/L sodium dihydrogen phosphate and 40mmol/L EDTA (pH 3.5). The protein was then dialyzed against 900 mL of deionzed water with at least 2 changes of the the deionised water. Then the entire content on the dialysis membrane was transferred into a beaker (usually the final volume was less than 100 mL), 1g of sodium carboxymethylcellulose was added to the dialysed protein and the final volume was then adjusted to 100 mL with distilled water (to produce a 2 % w/v suspension). The resulting slurry was spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature ranging from 98 – 99 °C. This procedure was repeated two more times to produce a total of three batches of co-spray dried apo lactoferrin with sodium carboxymethylcellulose dry powders.

2.2.2.2.8 Preparation of spray-dried tobramycin/gentamicin

A 2 % w/v solution of tobramycin or gentamicin was prepared by dissolving 2 g of tobramycin or gentamicin in a 50 mL of deionised water, upon complete dissolution the final volume was made up to 100 mL. The resulting solution was then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature

of 102 – 104 °C (for spray-dried tobramycin) and 99 – 102 (for spray-dried gentamicin). This procedure was repeated two more times to produce a total of three different batches of spray-dried tobramycin/gentamicin powders.

2.2.2.2.9 Preparation of co-spray dried lactoferrin with tobramycin/gentamicin

A 2 % w/v suspension of lactoferrin and tobramycin/gentamicin was prepared by mixing 1 g of lactoferrin and 1 g of tobramycin/gentamicin in 50 mL of deionised water, upon complete dissolution of the powders, the final volume was made up to 100 mL with deionised water. The resulting suspension was then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature of 104 – 106 °C (for co-spray dried lactoferrin with tobramycin) and 102 – 104 (for co-spray dried lactoferrin with gentamicin). This procedure was repeated two more times to produce a total of three batches of co-spray dried lactoferrin with tobramycin/gentamicin dry powders.

2.2.2.2.10 Preparation of co-spray dried apo lactoferrin with tobramycin/gentamicin

1g of lactoferrin was suspended in a final volume of 100 mL of deionized water (1 % w/v) then transferred to dialysis membrane and dialyzed for 24h at 4 °C against 20 mmol/L sodium acetate, 20mmol/L sodium dihydrogen phosphate and 40mmol/L EDTA (pH 3.5). The protein was then dialyzed against 900 mL of deionzed water with at least 2 changes of the deionised water. Then the entire content on the dialysis membrane was transferred into a beaker (usually this volume was less than 100 mL) and 1 g of tobramycin/gentamicin was added and the final volume made up to 100 mL (to produce

a 2 % w/v suspension). The resulting suspension was then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature ranging from 103 – 104 °C (for co-spray dried apo lactoferrin with tobramycin) and 93 – 103 °C (for co-spray dried apo lactoferrin with gentamicin). This procedure was repeated two more times to produce a total of three batches of co-spray dried apo lactoferrin with sodium carboxymethylcellulose dry powders.

2.2.3 Estimation of the iron content of Lf preparations

A standard calibration graph of spectrosol ferric nitrate was prepared by measuring the absorbance of stock concentrations (0.05 – 5 ppm) using a Perkin Elmer AAnalyst 100 atomic absorption spectrophotometer. The amount of iron bound to the various lactoferrin preparations was then estimated from the calibration curve following iron absorption measurements.

2.2.4 Moisture content determination

To determine the moisture content of the spray-dried powders, two methods were employed; gravimetric measurements and Karl Fischer titration.

2.2.4.1 Gravimetric measurements:

Thermogravimetric analysis was performed using Pyris 1 Thermogravimetric analyzer (Perkin elmer, Waltham, USA). Samples (weighing approximately 3 – 5mg) were placed into platinum pans and analysed under a nitrogen purge (20 mL/min) heated from 20 –

140°C at a heating rate of 10°C min^{-1} and the loss in mass as function of temperature were evaluated from triplicate assessments.

2.2.4.2 Karl Fischer (KF) titration:

Karl Fischer titration was carried out using a C20 Coulometric Karl Fischer titrator from Mettler Toledo (Leicester, UK). Analysis of samples weighing approximately 5mg was carried out using dried methanol KF Coulometric reagent CombiCoulomat fritless (Merck, Darmstadt, Germany). The polarising current for the KF coulometer was 5 µA and the endpoint (stop voltage) was 100mV. The absolute drift stop (end point criteria) value was 3 µg/min, relative drift stop was 2 – 5 µg/min and the maximum titration time was set to 10 minutes. The water content was calculated by predefined method function on the equipment.

2.2.5 Particle size analysis

Particle sizing of all the spray-dried powders utilised in the investigation were carried out in the dry state using the method of laser light diffraction within a Sympatec HELOS H1867 (Helium-Neon Laser Optical System, Sympatec Inc., Clausthal-Zellerfeld, Germany) with a measuring range of 0.1 – 87.5µm coupled to a RODOS dispersing unit and a VIBRI vibratory feeder. The powders were dispersed using compressed air while the RODOS dispersing unit was set at 2 bar. Dispersion pressures were carefully chosen following several titration experiments to determine the optimum pressure required for separation of the particles without causing their attrition. WINDOX 5.0 sensor control program 'HELOS Sensor control' (Sympatec GmbH, Clausthal–Zellerfeld, Germany) was utilized in particle size analysis.The average Volume median diameter (VMD) of three consecutive measurements was calculated.

2.2.6 Thermal analysis

The thermal behaviour of all the various powders was analysed using the Differential Scanning Calorimeter (DSC). DSC (Pyris Diamond DSC and Intracooler 2P: Perkin-Elmer, Wellesley, USA) analysis of a 3mg quantity of the powder was carried out in hermetically sealed aluminum pan using nitrogen purge at 20 mL/min (range: 0 – 250^{0}C, heating rate 10 ^{0}C/min).

2.2.7 Angle of repose

The angle of repose, which measures the inclination angle of the bulk powder to the free surface, was assessed as follows;

A funnel was clamped to a retort stand at a fixed height from the base. A pan of diameter 4cm was centered at the base directly underneath the funnel. The various spray-dried powders were poured through the funnel and allowed to flow. Care was taken to prevent vibration or movement of the funnel. The procedure was repeated three consecutive times to ensure reproducibility of the results.

The angle of repose was determined from the following equation;

$$Tan\ (\alpha) = \frac{height}{0.5\ base} \qquad \text{(Equation 2.4)}$$

2.2.8 Colorimetric detection and quantification of total protein

Quantitation of total protein was carried out according to the manufacturing instructions accompanying a bicinchoninic acid (BCA) assay kit. Briefly, bovine serum albumin standards were prepared by diluting the contents of a 1 mL ampoule containing 2 mg of

albumin (to prepare standard concentrations of 20 – 2000 µg/mL). The working reagent was prepared mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. Then a 200 µL aliquot of the working reagent was added into each of the wells already containing 25 µL of either standard or test protein. The plates were covered and incubated at 37 °C for 30 minutes, then cooled to room temperature and then the absorbance measured at 562 nm using a Multiskan Spectrum - UV/Vis Microplate Spectrophotometer.

2.2.9 Sodium dodecylsulphate – Polyacrylamide Gel Electrophoresis

The SDS-PAGE technique used in this investigation employed Laemmli sample buffer (prepared by mixing 4.4 ml of 0.5 M Tris (pH 6.8) with 4.4 mL of Glycerol, 2.2 mL of 20% (w/v) SDS, 0.5 mL of 1% (w/v) Bromophenol Blue, 0.5 mL of Beta-Mercaptoethanol) in the preparation of the protein samples. The β-mercaptoethanol in the sample buffer reduces protein disulphide bonds while the SDS denatures the proteins. The reduced and denatured proteins were heated at 70°C for 5 minutes (to enhance the denaturation process) then a 15 µl aliquot of each of the protein sample was loaded into each of the wells of a 4 – 20 % precast polyacrylamide gels (Bio-Rad Laboratories, Hercules USA). 5 µL aliquot of prestained protein ladder was also loaded into one of the wells. The gels were run at a constant current of 60 mA (200V, 12W) for 22 minutes in a Bio-Rad vertical electrophoresis tank until the dye had reached the lower edge of the gel. Gels were run in 1 L of 1x running buffer diluted from a 10x stock (prepared by dissolving 30.3 g (0.25 M) Tris Base, 144 g (1.92 M) Glycine, 10 g (1%) SDS in distilled water to make a final volume of 1000 mL).

2.2.9.1 Detection of proteins

2.2.9.1.1 Coomassie blue stain

The various protein bands in the gels were detected by staining with the staining solution (0.1 % w/v Coomassie brilliant blue R-250 dye, 50 % v/v methanol and 10 % v/v glacial acetic acid) for 30 minutes followed by destaining (40 % v/v methanol and 10 % v/v glacial acetic acid) for another 30 minutes.

2.2.9.1.2 Silver stain

Silver staining of the protein bands was performed on the SDS-PAGE using Pierce silver stain kit. This staining procedure is well suited for the detection of nanogram and subnanogram quantities of protein and hence makes this procedure a more sensitive technique than coomassie staining. The gel was washed with ultrapure water and fixed in a solution of 30% v/v ethanol: 10% v/v acetic acid solution for 15 mins. The fixative was decanted and the gel washed with a 10% ethanol solution for 5 mins, then with enough ultra pure water to just about cover the gel for another 5 mins. The gel was then incubated for 1 min at room temperature in the sensitising solution (prepared as directed by the manufacturer). After this, the gel was washed for 1 min with ultra pure water (twice). The gel was incubated for 30 minutes in the stain working solution (prepared as directed by the manufacturer). The working solution was decanted and the gel quickly washed with ultrapure water for 20 seconds and incubated with the developer working solution (prepared as directed by the manufacturer) until optimal protein bands are achieved (2 – 3 minutes). The developer working solution was decanted and replaced with stop solution (5 % v/v acetic acid) for 10 minutes. The gel images were captured using a G:BOX connected to a computer running GeneSnap version 7.07.

2.2.10 Western Blot

Western blotting is an analytical technique whereby specific antibodies are used to detect specific proteins in a given sample of tissue homogenate or extract. This was carried out to confirm the identity of the protein (lactoferrin) as supplied and following spray drying by using specific antibodies. Following protein quantification (section 2.2.8), 1 mg/mL of the protein was diluted 1:5 in volume with laemmli sample buffer as described in section 2.2.9 and boiled for 5 minutes. 4 – 20 % polyacrylamide gels were loaded with the various protein preparations at 15 µL per lane; each gel also contained a lane of prestained molecular weight marker. Gels were run as described above in section 2.2.9. Separated proteins were then transferred from the gel to a nitrocellulose membrane using the wet transfer technique (Lin and Kasamatsu, 1983; Towbin *et al.*, 1979). Briefly, the gel and membrane were sandwiched between two sponges and filter paper and dampened with electrotransfer buffer (prepared and contained 100 mL of 10x running buffer, 200 mL of methanol and sufficient distilled water to make up to 1000 mL) making sure to roll out any air bubbles between the gel and the membrane. The assembly was submerged in transfer buffer, placed into a TE22 mini transfer tank to which an electric field is applied (400 mA) for 30 minutes. Following transfer of the proteins (confirmed by visual inspection of the gel to verify that the prestained markers have moved from the gel onto the membrane and also by staining the gel post-transfer), the nitrocellulose membrane was removed from the sandwich and covered with block buffer - 0.3 % (v/v) Tween 20 in PBS –for 1 hour at room temperature with agitation. The nitrocellulose membrane was then washed with wash buffer – 0.05 % (v/v) Tween 20 in PBS and incubated for 2 hours at room temperature (or stored at 4°C overnight) with a 1:500 (v/v) dilution of the primary antibody (IG12, 4C6 and 4C3).The membranes were then washed with wash buffer - 0.05 % (v/v) Tween 20 in PBS –every 15 mins for 1 hour before incubating with a 1:5000 dilution of the secondary antibody (anti mouse) for 2 hours at room temperature. The membrane was once again washed with wash buffer

every 15 minutes for 1 hour and the antibody binding detected using Immobilon® Western Enhanced Chemiluminescent substrate (ECL) following the manufacturers instruction. Blots were drained and exposed to x-ray film for 2 minutes. x-ray films were developed using Kodak GBX developer and Kodak GBX fixer as per the manufacturer's instruction in order to allow visualisation of the protein bands.

2.2.11 Content uniformity determinations

Following blending, the various powder formulations were assessed for content uniformity. Each formulation was spread on a clean petri dish surface. Ten samples each weighing 20 ± 2 mg were randomly taken from different areas within the spread-out powder and dissolved in 10mL distilled water to give a concentration of 0.2 mg/mL. The enzyme activity of 500 µL was evaluated using methods described in section 2.2.1.1.2 and absorbance values obtained were then compared to the standard calibration plot of absorbance against concentration (by extrapolation and intrapolation of the standard calibration curve) and their content uniformity expressed as relative standard deviation.

2.2.12 Capsule filling for DPI device

For spray-dried alkaline phosphatase: One empty size 3 gelatin capsule was weighed and 20 ± 2 mg of spray-dried alkaline phosphatase was measured and manually loaded into the capsule. This was repeated two more times to enable triplicate measurement of the aerosolisation performance of spray-dried alkaline phosphatase.

For spray-dried NaCMC modified formulations of alkaline phosphatase: For each of the formulations, five empty size 3 gelatin capsules were weighed and 20 ± 2mg of each formulation blend was measured and manually loaded into the 5 capsules.

For spray-dried lactoferrin and combinations of lactoferrin with aminoglycosides: Empty size 3 gelatin capsules were weighed and 30 ±2 mg of the various formulations was measured and manually loaded into individual capsules.

Following filling, each of the capsules was put into Cyclocaps™ Cyclohaler™ DPI device (Pharmachemie BV, The Netherlands) and aerosolised immediately.

2.2.13 Preparation of pMDI canisters

Calibration of the pamasol P2011 propellant filler: Empty aluminium MDI canisters with cap were weighed and crimped. The fill dial on the pamasol was set to deliver 6g of HFA-134a per fill was pressure filled into the canisters through the valve using a Pamasol P2011 propellant filler (Pamasol Willi Mäder AG, Pfäffikon, Switzerland). The mean actual fill weight of five MDI canisters was calculated.

Preparing alkaline phosphatase pMDIs: Following calibration of the pamasol small scale crimper, empty aluminium MDI canisters with caps were weighed and 20mg of spray-dried alkaline phosphatase, 5 x 20 mg per canister of co-spray dried sodium carboxymethylcellulose and alkaline phosphatase were put into the aluminium canisters. All the canisters were crimped with a BK357 30 µL valve and approximately 6 g of HFA-134a was pressure filled through the valve using a Pamasol P2011 propellant filler. Three pMDI canisters were prepared for each of the spray-dried formulations. All

formulations were vortex-mixed for 1 minute tested for *in vitro* aerosolisation properties using a next generation impactor for 2 x 5s and stored valves up at room temperature.

2.2.14 *In vitro* aerosol characterisation

An in *vitro* technique which is useful to some extent in the prediction of in *vivo* powder deposition was developed. Assessment of aerodynamic deposition was carried out using the Next Generation Impactor (NGI). The NGI (Model 170, Copley Scientific Limited, Nottingham, UK) had been calibrated by the manufacturers. A flow rate of 60 L/min was maintained throughout the entire investigation.

The detailed procedure for use of NGI in section 2.9.18 of supplement 5.1 of the European Pharmacopoeia (Ph. Eur.) was employed throughout the investigation.

2.2.14.1 Assembly method for the NGI

15mL of the collection solution (distilled water) was accurately measured and transferred into the pre-separator using a pipette. Care was taken to ensure that a good seal was obtained between the throat, the pre-separator and the vacuum during assembly of the NGI and by attaching a flow rate meter (Copley Scientific Limited, Nottingham, UK) to the induction port it was ensured that the flow rate was maintained at 60L/min.

2.2.14.2 Operational method for the NGI

The cyclohaler™ device fitted with the filled size 3 capsule, was attached to the induction port through a specially designed adaptor to provide a very good seal. The capsules were punctured by pressing the push buttons on the inhaler device. The

vacuum pump was started and the powder formulation was allowed to aerosolise for 2 x 5 seconds.

For the pMDI formulations, 20 actuations were aerosolised through a plastic actuator attached to the NGI via the USP throat. After each actuation the canister was removed and shaken to provide a homogenous mixture for the next actuation. Each deposition experiment was repeated in triplicate.

2.2.14.3 Sample recovery method for the NGI

Following aerosolisation of the dry powder inhaler (DPI) and pMDI formulations, the NGI was dissembled and deposits collected from all the different components using the collecting solution (distilled water). The powders which deposited in the throat were recovered by rinsing the throat with 20mL of distilled water; the pre-separator was properly washed with 15mL of collecting solution. 20mL volume of collecting solution was placed in stage 1 and MOC while 10mL volume of collecting solution was placed in stages 2 - 7, the resulting solutions were each swirled and transferred to 50mL beakers. Complete dissolution was facilitated in all solutions by placing the beakers containing the solutions on magnetic stirrers (Fisher Thermix Stirring Hot Plate, Model 201T). The resulting solutions were visually inspected to confirm the complete dissolution of the powders. A sample of the solution was then removed for UV analysis.

Table 2.5: Operating conditions and theoretical cut-off diameters of Next generation impactor

Next generation impactor	
Flow rate (L/min)	60
Time per actuation (s)	2 x 5
Volume per actuation (L)	10
Cut-off diameters (µm)	
Stage 1	8.1
Stage 2	4.4
Stage 3	2.8
Stage 4	1.7
Stage 5	0.94
Stage 6	0.55
Stage 7	0.34

2.2.14.4 Validation of dry powder recovery from the NGI device

The method employed in the collection of samples from the NGI was validated through the following procedure; 1mg each of both the commercially available alkaline phosphatase and the spray-dried alkaline phosphatase were each directly sprinkled on the cups, the pre-separator and the throat. The samples were recovered and quantified by rinsing the various impaction stages with the collecting solution (distilled water) and resulting solution analysed using the spectrophotometer.

2.2.14.5 Determination of RD, ED, FPD, FPF, MMAD, GSD and Dispersibility

The calibration curve was used to determine the concentration hence, the mass of the sample recovered from the throat, pre-separator and each of the cups. The recovered

dose (RD) denotes the sum total of all the impactor sections per aerosolised capsule. The emitted dose was taken as the amount of alkaline phosphatase that was emitted from the capsule. The fine particle dose (FPD) denotes the amount of alkaline phosphatase deposited in the lower stages of the NGI (cut-off diameter <5 μm). This was determined from a plot of cumulative mass against effective cut-off diameter and interpolating the activity of enzyme with a diameter of ≤5 μm. The fine particle fraction denotes the ratio of the FPD to total dose corrected for the actual amount of enzyme present in the formulation. Mass median aerodynamic diameter (MMAD) was determined by calculating the ECD at cumulative 50%. The dispersibility of the powders is the percentage of the FPD to ED. For the pMDI formulations, the pMDI vial was weighed before and after each run, to enable the computation of the total dose (TD) based on the proportion of alkaline phosphatase enzyme in each suspension.

2.2.15 Bacterial viable counts

All viable counts carried out in this study were based on the Miles and Misra method (surface viable count) with some modifications. Summarily, 10 µL aliquots of serially diluted bacterial suspensions taken from overnight cultures (10-fold, 100-fold, 1000-fold and in some cases 10,000-fold dilutions in sterile distilled water) were placed on the surface of 'sufficiently dried Mueller-Hinton Agar plate', evenly spread out and allowed to dry before incubating at 37 °C for 18 – 24 hours. Each plate (or sector if divided) was observed for growth. Dilutions containing less than 300 discrete colonies were counted and the viable count determined by using the following equation

$$Colony\ forming\ unit\ per\ mL = \frac{Number\ of\ colonies}{Volume\ tested} \quad \text{x} \quad Dilution\ Factor$$

2.2.16 Bacterial Killing assays

Minimum bactericidal concentration (MBC) determinations for the various antimicrobial test agents was performed as previously described by Xu et al., (2010) with some modifications. Briefly, 10 µL of overnight bacterial cell suspension was diluted to approximately 10^8 cfu/mL and incubated in a 96-well microtitre plate containing 90 µL of the various test agents in casamino acid (CAA) media for 1 hour. Bacterial growth was determined at 10 minutes intervals by plating out serially diluted aliquots from each well onto Mueller-Hinton agar which had been allowed to set in petri dishes. Plates were incubated overnight at 37°C. The number of colony forming unit per millilitre was then determined as described above (in section 2.2.15.)

2.2.17 Biofilm quantification assay

The ability of *P. aeruginosa* PAO1 to form biofilms was investigated using methods previously described by Eckhart et al., (2007) and Merritt et al., (2005) with some modifications. Overall, the protocol involves growing bacterial cells in microtitre dishes for a desired period of time and then the wells are washed to remove planktonic bacterial cell. However, cells which remain adherent in the wells are stained with a dye (crystal violet in this case), washed again after a given period of time and the surface associated dye solubilised in an appropriate solvent (in this case 30 % v/v glacial acetic acid)

2.2.17.1 Initiation of biofilm experiments

100 µL aliquots of overnight cultures of planktonic bacteria culture were put into individual wells of a 96-well microtitre plate and then 100 µL of the test compounds; Lactoferrin (Lf), Spray-dried lactoferrin (SDLf), Apo Lactoferrin (aLf), Spray-dried apo

Lactoferrin (SDaLf), Tobramycin (Tobi), Spray-dried Tobramycin (SD Tobi),), Spray-dried Tobramycin + Lactoferrin (SD Tobi + Lf), Spray-dried Tobramycin + Apo Lactoferrin (SD Tobi + aLf), Gentamicin (Genta), Spray-dried Gentamicin (SD Genta),), Spray-dried Gentamicin + Lactoferrin (SD Genta + Lf) and Spray-dried Gentamicin + Apo Lactoferrin (SD Genta + aLf), were added at a concentration of 1mg/mL. Control wells were also setup with no antibacterial agents included (antibacterial agents were replaced with sterile distilled water). The microtitre plates were incubated at 37°C for 24 hours after which biofilms grown by the bacteria were then quantified.

2.2.17.2 Quantifying biofilms

Bacterial suspensions were removed and the wells washed twice with 250 µL of distilled water. Bacterial biofilms (usually consisting of microcolonies encased in extracellular polysaccharide material (Costerton *et al.*, 1999)) adhering to the walls of the 96-well plate were stained with 200 µL of 0.1 % w/v crystal violet (CV) solution in water for 15 mins. Excess CV was removed and the wells rinsed with distilled water. The dye (CV) which was still adherent to the biofilm was eluted with 200 µL of a 30 % v/v solution of glacial acetic acid. The quantity of biofilms in each well was then quantified by measuring the optical density of the eluate at 550 nm. The absorbance values of control wells containing only 30 % acetic acid was subtracted from the test wells in order to minimise background interference. All experiments were repeated in triplicates using new 96-well microtitre plates every time. The absorbance values obtained at 550 nm (A_{550}) are proportional to the amount of biofilm present.

2.2.17.3 Persistence of biofilms experiments

In order to assess the effects of the test compounds on the persistence of bacterial biofilms, 100 µL aliquots of overnight planktonic bacteria cultures were put in wells of a 96-well microtitre plate and incubated at 37°C for 24 hours. Then all the various

antibacterial agents listed in section 2.2.17.1 was then added at the same concentrations (1mg/mL). Control wells were setup as well which contained only sterile distilled water and the plates incubated at 37°C for 24 hours. The biofilms were quantified using the procedure described above in section 2.2.17.2.

2.2.18 Growing biofilms on cover glass

A 1 mL aliquot of overnight bacterial cell suspension (~ 10^7 cfu/mL) of *P.aeruginosa* PAO1 grown at 37°C in a media containing 0.5 % w/v casamino acids and 1 mM $MgCl_2$ (casamino acid rich media) was put into each chamber of an 8-well chamber glass slide and incubated at 37°C for 72 hours. The bacterial cell suspension was removed and replaced with 0.5 mL of fresh casamino acid rich medium every 16 hours. Following 72 hours growth of bacterial biofilms on the glass slide, the wells were carefully washed with phosphate-buffered saline (PBS) to remove any remaining planktonic bacteria that may be surface attached to the biofilms, without detaching the biofilm from the glass. The surface attached biofilms were exposed to 200 µg of the various test agents in solution and incubated at 37°C for 24 hours. Both positive and negative controls were setup. While the positive controls contained 200 µL of the CAA rich media without any antibiotic included, the negative control contained 200 µL of Isopropanol (to ensure the complete destruction of bacterial micro-colonies in the biofilms).

2.2.18.1 Staining of biofilms to enable visualisation of live/dead biofilms

Following treatment with the antimicrobial agents, the glass slides were washed again with 0.5 mL of PBS and stained with a LIVE/DEAD kit (Invitrogen Molecular Probes, Eugene, OR, USA) using a mixture of 3 µL of syto 9 (green fluorescent) with 3 µL of propidium iodide (red fluorescent) per mL of saline. Both syto 9 and propidium iodide are

nucleic acid stains. While syto 9 stains all nuclei acid in a population (both those with intact and damaged membranes), propidium iodide on the other hand stains only bacteria with damaged membranes. It is important to note that when used together, propidium iodide causes a reduction in the green fluorescence of syto 9.

2.2.18.2 Visualisation of biofilms

Visualisation of both the treated and untreated biofilms was carried out using Leica TCS SP5 II (Leica Microsystems, Milton Keynes, UK) confocal laser scanning microscope (CLSM). Images were obtained using 20x HC PL APO20x/0.70 CS lens. For detection of SYTO 9 (green channel), 488 nm line of the argon laser was used and a detection bandwidth of 495 – 515 nm. For detection of propidium iodide (red channel), 561 nm line was used and a detection bandwidth of 615 – 600 nm. Image analysis was carried out using a Leica LAS AF Version 2.2.1 build 4842.

2.2.19 Antibiotic permeability assay

The penetration of tobramycin, gentamicin and their various combinations through *P.aeruginosa* biofilms was investigated using the method previously described by Singh et al., (2010) with some modifications.

2.2.19.1 Antibiotic disc preparation

Antibiotic susceptibility disks containing; tobramycin, gentamicin and combinations of both antibiotics and the different preparations of lactoferrin were all prepared locally by impregnating a sterile blank paper disk cut-out with a diameter of 6.3 mm with 0.02 mL of 1mg/mL standard antibiotic solutions (to prepare a 20 µg antibiotic disc). The procedure employed was as follows; using an ordinary two-hole puncher, paper disk with approximate diameter of 6.3 mm were cut out. Then the curled surfaces were

flattened by spreading the discs on a clean smooth surface and then pressing them by rolling a bottle repeated over them. The disk were then sterilised by autoclaving. To load various concentrations of the antibiotics onto the disks, antibiotic solutions containing the required mass of the antibiotic agent in 0.02 mL was prepared (since 0.02 mL is the optimum volume that a paper disk that size could absorb). For example, for the preparation of tobramycin disk with a potency of 10 µg, the antibiotic solution prepared contained 500 µg of tobramycin per mL. Using a micropipette, 0.02 mL aliquots were delivered onto the sterile disks place on a dry petri dish making sure to allow the tip pipette to make light contact with the disk. The petri dishes were then placed in a clean incubator set at 37 °C for 2 hours. Upon drying the locally made antibiotic disks were used immediately or stored at -20 °C. *Escherichia coli* NCTC 10418 was used as the organism for quality control of the antibiotic discs as well as in preparing a calibration curve of the diameter of the zone of inhibition (mm) against log concentration.

2.2.19.2 Growing the biofilms on track-etched polycarbonate membranes

Sterile track-etched ploycarbonate membranes were inoculated with 5 µL of *P.aeruginosa* bacterial suspensions and incubated on Muller-Hinton agar plates at 37 °C for 48 hours with the membrane-supported biofilms transferred to a new agar plate every 18 hours.

The quality control organism *Escherichia coli* NCTC 10418 was cultured overnight in Luria broth. The growth of the control organism was adjusted to the density of a 0.5 McFarland standard (prepared by adding 0.5 mL of 0.048 M $BaCl_2$ to 99.5 mL of 0.18 M H_2SO_4 with constant stirring, absorbance range; 0.08 – 0.13) by adding sterile distilled water while visually inspecting both the test and the standard suspension against a white background with a contrasting black line. A sterile cotton swab was dipped into the culture medium containing the *E. coli* and spread on Mueller-Hinton agar plate in order

to permit the growth of a confluent lawn of bacteria following 18 hours of incubation at 37 °C.

2.2.19.3 Penetration of antibiotics through biofilms

The control organism (*Escherichia coli* NCTC 10418) which was applied on the surface of the agar was allowed to dry and nitrocellulose membranes supporting biofilm growth were then placed on top of the agar already impregnated with the control organism. A 6 mm nitrocellulose membrane was then placed on top of each biofilm and the antibiotic disc placed on the top of this.

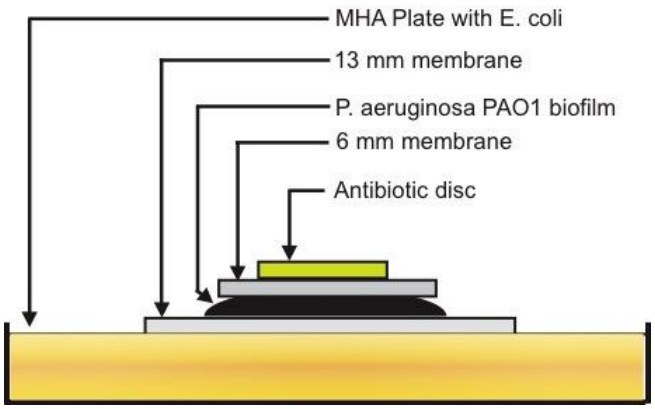


Figure 2 -2: Schematic depiction of experimental setup used to track the penetration of antibiotics through *Pseudomonas aeruginosa* biofilms. The Mueller-Hinton agar plate is covered with a confluent lawn of the control strain (*E. coli* NCTC 10418). A sterile track-etched polycarbonate membrane (0.2 µm pore size) that supports biofilm growth is placed on MHA with a smaller diameter polycarbonate membrane and a moistened paper disk containing the test compound place on the biofilm.

Both locally prepared and commercially supplied antibiotic disks were moistened with 20 µL of sterile distilled water to prevent capillary action of the antibiotics (as it may adversely affect the zone diameter). Controls, which comprised of sterile polycarbonate membranes and antibiotic disc without biofilms were also set up. All plates were incubated at 37°C for 24 hrs and the clear zones visible on the plates indicating the inhibition of growth by the antibiotic (tobramycin or gentamicin) were measured. A calibration plot of the zone diameter measurements against log concentration (for antibiotic discs containing 1 – 10,000 µg) was constructed and used to estimate the concentration of the antibiotic that passed through the biofilms. The diameter of the zones of inhibition in the controls were taken to signify 100% penetration and used to determine the percent penetration and percent retardation of the antibiotics through the *Pseudomonas* biofilms. All experiments were performed in triplicates and a two-tailed, paired t-test was used for statistical analysis. In order to avoid false negative results arising from side diffusion of the antibiotic around the biofilm rather than through the biofilm, biofilms were only used if they were <13 mm in diameter and were therefore contained within the larger membrane.

2.2.20 Testing the compounds on airways epithelial cells *in vitro*

2.2.20.1 Culturing cells from frozen stock

As all cell stocks were stored in liquid nitrogen prior to commencement of experiments, there was a need to properly thaw all cell types to ensure that they maintain their viability.

Frozen cells (BEAS-2B, C38 and IB3) were removed from liquid nitrogen and put into a water bath set to 37 °C and allowed to thaw. Once the cells had thawed, the 1 mL content of the vials was put into a tube containing 5 mL of cell culture medium (DMEM

supplemented with 10% v/v fetal bovine serum, 2mM L-glutamine and antibiotics) and centrifuged at 2000 rpm for 5 minutes. Following centrifugation, the supernatant was discarded while the cell pellet was resuspended in 10 mL of cell culture medium. The entire contents of the tube was then transferred into a tissue culture flask, the flask was appropriately labelled (cell type, passage number and date) and flasks were incubated at 37 °C, 5 % CO_2 in a humidified incubator.

2.2.20.2 Subculturing cells

The cell culture medium was warmed to 37 °C in a water bath. The cells were washed with phosphate buffered saline (PBS) to remove the FBS which would have inhibited the activity of trypsin. The PBS was discarded and 2 mL of trypsin-EDTA (0.25%) was added to lift adherent cells off the tissue culture flask. Following the addition of trypsin, the tissue culture flask was incubated at 37 °C for 10 mins as this is the optimum condition required for trypsin's enzymatic activity. Following detachment of the cells from the tissue culture flask, 3 mL of the cell culture medium was added to the tissue culture flask to neutralize the trypsin. All the cells contained in the tissue culture flask were then collected into a falcon tube and centrifuged at 2000 rpm for 5 mins. The supernatant was discarded and the bottom of the falcon tube flicked to loosen the cells. Enough cell culture medium was added to make 1 mL for each of the tissue culture flask to be used- flasks were usually subcultured at a 1:3 ratio. Finally 9 mL of cell culture medium was added to each tissue culture flask and incubated 37 °C, 5 % CO_2. For setting up plates of cells for experiments, cells were resuspended in 1ml and counted using a haemocytometer.

2.2.20.4 Counting of cells

Cell counts were carried out to ensure consistency in the number of cells seeded in every experiment. After the cells have been trypsinized, centrifuged and resuspended in 1ml fresh cell culture medium as described previously, 10 µL of a homogenously mixed cell suspension was withdrawn from the falcon tube and put into an eppendorf tube containing 10 µL of trypan blue and mixed properly (1:1 dilution). 10 µL of the mixture was then added to opposite sides of an improved Neubauers haemocytometer and a cover slip placed over the counting chamber. Cells in the four quadrants and the middle of each of the grids of the haemocytometer were counted and the average calculated number of cells multiplied by the dilution factor and then by 10^4 to obtain the number of cells per mL.

2.2.20.3 Submerged cell culture

Airway epithelial cells were counted and seeded at a density of 100,000 cells per well in a 24-well plate. The cells were grown under submerged conditions (i.e. airway epithelial medium inundates the epithelial cells) maintained in DMEM/F12 medium supplemented with (5 % v/v FBS, and 0.1 U/mL penicillin and 0.1 mg/mL streptomycin) and incubated in tissue culture flasks at 37 °C, 5 % CO_2 until they had attained approximately 90 % confluency. A light microscope was used to monitor cell confluency, and to make sure that there was no bacterial or fungal contamination.

2.2.20.5 Culturing cells on Transwell® (TW) Inserts

Culturing epithelial cells on either Transwell or inserts permits growth at the air-liquid interface and allows for *in vitro* simulation of airway epithelial cells from either the apical (lumen-facing) or basal (blood-facing) aspect of the cultures. The Transwell insert used

comprised a 24-well translucent PET membrane with the following specifications; membrane diameter – 6.4 mm, pore size – 0.4 µm, an effective growth area of 0.3 cm^2.

Airway epithelial cells were seeded at a density of 100,000 cells per 300 µL of airway epithelial cell growth medium into the apical portion of the insert. 600 µL of epithelial cell growth medium was ten added to the basal compartment of the Transwell®.

The whole setup was incubated at 37 °C, 5 % CO_2 for four days. On the fourth day, the epithelial growth medium on the apical portions of the Transwell® was removed and the epithelial cells were now said to be at air-liquid interface (ALI – this has been shown to permit the induction of mucociliary differentiation). The epithelial cell growth medium in the basolateral compartment was also replaced concurrently. Subsequently, all the liquid permeating to the apical portion of the inserts (apical liquid) was removed and the basolateral medium replaced every other 4 or 5 days until the cells have assumed the required tight junction. This had been previously reported by Willets, (2012) and Bielemeier (2012) to be around 11 to 14 days post apical medium removal.

2.2.20.6 Measurement of transepithelial electrical resistance (TEER)

Trans epithelial electrical resistance (TEER), which is a measure of the resistance of electrical current across an epithelial cell layer, informs on the confluence of a cellular monolayer as well as the presence of tight junctions. To measure the TEER of cell cultures growing at ALI, 300 µL of airway epithelial medium was added to the apical compartment of the inserts containing the various epithelial cells growing at ALI. The trans epithelial electrical resistance was then measured using Epithelial Voltohmmeter with STX2 chopstick electrodes (World precision instruments, Sarasota FL, USA). The shorter portion of the chopstick electrode was introduced into the apical compartment while the longer portion was put into the basolateral compartment. Three independent

measurements were made for each well including a blank (empty) well containing no cells. The resistance of the empty well was subtracted from the mean resistance of the wells containing epithelial cells and reported as ohm (Ω) • cm^2.

2.2.20.7 In vitro toxicity assessments

Airway epithelial cells were seeded at a density of 100,000 cells per 1 mL of airway epithelial cell growth medium per well and left for 24 hours. Following achievement of 90 % confluency of submerged cells (BEAS-2B, C38, IB3 and CALU) in the 24 well plates the cell culture media in the various wells were replaced with 0.5 mL per well of a serum (FBS) and antibiotic free media and incubated at 37 °C, 5 % CO_2 for 24 hours. The wells were visually inspected for bacterial or fungal growth under the light microscope, in the absence of which 500 µL aliquot of insulin transferring and selenium (ITS) cell culture medium containing 5 mg of the individual agents (either alone or in combination) undergoing test was added and incubated for a further 24 hours at 37 °C, 5 % CO_2.

2.2.20.7.1 Cell viability assay using Cell Titer Blue™ (CTB)

Cell viability assessments whose primary goal was to determine if the metabolic processes of submerged cells (previously exposed to the various antimicrobial agents) was still active.

Cell Titer-Blue™ assay is an endpoint assay that provides a homogenous, fluorometric method for monitoring cell viability. The assay is based on the ability of living cells to convert resazurin (a redox dye) into resorufin (a fluorescent product)

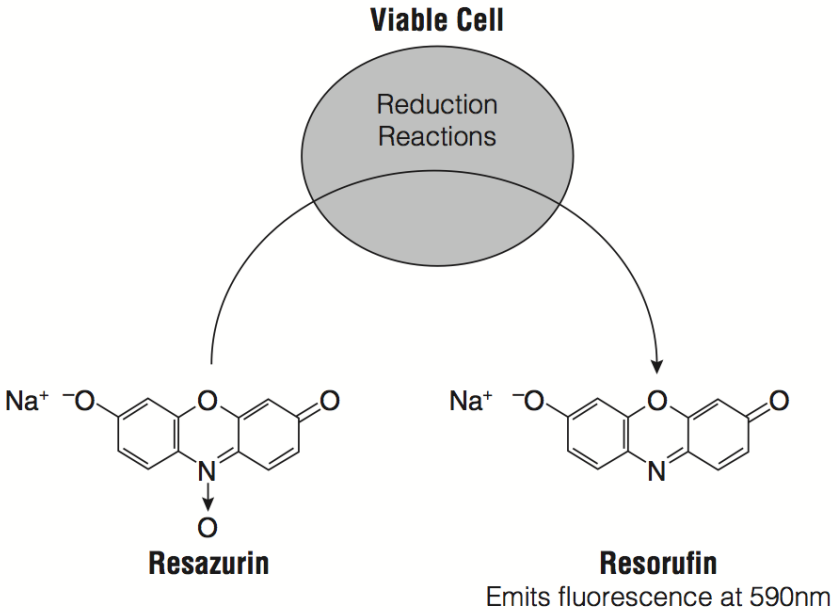


Figure 2 -3: Conversion of resazurin to resorufin by metabolically active cells.

Following exposure of submerged cells to the various agents for 24 hours as described in section 2.2.20.7,100 µL of CTB was added to each well and cells were incubated at 37 °C for 3 hours. A 200 µL aliquot from each well was transferred to a black 96 well plate and the fluorescent intensity measured using a standard multi-well fluorescent plate reader (Spectramax Gemini XS, Molecular Devices, Berkshire UK) with 560 nm excitation and 590 nm emission wavelength.

2.2.20.7.2 Quantification of IL-8 using Enzyme-linked immunosorbent assay

For ELISA studies, after 24 hour incubation, the supernatant was harvested, put into 1.5 mL eppendorf tubes, labelled and centrifuged at 2000 rpm for 5 min to remove any dead cells or cellular debris from the supernatant so as to prevent interference with the ELISA results. The resulting supernatant was put into a 1.5 mL eppendorf and used immediately for cell viability or IL-8 investigations or stored at -80 °C.

The ELISA was carried out according to the manufacturer's instructions, with some minor modifications. Briefly, high affinity binding microtitre plates (Nunc MaxiSorp™) were coated with capture antibody in coating buffer. This was prepared by adding 50 µL of reconstituted capture antibody (100 µg/mL) in 10 mL of carbonate bicarbonate buffer (reconstituted by dissolving one capsule of buffer (Sigma) in 70 mL of deionised water, upon complete dissolution the final volume was made up to 100 mL with distilled water to produce 0.05 M carbonate-bicarbonate buffer (pH 9.6). 50 µL of capture antibody was dispensed into each well, plates were covered with adhesive seals and incubated overnight at a temperature of 4 °C. Following incubation, the contents of the plates were discarded and the microtitre plates washed three times with the wash buffer – PBS with 0.05 % v/v Tween-20 and vigorously tapped to dry. 200 µL of block buffer – wash buffer with 1% w/v BSA was added into each well and plates were re-sealed and incubated for 1 hour at room temperature. The microtitre plates were once again washed with the wash buffer and tapped dry, then, 50 µL of standard dilutions of IL-8 (0 – 1000 pg /mL) and test samples were added into individual wells in triplicates and incubated at 37 °C for 1 hour. Following incubation, the microtitre plates were once again washed three times and slapped dry, 50 µL of detection antibody (anti-human IL-8) was then put into each well and incubated at 37 °C for 1 hour (2 hours at room temperature). Excess antibody was removed and the wells washed again. 50 µL of reconstituted Avidin-HRP enzyme (6 µL stock diluted in 12 mL of the diluent) was put into each well and the microtiter plate incubated at room for 30 minutes. The microtitre plate was again washed and 50 µL of SigmaFAST™ OPD substrate (prepared according to manufacturers direction) was put into each well and the absorbance measured after 10 minutes. The IL-8 content of each of the test samples were determined from a calibration plot of absorbance against log concentration of standard IL-8.

2.2.21 Statistical analysis

Statistical analyses were performed on the data obtained. Data is presented as the mean ± standard deviation of the mean. GraphPad InStat version 3.06 software was used in analyzing data. One way ANOVA with Tukey multiple comparison and post test was employed.

Chapter Three

Developing a method for spray drying a model potein (alkaline phosphatase)

3.1 Summary

The purpose of this study was to investigate the effects that different excipients and solvents have during the process of spray drying proteins. Alkaline phosphatase was chosen as the model protein because it is readily available, economical to use and spray-drying induced damage can be assayed using a straight forward enzyme activity assay. Specifically, the suitability of using the excipient, sodium carboxymethylcellulose, as a thermoprotectant with or without employing different concentrations of cosolvent during spray drying were examined. This investigation also sought to assess the *in vitro* suitability of utilising either pressurised metered dose inhaler (pMDI) or dry powder inhaler (DPI) in the delivery of the various spray-dried powders to the airways. Four spray-dried powders were investigated: a control comprising of 2% w/v spray-dried enzyme alone, and three test formulations comprising of combinations of 4:1 (w/w) sodium carboxymethylcellulose/enzyme spray-dried from a water based suspension feedstock and two water:ethanol (70:30 and 90:10) co-solvent system, with suitably optimised spray drying parameters. The alkaline phosphatase activity, molecular weight, particle size, moisture content, crystallinity and yield of the spray-dried formulations were assessed. Both DPI and pMDI formulations were prepared. *In vitro* deposition studies using the next generation impactor (NGI) indicates that higher units of alkaline phosphatase were delivered using the DPI (130 – 242 units/mL compared to 20 shots of pMDI delivering 19 – 124 units/mL) with the co-solvent, sodium carboxymethylcellulose modified formulations delivering a significantly higher fine particle fraction (< 5 µm) ($p < 0.001$) than the excipient-free spray-dried formulations. *In vitro* deposition studies of the various spray-dried powders using the pMDI device showed that a high percent respirable fraction of the enzyme was consistently delivered, although the water-ethanol (90:10) cosolvent and sodium carboxymethylcellulose modified formulation delivered significantly ($p < 0.01$) higher respirable fraction. While the selection of appropriate excipients and solvent systems may offer the ability to prepare spray-dried powders

suitable for the pulmonary delivery of peptides and proteins (since they possess very low inter-particulate cohesion and the requisite particle size needed for deposition in the lower respiratory tract), they can also have negative effects. The data shows that proteins which are spray-dried without excipients may also retain >70% enzymatic activity compared to the native activity, while also producing respirable particles which can be delivered to the lower airways.

3.2 Introduction

The inhaled route has been widely used to deliver low molecular weight active pharmaceutical ingredients (APIs), which aids the management of local airway diseases such as asthma, chronic obstructive pulmonary diseases and infection. However, recent developments in biotechnology means that therapeutic protein drugs are now more readily used in the management of various diverse diseases including; anaemia (erythropoietin and analogues) , chronic hepatitis B or C (interferon alfa), prostate cancer (Luteinizing Hormone Releasing Hormone; LHRH), rheumatoid arthritis and Crohn's disease (infliximab) (Li *et al.*, 2002; Fukuda *et al.*, 1989). Even though protein formulations are now commonplace amongst the repertoire of medicines available to medical practitioners, it is still the case that the parenteral route remains the major route utilised in the delivery of these therapeutic peptides. This is due to formulation challenges commonly encountered with protein APIs including; inactivation of the protein during or after processing and poor long term stability. The two most common stabilisaton methods employed to stabilise proteins are; 1) the use of excipients and 2) dehydration of the protein formulation to produce dry protein powders (Ameri and Maa, 2006).

While, the inclusion of excipients enhances the physical and chemical stability of the active pharmaceutical ingredient and improves the aerosolisation characteristics (Lechuga-Ballesteros *et al.*, 2008; Ohtake *et al.*, 2011), the removal of water (dehydration or drying) from protein formulations that are insufficiently stable in aqueous environments and greatly increases protein stability due to reduced mobility of the protein and the absence of certain degradation pathways facilitated by water (Maltesen and Van De Weert, 2008).

3.2.1 The use of stabilising excipients in pulmonary delivery of proteins

Pharmaceutical excipients tend to be inert substances that are added to protein formulations for various reasons including; stabilisation of protein formulations, assisting the manufacture of the dosage form, controlled or targeted drug delivery and as preservatives within the protein formulation. Some of these excipients categories include – Polymers (e.g., polyethylene glycol, dextran, hydroxyl ethyl starch), salts (e.g., sodium chloride, potassium chloride, sodium sulphate), sugars (e.g., lactose, sucrose, trehalose, glucose, sorbitol), buffering agents (e.g., citrate, acetate,Tris, phosphate), amino acids (e.g., histidine, arginine, lysine, methionine), surfactants (e.g., polysorbate 20 and 80) and preservatives (e.g, benzyl alcohol, m-cresol, phenols). It is important to note that, even though there are a lot of excipients under study for liquid protein formulations, only a few are currently approved by the FDA for inclusion in dry protein formulations due to a lack of toxicity studies that are yet to be conducted with inhaled excipients. Currently, the only accepted excipients used for inhalation are the sugars: glucose, lactose and mannitol, with lactose being the most commonly employed in currently marketed DPIs, as they are good carriers (i.e. facilitate deaggregation of micronized powders), have an established safety and stability profile, are inexpensive and easily available at different grades (Pilcer *et al.*, 2012).

In particular, the use of polymers as excipients in pulmonary drug delivery systems has also been gaining a lot of interest among researchers (Amidi *et al.*, 2010; Charman *et al.*, 1993; Manning *et al.*, 1995; Li *et al.*, 2010). This is because they are good at stabilising proteins during processing (Tiwari *et al.*, 2010; Pisal *et al.*, 2010) as well as for controlling the release of protein drugs (Zeng *et al.*, 1995). Polymers have also been reported to preserve biological activities of proteins (Liao *et al.*, 2005; Tawfeek *et al.*,

2013; Tewes *et al.*, 2010) as well as improve and maintain fine particle fraction of encapsulated protein above 50% (Liao *et al.*, 2005).

3.2.2 Mechanism of polymer protection of proteins

In solution, polymers stabilize proteins through specific and non specific mechanisms. While hydrophilic polymers (e.g. polyethylene glycols, polysaccharides and inert proteins) stabilize proteins independent of their chemical nature, charged polymers particularly function in a protein-specific manner. Several mechanisms have been reported by various aurthors to explain how hydrophilic polymers are able to stabilize proteins (Vrkljan *et al.*, 1994; Arakawa and Timasheff, 1985; Gombotz *et al.*, 1994; Cleland *et al.*, 1992) but what stands out amongst them is the 'crowding effect' which plays a role in stabilizing proteins (Laurent, 1963). The 'crowding effect' is the ability of the included polymers to cause a spatial reduction in the radius of the native protein structure, a phenomenon that is more thermodynamically favourable to maintain, as confined protein possess a smaller surface area for exclusion (Zhou, 2004b; Zhou, 2004a). As polymer exclusion increases with polymer size, large polymers have been reported to be more effective at stablising proteins in solution (Minton, 2005). A second mechanism by which polymers can stabilize proteins is though specific binding, for example, the binding of acid fibroblast growth factor (aFGF) to heparin stabilizes it (Volkin *et al.*, 1993). In addition, polymers have also been shown to prevent instabilities that may lead to protein aggregation, for example, dextran, a high molecular weight polysaccharide inhibits metal-ion catalyzed oxidation through metal ion complexation (Li *et al.*, 1996).

3.2.3 The use of sodium carboxymethylcellulose as a thermoprotectant for proteins during spray drying

Excipients such as sodium carboxymethylcellulose – an anionic water soluble polymer – have been successfully employed in the development of non-invasive systems for delivery of proteins to the lungs. Particularly, Li and Seville (2009) and Li et al., (2010) have co-spray dried formulations of proteins and sodium carboxymethylcellulose with the view of developing respirable pMDI and DPI formulations with improved stability. From these pilot investigations, sodium carboxymethylcellulose was shown to be especially suited as an excipient in the formulation of respirable spray-dried proteins due to its ability to significantly ($p < 0.05$) increase the FPF compared to NaCMC free spray-dried protein formulations (Li and Seville, 2009), provide significant ($p < 0.01$) protection to the enzymatic activity of spray-dried alkaline phosphatase, it is also non toxic (Greig, 1999), high soluble and possess colloidal properties (Hooton, 2009). However, their non degradability and irritating nature may present a problem during routine use in formulations intended for pulmonary delivery. The use of sodium carboxymethylcellulose in formulations intended for lung delivery has yet to be fully characterised therefore, this chapter will investigate the potential application and limitations of the excipient in the formulation of inhalable protein particles.

3.2.4 Drying techniques used for the production of protein particulates

Drying is a very important unit operation within the pharmaceutical industry this is because a dry product is generally more stable than a liquid preparation. In view of this, various drying techniques are currently being used to produce protein powders with desirable stability properties. The most commonly used drying technique for producing dry protein pharmaceuticals is freeze drying, followed by spray drying with supercritical

fluid (SCF) and spray freeze drying still emerging as a viable alternatives. The different techniques utilise different physical principles; sublimation (freeze drying), evaporation (spray drying), precipitation (SCF) and a combination of evaporation and sublimation (spray freeze drying) to produce protein powders with significantly different powder characteristics (Vehring, 2008; Abdul-Fattah *et al.*, 2007; Shoyele and Cawthorne, 2006; Amorij *et al.*, 2008). While freeze drying is widely employed for the production of parentral formulations (Carpenter *et al.*, 1997), spray drying is better suited for the production of pulmonary formulations (Maa and Prestrelski, 2000). Regardless of the drying method employed to produce powdered proteins the outcome is usually the production of an amorphous material with a somewhat better stability than the liquid preparation

3.2.5 Spray drying technique

Spray drying is a widely employed technique in industrial chemistry and food science due to its ability to rapidly dry aqueous or organic solutions, emulsions and suspensions producing dry powders. In the pharmaceutical industry, spray drying is being used as a micronisation technique for the development of highly respirable powders whose release properties and morphological characteristics can be varied by incorporating suitable excipients into the spray drying feedstock (Seville *et al.*, 2007). The spray drying technique has remained popular especially due to the increasing demand for the design of sophisticated particles such as: large porous particles (i.e. polymer particles made by double- and single emulsification solvent evaporation or consisting of dipalmitoylphosphatidylcholine (DPPC) albumin and saccharides spray-dried from ethanol water co-solvent systems) (Vanbever *et al.*, 1999a; Vanbever *et al.*, 1999b; Ben-Jebria *et al.*, 1999; Wang *et al.*, 1999); particles with foam-like morphology (e.g. PulmoSpheres™) (Dellamary *et al.*, 2000; Weers, 2000a); encapsulated particles

including proteins (Quaglia *et al.*, 2003; Bittner *et al.*, 1998; Burke *et al.*, 2004); hollow, low density particles with controllable surface characteristics (Edwards *et al.*, 2003; Kuo and Lechuga-Ballesteros, 2003; Platz *et al.*, 2002; Weers *et al.*, 2009); particles with single or multiple functional layers (Learoyd *et al.*, 2009) and nano sized particles (Pilcer *et al.*, 2009; Sham *et al.*, 2004).

The basic principle of operation behind spray drying involves the atomisation of the liquid feedstock leading to the creation of high frictional forces over the liquid surfaces with a large contact surface area, subsequent injection of the liquid feedstock into a drying chamber containing hot gaseous medium (typically air or nitrogen), leads to instant drying of the spray droplets into solid droplets (Cal and Sollohub, 2010). It is important to mention that the temperature of the material being dried never reaches the inlet temperature of the drying gas due to the short period (10^{-4} – 10^{-3} s) of drying and the short residence time of the particle in the drying chamber (Elversson and Millqvist-Fureby, 2005), making spray drying a very useful method for drying heat sensitive substances such as proteins (Cal and Sollohub, 2010). However, there is a need to optimise the spray drying parameters for each individual protein as thermal stability of proteins vary (Maltesen and Van De Weert, 2008).

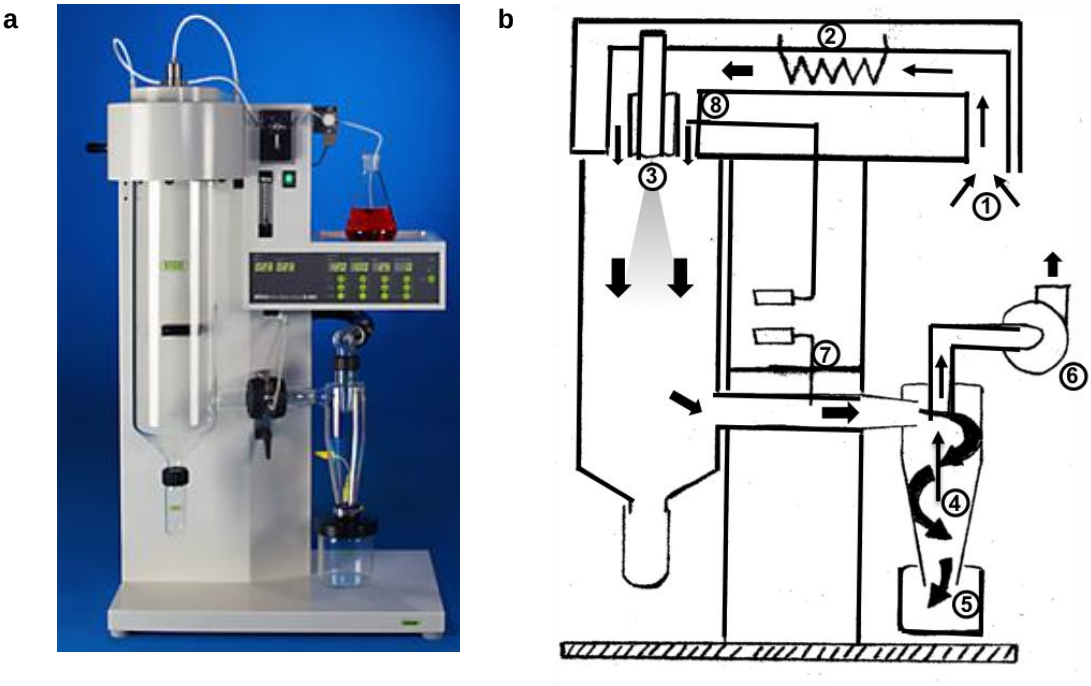


Figure 3 -1: Buchi B290 laboratory spray dryer. (a) laboratory scale equipment (b) schematic diagram of the dry airflow: (1) air intake (2) heater (3) flow stabiliser intake into the drying chamber (4) cyclone separator (5) product collector (6) Aspirator (7) outlet temperature sensor (8) inlet temperature sensor.

The newly-formed particles are separated from the gas by means of a cyclone separator (fig 3 -1), bag filter or electrostatic precipitator (Sou *et al.*, 2011). Amongst the many potential advantages offered by spray drying, the ability to effectively control the particle size of the resultant powder by modifying spray-drying parameters seems to be the most obvious. The typical particle diameter usually produced following spray drying, range between 0.5 – 50 µm (Vehring, 2008).

Nothwithstanding all the advantages of spray drying already outlined, the use of the spray drying technique to produce protein-microparticulates has not been without challenges. Spray drying is associated with various stresses including; pressure effects, exposure to air-liquid interfaces during atomisation and dehydration. Together, this stressess may result in disruptions to the native structure of proteins thereby decreasing

protein activity considerably (Tzannis and Prestrelski, 1999a; Prestrelski *et al.*, 1993; Mumenthaler *et al.*, 1994; Broadhead *et al.*, 1994).

3.2.6 Alkaline phosphatase as a model protein

Alkaline phosphatases (EC 3.1.3.1) (APs), are dimeric enzymes present in most organisms. They are non specific phosphomonoesterases that catalyze the hydrolysis of monoesters of phosphoric acid as well as facilitate the transphosphorylation reactions that occur in the presence of large concentrations of phosphate acceptors (Millan, 2006). In humans, four distinct variants of alkaline phosphatase exist, three isoenzymes are tissue specific (i.e intestinal AP, placental AP and germ cell AP) while the fourth isoezyme is tissue-nonspecific (expressed in a variety of tissues including liver, bone and kidney). The tissue specific isoenzymes are 90 – 98% homologous between themselves and 50% homologous with the tissue-nonspecific isoenzymes. Functional properties of mammalian alkaline phosphatase indicate that while the tissue-nonspecific alkaline phosphatases have bone mineralisation functions, intestinal alkaline phosphatase likely facilitate intestinal absorption (Millan, 2006), the biological function of placental AP has not yet been unravelled but it has been suggested that it may have a role to play in the transfer of maternal IgG to the foetus (Makiya and Stigbrand, 1992a; Makiya and Stigbrand, 1992b; Stefaner *et al.*, 1997).

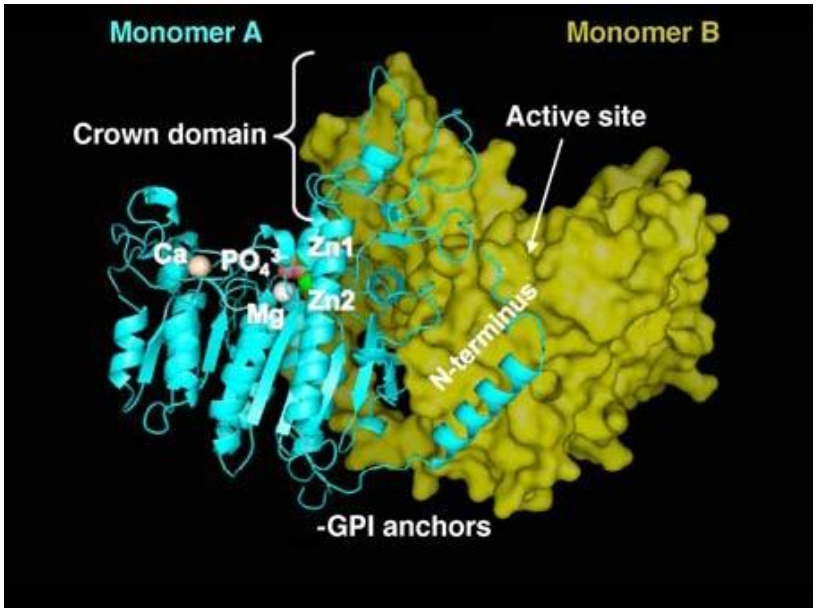


Figure 3 -2: Schematic representation of the three-dimensional structure of alkaline phosphatase. Monomer A is shown in ribbon representation and in cyan, while monomer B is shown in surface representation in yellow. Also indicated are the active site metals, Zn1, Zn2 and Mg, the novel fourth metal site occupied by Ca, the crown domain and the amino terminal arm. The image is adapted from Millan (2006)

For the purposes of this pilot study, bovine intestinal alkaline phosphatase was used and it comprised of two identical subunits with a molecular weight of 160,000 kDa (Neumann and Lustig, 1980). Each unit contains two (one loosely bound and one tightly bound) Zn^{2+} molecules, one magnesium binding site and one calcium binding site which aids the structural stability of the protein. The enzyme has a broad specificity for phosphate esters of alcohols, amines, pyrophosphate and phenols. However in research laboratories, the enzyme is commonly used to dephosphorylate proteins and nucleic acids (Morton, 1955b; Morton, 1955a)

The optimal pH range for alkaline phosphatases activity is between 8 – 10.5, and is dependent on the substrate, substrate concentration and ionic concentration. For the purpose of this study, the enzyme activity was determined at pH 9.8 (i.e. diethanolamine assay). The choice of alkaline phosphatase as the model protein for this study is based on the availability of the purified protein, the existence of a suitable assay method for assessing the activity of the enzyme and its relatively cheap cost compared to most commercially available proteins.

3.3 Aims and objectives

The aim of this study was to investigate the feasibility of developing spray-dried protein particles, which are suitable for delivery to the lower respiratory tract without loss of biological activity either by using optimised spray drying parameters alone, or by modifying the protein formulation by the inclusion of sodium carboxymethyl cellulose. In order to improve the percentage fine particle fraction of the sodium carboxymethyl cellulose modified protein formulation, the use of water-ethanol (90:10 and 70:30) co-solvent system in the preparing the NaCMC modified formulation will be investigated. Alkaline phosphatase was selected as a model protein for assessing the biological activity of the various formulations following spray drying.

3.4 Materials

Phosphatase alkaline from calf intestinal mucosa (1.46 U/mg), bromophenol blue indicator reagent, Coomassie Brilliant blue R, sodium dodecyl sulphate all purchased from sigma-aldrich (Poole, UK), 1-Step™ PNPP and BCA protein assay reagent both purchased from Thermo Scientific (Rockford, IL USA), analytical grade, absolute ethanol was purchased from Fisher Scientific (Loughborough, UK), 98% 4-Nitrophenol from Acros organics (New Jersey, USA), Mini-PROTEAN®TGX™ Precast gels was purchased from Bio-Rad laboratories (Hercules USA), PageRuler™ Plus and pre-stained protein ladder was purchased from Fermentas (York, UK), Hydrofluoroalkane (HFA)-134a (pharmaceutical grade) was purchased from INEOS Fluor (Cheshire, UK).

3.5 Methods

3.5.1 Preparation of spray-dried alkaline phosphatase

Alkaline phosphatase was spray-dried as a 2 % w/v aqueous solution. The spray drying parameters utilised in the micronisation of the enzyme were: Inlet temperature, 180 ^{0}C; spray flow rate, 606 L/hr; pump setting, 3.2 mL/min; aspirator setting, 85 % (34 m^3/hr); to produce an outlet temperature of 99 – 101 ^{0}C.

3.5.2 Preparation of spray-dried alkaline Phosphatase – Sodium carboxymethylcellulose combinations

2 % w/v NaCMC:alkaline phosphatase (80:20) mixture was co-spray dried from water-only and a water:ethanol (90:10 and 70:30) suspension feedstocks using the following spray drying parameters; Inlet temperature, 180 ^{0}C; spray flow rate, 606 L/hr; pump

setting, 3.2 mL/min; aspirator setting, 85 % (34 m^3/hr); to produce varying outlet temperatures

3.5.3 Characterisation of the enzymatic activity of alkaline phosphatase

The alkaline phosphatase content of the spray-dried powders was determined using a quantitative assay described in section 2.2.1.1.2. Briefly, 100 µg/mL of the various spray-dried powders (SD-AP, SD-N-AP, SD-N-AP 1, SD-N-AP 2) was prepared and 10 µL aliquot of the sample solution was subsequently added into 1mL of 1-Step PNPP and incubated at room temperature for 5 minutes, during which time the PNPP was converted to PNP through the action of alkaline phosphatase. Following completion of the incubation period, the reaction was quenched by the addition of 500 µL of 2 N NaOH and the absorbance values of the solution determined by UV–vis spectrometry at 405 nm. The activity of alkaline phosphatase was defined as follows: one unit of alkaline phosphatase generates 1 µmol PNP in 1min at 37 °C. The amount of PNP generated was calculated by reference to a PNP calibration curve, prepared using a series of standard solutions of PNP (10–50 µM) in diethanolamine–HCl solution (section 2.2.1.1.1)

3.5.4 Determination of moisture content

The moisture content of the spray-dried powders was determined using thermogravimetric analysis. Samples (approximately 5 mg) were placed into platinum pans and analysed under a nitrogen purge (20 mL min^{-1}), heated from 20 to 140°C at a heating rate of 10°C min^{-1}.

3.5.5 Particle size distribution (PSD)

Particle size distributions of the various spray-dried powders were determined using a Helos H 2178 laser diffraction instrument in combination with a Rodos dry dispersing unit and a Vibri vibratory feeding unit. Approximately 5 – 10 mg of each sample was dispersed at 2.0 bars using an R2 lens which covered sample size range between 0.45 – 87.5 µm. Triplicate measurement was carried out on all the sample batches and data are expressed as the percentage of particles sized in the lower 10, middle 50, upper 10th percentile ($d[v,10]$, $d[v,50]$ and $d[v,90]$ respectively) and the volume mean diameter (VMD). The span of the volume distribution, a measure of the width of the distribution relative to the median diameter ($d[v,50]$), was also calculated using equation 3.1. A large span is indicative of a heterogenous size distribution

$$Span = \frac{d[v,90] - d[v,10]}{d[v,50]} \quad \text{(Equation 3.1)}$$

3.5.6 Scanning Electron Microscopy (SEM)

The morphology of the micro-particles making up the bulk of the various spray-dried alkaline phosphatase powders were investigated using scanning electron microscopy (SEM, STEREOSCAN 90, Cambridge instruments). Samples were mounted on adhesive black carbon tabs (premounted on aluminium stubs) and sputter-coated (Polaron SC500, Polaron Equipment) with a thin layer of gold for 3 mins at 20 mA prior to analysis. The samples were studied under high magnification. This analysis was repeated three times, once for each of the spray-dried powder batches.

3.5.7 Preparation of DPI formulations

For spray-dried alkaline phosphatase: One empty size 3 gelatin capsule was weighed and 20 ± 2 mg of spray-dried alkaline phosphatase (theoretically equivalent to 196 units of the alkaline phosphatse enzyme) was manually measured into the capsule and subsequently loaded into a cyclohaler™. This was repeated two more times to enable triplicate measurement of the aerosolisation performance of spray-dried alkaline phosphatase.

For spray-dried NaCMC modified formulations of alkaline phosphatase: For each of the formulations, five empty size 3 gelatin capsules were weighed and 20 ± 2mg (theoretically equivalent to 240 units of the enzyme for the SD AP, 155 units of the enzyme for the SD-N-AP 1 and 135 units of the enzyme for the SD-N-AP 2 and contained in the 5 capsules) of each formulation blend was measured and manually loaded into the 5 capsules.

Preparing alkaline phosphatase pMDIs: pMDI formulations of the spray-dried powders were prepared by adding either 100mg of spray-dried powder (theoretically equivalent to 995 units enzyme for the SD AP, 235 units of the enzyme for SD-N-AP, 170 units of the enzyme for SD-N-AP 1 and 148 units of the enzyme for SD-N-AP 2) into pre-weighed 15 mL polyethylene terephthalate (PET) uncoated pMDI vials. A BK357 30 µL valve was crimped onto the vials using a Pamasol P2002 small-scale crimper and approximately 6 g HFA- 134a was pressure-filled through the valve using a Pamasol P2011 propellant filler to form suspensions with an approximate concentration of 1.6% (w/w) powder in propellant. The formulations were vortex-mixed for 1 minute and tested for *in vitro* aerosolisation properties using a next generation impactor for 2 x 5s. The theoretical dose contained in 20 shots from each formulation was calculated to be ~125 units of enzyme for the SD AP pMDI formulation, ~30 units of enzyme for the SD-N-AP pMDI formulation, ~22 units of enzyme for the SD-N-AP 1 pMDI formulation and ~19

units of enzyme for the SD-N-AP 2 pMDI formulation. The actual dose released per test was calculated from the mass loss following actuation. A total of three pMDI formulations were prepared for each batch of spray-dried powder (n = 3).

3.5.8 *In vitro* aerosolisation testing of the various DPI and pMDI formulations

The aerosolisation properties of the different DPI and pMDI formulations were investigated using Next Generation Impactor (NGI). The flow rate through the NGI was adjusted to 60 L/min using an electronic digital flow meter (Model DFM2: Copley Scientific). The cyclohaler™ device fitted with size 3 capsule, was attached to the induction port of the NGI using an adapter, the vacuum pump was switched on and the powder formulation was allowed to aerosolise for 2 x 5 seconds. Following aerosolisation of the dry powder formulations, the NGI was dissembled and deposits collected from all the different components using the collecting solution (diethanolamine-HCl solution). A flow rate of 60 L/min was maintained throughout the entire investigation. The powders which deposited in the throat were recovered by rinsing the throat with 20mL of distilled water; the pre-separator had 15mL of collecting solution in it during aerosolisation. 20mL volume of collecting solution was placed in stage 1 and MOC while 10mL volume of collecting solution was placed in stages 2 - 7, the resulting solutions were each swirled and transferred to 50mL beakers. Complete dissolution was facilitated in all solutions by placing the beakers containing the solutions on magnetic stirrers (Fisher Thermix Stirring Hot Plate, Model 201T). The resulting solutions were visually inspected to confirm the complete dissolution of the powders. A sample of the solution was then removed for UV analysis making sure to dilute even further when necessary. For the pMDI formulations, 20 actuations were aerosolised through a plastic actuator attached to the NGI via the USP throat. After each actuation the canister was

removed and shaken to provide a homogenous mixture for the next actuation. Each deposition experiment was repeated in triplicate

3.5.9 Determination of RD, ED, FPD, FPF, MMAD, GSD and Dispersibility

The method for determining RD, ED, FPD, FPF, MMAD, GSD are outlined in section 2.2.14.5. For the pMDI formulations, the pMDI vial was weighed before and after each run, to enable the total dose (TD) to be calculated, based on the proportion of alkaline phosphatase in each suspension. For the DPI formulations, the TD was defined as the activity of alkaline phosphatase present in the powder loaded into the capsule. In each case, the TD was corrected for the alkaline phosphatase activity in each powder. The recovered dose (RD) was defined as the total activity of alkaline phosphatase detected (i.e. device + throat + Stages 1–8), expressed as the percentage of TD. The fine particle dose (FPD) was defined as the activity of alkaline phosphatase recovered from Stage 3 to stage 8 of the NGI (effective cut-off diameter 4.4 µm). The fine particle fraction (FPF) was calculated as the ratio of FPD to RD, expressed as a percentage. A total of four pMDI and four DPI formulations were prepared for each batch of spray-dried powder (n = 3) to enable the aerosolisation performance of the formulations to be determined.

3.5.10 SDS-PAGE analysis of the various alkaline phosphatase formulations

Analysis of samples by SDS-PAGE was carried out using a 4 – 20 % precast polyacrylamide gel following the procedure described by Laemmli (1970) and detailed in section 2.2.9. A lane of prestained molecular weight marker was included in each gel to

allow determination of the apparent molecular weight of the lactoferrin in each preparation. Following electrophoresis, gel protein staining was performed using Coomassie brilliant blue R-250, following the procedure detailed in section 2.2.9.1.1.

3.5.11 Statistical analysis

Statistical analysis were performed on the data obtained. Data is presented as mean ± standard deviation (n = 3). GraphPad InStat version 3.06 software was used in analyzing data. One way ANOVA with Tukey multiple comparison and post test was employed.

3.6 Results

3.6.1 Determining the unit of alkaline phosphatase through diethanolamine

One unit of AP was defined as the amount of AP required to generate 1 µM PNP per minute at 37 °C. Based on this, three calibration curves of PNP were prepared on three different days (as described in section 2.2.1.1.1). A calibration plot of absorbance at 405 nm against molar concentration of PNP (10 – 50 µM) yielded a linear relationship (y= 0.0189x 0.0026, $r^2 > 0.999$). Using Beer-Lambert's law, A = ε c l (where A – absorbance, ε – molar absorbtivity, c – concentration and l – path lenght) the molar absorbtivity for PNP was calculated to be 18.9 mM^{-1} cm^{-1} for this study. This value was then used to determine the units of AP in throughout this study.

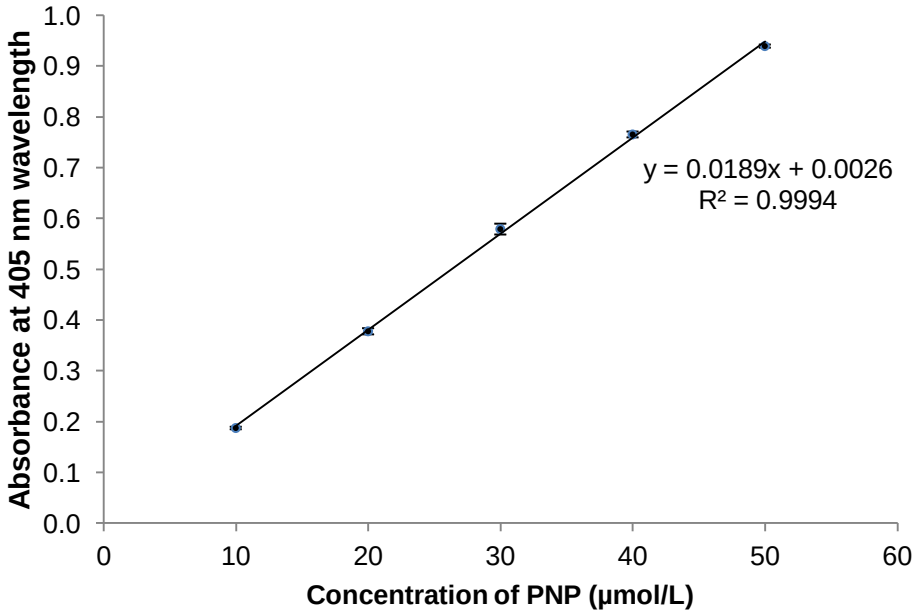


Figure 3 -3: Calibration curve showing a plot of absorbance versus concentration for p-nitrophenol. The mean absorbance of triplicate measurements obtained on 3 different days are plotted against nominal concentration values (n = 9). Error bars representing standard deviation is displayed for each point although they are within the limits of the symbols.

All calibration curves obtained on different days and constructed for 4-nitrophenol were linear within the measured concentration range of 10 – 50 µM (Day 1: y= 0.0188x + 0.0069, r^2 = 0.999; Day 2: y= 0.019x + 0.0008, r^2 > 0.999; Day 3: y= 0.019x + 0.0001, r^2 > 0.999). The assay method generally showed good reproducibility with similar inter-day results.

3.6.2 Validating the p-nitrophenol assay

In order to ensure the validity of the assay method and the calibration graph produced, especially since it was going to be used to estimate the units of alkaline phosphatase in the entire study, some validation parameters were determined including; specificity, precision, detection limit, quantitation limit and linearity. The specificity of the assay method in estimating the absorbance of PNP in the presence of sodium carboxymethylcellulose was assessed and found not to be different from calibration plot in figure 3 -3, the linearity of the assay method was found to be > 0.999 on all three days, the precision of the assay which is the degree of scatter between absorbance measurements obtained on three differenet days was determined by evaluating the standard deviation (SD) and relative standard deviation (RSD) (table 3 -1). The detection limit, which is the lowest amount of the analyte in a sample that can be detected but not necessarily quantified using the calibration plot was determined (table 3 -1) and finally, the quantitation of the graphical plot of absorbance against, micromolar concentration of PNP. All results obtained showed that the assay method was appropriate for use.

Table 3.1: Calculated and measured mean absorbance, standard deviation, % RSD, r^2, LOD. LOQ and nominal concentration of *p*-nitrophenol. Values were calculated or measured on three separate days and nominal concentrations, absorbances and calculations are shown for each day.

	Standard conc. (µmol/L)	Mean absorbance	Standard deviation	Relative standard deviation (%)	Linear regression (r^2)	Limit of detection (µmol/L)	Limit of quantitation (µmol/L)
Day 1	10	0.187	0.003	1.34			
	20	0.383	0.006	1.68			
	30	0.587	0.007	1.23	0.9988	0.7021	2.1277
	40	0.765	0.004	0.53			
	50	0.938	0.003	0.28			
Day 2	10	0.188	0.004	1.92			
	20	0.378	0.006	1.55			
	30	0.579	0.012	2.00	0.9994	1.0421	3.1579
	40	0.766	0.009	1.17			
	50	0.940	0.002	0.22			
Day 3	10	0.188	0.002	1.11			
	20	0.377	0.005	1.22			
	30	0.572	0.010	1.79	0.9996	0.5211	1.5789
	40	0.767	0.006	0.73			
	50	0.941	0.005	0.48			

The highest %RSD for inter-day precision was determined to be 2 % at nominal concentration of 30 µmol/L on day 2 (Table 3.1) indicating good inter-day precision. Analysis also showed that the lower limits of detection and quantification were approximately 0.52 µmol/L and 1.58 µmol/L respectively. Both were obtained on day 3.

3.6.3 Determination of the unit of alkaline phosphatase in the various spray-dried formulations

In order to determine the effect of spray drying on the activity of alkaline phosphatase the units per millilitre of the enzyme was calculated using the equation 3.2;

$$Units\ per\ mL\ of\ enzyme = \frac{\left(\frac{Absorbance}{min}\right)(1.01)(DF)}{(18.9)(0.01)} \quad \text{(Equation 3.2)}$$

where; 1.01 = Volume (in millilitres) of assay; DF = Dilution factor; 18.9 = millimolar extinction coefficient of p-Nitrophenol at 405 nm; 0.01 = volume (in millilitres) of enzyme used.

Table 3.2: The calculated units per millilitre and percent relative activity of various alkaline phosphatase powders. The percent relative activity was calculated by assuming the activity of the supplied alkaline phosphatase enzyme to be 100%. The values are mean ± SD of triplicate measurments.

Formulations	Before spray drying		After spray drying	
	Units/mL enzyme	% relative activity	Units/mL enzyme	% relative activity
SD-AP	135.71 ±0.8	100 ±0.6	99.50 ±5.3	73.3 ±3.9
SD-N-AP	138.83 ±2.7	102.3 ±2.0	117.89 ±4.8	86.9 ±3.5
SD-N-AP 1	135.20 ±1.6	99.6 ±1.2	85.22 ±2.8	62.8 ±2.1
SD-N-AP 2	131.89 ±1.1	97.2 ±0.8	73.96 ±4.1	54.5 ±3.0

The enzymatic activity of the preparations were measured before and after spray drying in order to assess the impact of the spray drying technique on the activity of the protein. Prior to spray drying, SD-N-AP 2 had the lowest mean enzymatic activity of 97.2 ±0.8% of nominal alkaline phosphatase activity and also the lowest mean pH value of 8.5 (Table 2.4). Following spray drying, the alkaline phosphatase activity of the spray-dried

powders: SD-AP, SD-N-AP, SD-N-AP 1 and SD-N-AP 2 were found to be 73.3 ±3.9%, 86.9 ±3.5%, 62.8 ±2.1% and 54.5 ±3.0% respectively of the original activity of the non-spray-dried enzyme which was arbitrarily assigned as 100%.

3.6.4 Spray-dried powder characteristics

3.6.4.1 Spray drying parameters

A total of 4 different spray-dried powders of alkaline phosphatase were generated by using the spray drying parameters outlined in table 3.3.

Table 3.3: Spray drying parameters employed to produce the four different alkaline phosphatase powders. Three batches of powders were produced using these parameters**.**

Abbreviations	Concentration of feedstock (% w/v)	Solvent	Inlet temp (^{0}C)	Outlet temp (^{0}C)	Airflow (L/hr)	Pump (mL/min)	Aspirator (m^3/hr)	Yield (%)
SD-AP	2	Water	180	99 - 101	606	3.2	34	67.2 ±2.7
SD-N-AP	2	Water	180	99 - 101	606	3.2	34	76.1 ±1.0
SD-N-AP 1	2	Water-Ethanol (9:1)	180	102 - 106	606	3.2	34	71.3 ±3.2
SD-N-AP 2	2	Water-Ethanol (7:3)	180	101 - 104	606	3.2	34	71.1 ±2.8

The data shown in table 3.3 illustrates that spray drying alkaline phosphatase alone resulted in a yield of 67.2 ±2.7 % of the initial starting mass while the inclusion of NaCMC in the water-only feedstock resulted in a significantly greater yield of 76.1 ±1%

($p < 0.01$). However, no statistical difference was found between yield of the co-solvent modified NaCMC-AP and the excipient-free spray-dried alkaline phosphatase.

3.6.4.2 Moisture content and particle size distribution of the various spray-dried formulations

The residual moisture content of the various formulations are shown in table 3.4. Excipient-free spray-dried alkaline phosphatase contained a residual moisture level of 5%, whereas the residual moisture content of NaCMC modified was 6.5 % when spray-dried from water and 3.0 % when spray-dried from a water-ethanol cosolvent. The particle size distribution of the various spray-dried formulation shows that SD-N-AP had highest VMD, while the excipient free spray-dried alkaline phosphatase produced the least VMD (Table 3.4).

Table 3.4: Moisture content and particle size distribution of spray-dried alkaline phosphatase powders and sodium carboxymethylcellulose modified formulations. The values are mean ± SD. Statistical analysis of the various formulations were carried out by one-way analysis of variance

(ANOVA) using GraphPad prism 5. † denotes $p < 0.05$ statistical difference compared to the spray-dried alkaline phosphatase only formulation, and ‡ denotes $p < 0.01$ statistical difference compared to the spray-dried alkaline phosphatase only formulation

		Particle size distribution (µm)				
Formulations	Moisture content (%)	*d*[*v*,10] (µm)	*d*[*v*,50] (µm)	*d*[*v*,90] (µm)	Span	Volume mean diameter (µm)
SD AP	5.0 ±1.8	1.10 ±0.3	4.04 ±0.6	8.86 ±1.0	1.97 ±0.5	4.56 ±0.8
SD-N-AP	6.5 ±1.1	1.23 ±0.4	5.39 ±0.5	20.86 ±0.8	3.67 ±0.4‡	8.54 ±1.0‡
SD-N-AP 1	3.3 ±0.3	1.14 ±0.2	5.06 ±0.4	17.18 ±0.5	3.19 ±0.3†	7.48 ±0.3‡
SD-N-AP 2	3.0 ±0.8	1.31 ±0.3	5.51 ±0.5	19.74 ±0.5	3.38 ±0.4‡	8.33 ±0.5‡

The spray drying parameters utilised in producing the various dry powders shows that powders generated by spray drying from co-solvents exhibited lower moisture content (analysed by thermogravimetric analysis) although possessed a higher particle size compared with excipient free spray-dried alkaline phosphatase powders (Table 3-11). The NaCMC-modified formulations showed greater heterogenicity with significantly higher span than excipient-free spray-dried alkaline phosphatase.

3.6.5 Aerodynamic assessment of powder blends

A total of eight products were evaluated for their aerodynamic properties using the next generation impactor. Four of the products were dry powders delivered from a cyclohaler and the other four products were delivered from a pressurised metered dose inhaler.

3.6.5.1 *In vitro* deposition data

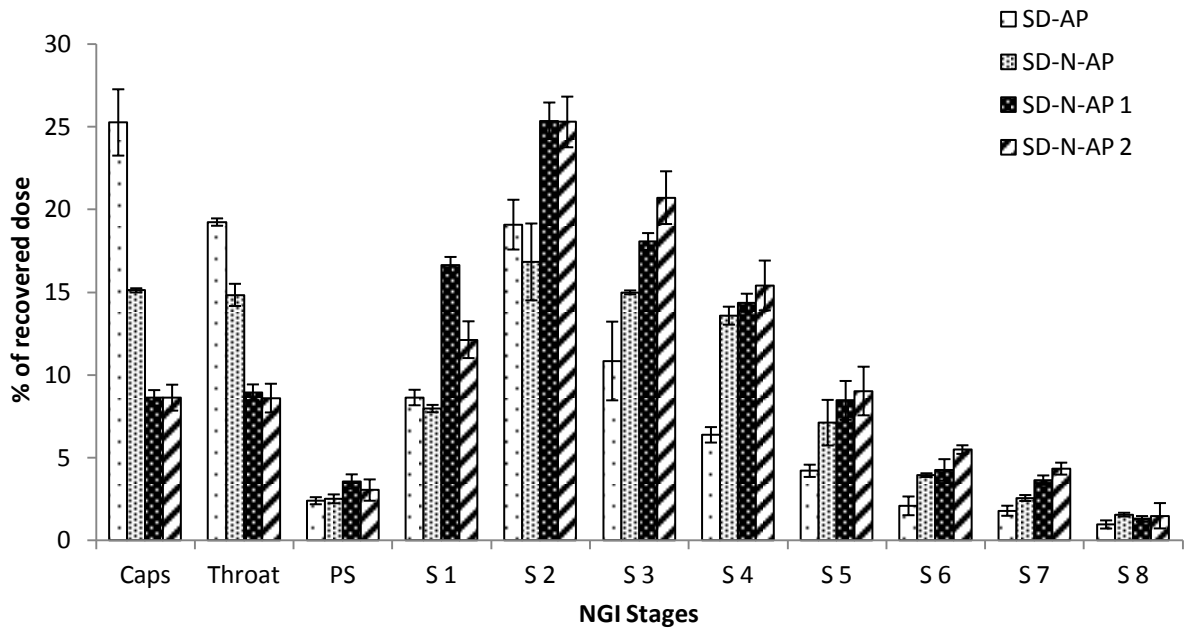


Figure 3 -4: In vitro deposition profile of four DPI formulations following aerosolisation into the NGI. Following preparation of the spray-dried powders, 20 mg of SD AP and 5 x 20 mg of SD-N-AP, SD-N-AP 1 and SD-N-AP 2 were placed into size 3 gelatin capsules and aerosolised from a cyclohaler device. Values are mean ± SD, n = 3 of percent nominalised units per mL of alkaline phosphatase.

Following aerosolisation of 20 mg of SD AP (theoretically equivalent to 188 ±3.5 units of the enzyme of spray-dried alkaline phosphatase) from the cyclohaler, about 47±3.8 units of alkaline phosphatase (25.3%) was retained in the device with a further 36 ±0.4 units alkaline phosphatase (19.23%) depositing in the throat of the NGI. However, 49.14 ±4.8 units of alkaline phosphatase (26%) deposited on stages 3 – 8.

Upon aerosolisation of 100 mg of SD-N-AP (theoretically equivalent to 242 ±5.9 units of the enzyme of NaCMC modified alkaline phosphatase) from the cyclohaler, about 37 ±0.3 units of alkaline phosphatase (15.1%) was retained in the device with a further 36 ±0.4 units alkaline phosphatase (14.83%) depositing in the throat of the NGI. However, 105.9 ±3.1 units of alkaline phosphatase (43%) deposited on stages 3 – 8.

Upon aerosolisation of 100 mg of SD-N-AP 1 (theoretically equivalent to 159 ±3.1 units of the enzyme of NaCMC modified alkaline phosphatase prepared for 90:10 water-ethanol co-solvent) from the cyclohaler, about 14 ±0.8 units of alkaline phosphatase (8.6%) was retained in the device with a further 14 ±0.8 units alkaline phosphatase (8.94%) depositing in the throat of the NGI. However, 79.6 ±1.6 units of alkaline phosphatase (50%) deposited on stages 3 – 8.

Upon aerosolisation of 100 mg of SD-N-AP 2 (theoretically equivalent to 130 ±1.5 units of the enzyme of NaCMC modified alkaline phosphatase prepared for 70:30 water-ethanol co-solvent) from the cyclohaler, about 11 ±1.0 units of alkaline phosphatase (8.6%) was retained in the device with a further 11 ±0.8 units alkaline phosphatase (8.6%) depositing in the throat of the NGI. However, 73 ±4.6 units of alkaline phosphatase (56%) deposited on stages 3 – 8.

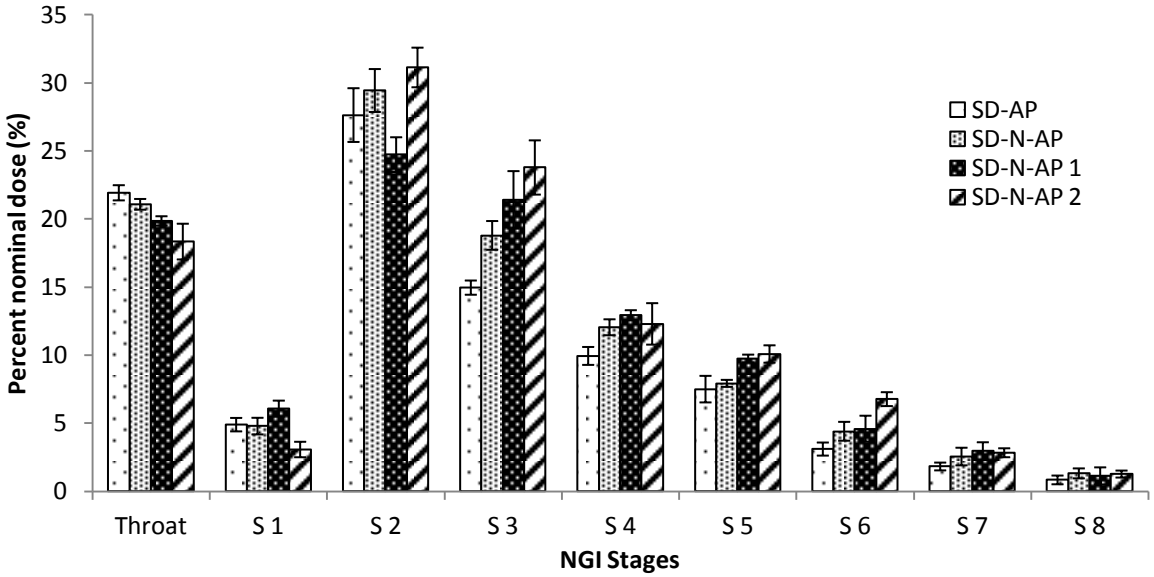


Figure 3 -5: In vitro deposition profile of four pMDI formulations following aerosolisation into the NGI. Following preparation of the spray-dried powders, 20 actuations of SD AP, SD-N-AP, SD-N-AP 1 and SD-N-AP 2 were aerosolised from the pMDI device into the NGI. Values are mean ± SD, n = 3 of percent nominalised units per mL of alkaline phosphatase.

Following aerosolisation of 20 actuations of SD AP (theoretically equivalent to 124 ±0.3 units of the enzyme of spray-dried alkaline phosphatase) from the cyclohaler with about 27 ±0.7 units of alkaline phosphatase (21.92%) depositing in the throat of the NGI while 47.58 ±3.4 units of alkaline phosphatase (38%) deposited on stages 3 – 8.

Upon aerosolisation of 20 actuations of SD-N-AP (theoretically equivalent to 30 ±0.2 units of the enzyme of NaCMC modified alkaline phosphatase) from the cyclohaler with about 6 ±0.1 units of alkaline phosphatase (21%) depositing in the throat of the NGI while 14 ±0.7 units of alkaline phosphatase (47%) deposited on stages 3 – 8.

Upon aerosolisation of 20 actuations of SD-N-AP 1 (theoretically equivalent to 21 ±0.6 units of the enzyme of NaCMC modified alkaline phosphatase prepared for 90:10 water-ethanol co-solvent) from the cyclohaler with about 3.84 ±0.3 units of alkaline phosphatase (18.3%) depositing in the throat of the NGI while 12 ±1 units of alkaline phosphatase (57%) deposited on stages 3 – 8.

Upon aerosolisation of 100 mg of SD-N-AP 2 (theoretically equivalent to 19 ±0.5 units of the enzyme of NaCMC modified alkaline phosphatase prepared for 70:30 water-ethanol co-solvent) from the cyclohaler, about 3.8 ±0.1 units of alkaline phosphatase (20%) depositing in the throat of the NGI while 10 ±0.7 units of alkaline phosphatase (53%) deposited on stages 3 – 8.

3.6.5.2 The fine particle fractions of the aerosolised powder formulations

The total dose of particles with aerodynamic diameters smaller than 5 µm was calculated by interpolation from the cumulative mass against the cut-off diameter of respective stages and considered as the fine particle dose (FPD), with a ratio of FPD to the recovered dose representing the fine particle fraction (FPF).

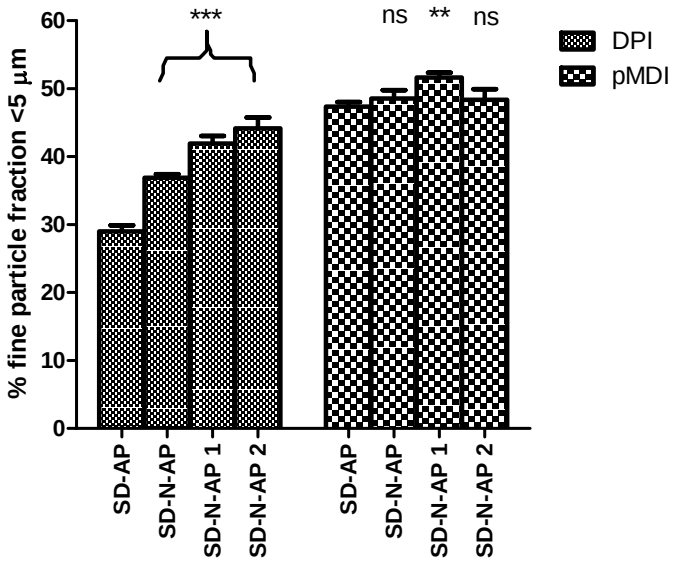


Figure 3 -6: Fine particle fraction < 5 µm of spray-dried alkaline phosphatase and sodium carboxymethylcellulose and co-solvent modified DPI and pMDI formulations evaluated using the NGI. Values are mean ±SD, n = 3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; *p* values were considered significant and the *** represents *p value* < 0.001 between SD AP and the sodium carboxymethylcellulose modified DPI formulations; †, *p* < 0.01 between SD AP and the sodium carboxymethylcellulose modified pMDI formulations. ns indicates no significance between SD AP and the sodium carboxymethylcellulose modified pMDI formulations.

The FPF of the DPI formulations ranged from 29% to 44% of the recovered dose, while the FPF of the pMDI formulations ranged from 47% to 51% (figure 3 -6). Sodium carboxymethylcellulose modified DPI formulations generally had significantly improved FPF while the FPF of the various pMDI formulations were found to be independent of sodium carboxymethyl cellulose content or the solvent used for preparation. However,

SD AP, SD-N-AP and SD-N-AP 2 were found to deliver statistically the same FPF, while SD-N-AP 1 delivered a significantly ($p < 0.01$) higher FPF.

3.6.5.3 Powder emission from the DPI device

The percent of powder emitting from the DPI increased with incorporation of sodium carboxymethylcellulose into the formulations as well as by spray drying from a co-solvent (Figure 3 -7)

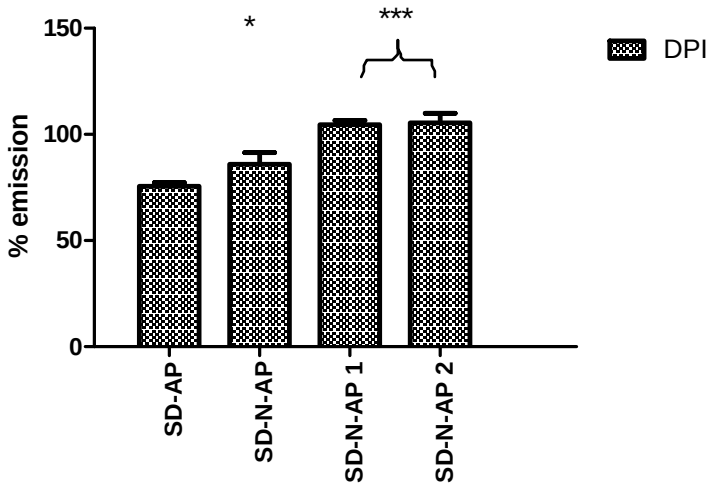


Figure 3 -7: Total powder emission of spray-dried alkaline phosphatase and sodium carboxymethylcellulose and co-solvent modified DPI at 60 L/min. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; *p* values were considered significant and *** represents *p value* < 0.001 between SD AP and the sodium carboxymethylcellulose modified formulations; * represents *p value* < 0.05 between SD AP and the sodium carboxymethylcellulose modified formulations.

Impaction studies from the DPI device shows that the highest average % emission of 105.4 ±4.6% was measured for SD-N-AP 2. The lowest average % emission of 75.6 ±1.8% was measured for the SD AP dry powder inhaler. No statistical difference was measured between the %emission of SD AP and SD-N-AP, also between SD-N-AP 1 and SD-N-AP 2 (ANOVA, *p* value is >0.05). However, the %emission of SD-N-AP 1 and SD-N-AP 2 compared to SD-AP were found to be significantly different (ANOVA, p value is < 0.001).

3.6.5.4 Dispersibility of the powder formulations from both DPI and pMDI

The dispersibility of the formulation is the ratio of the fine particle dose to the emitted dose. Dispersibility measures the adherence of the dispersed powders (released from the capsule or pMDI device) to the air stream.

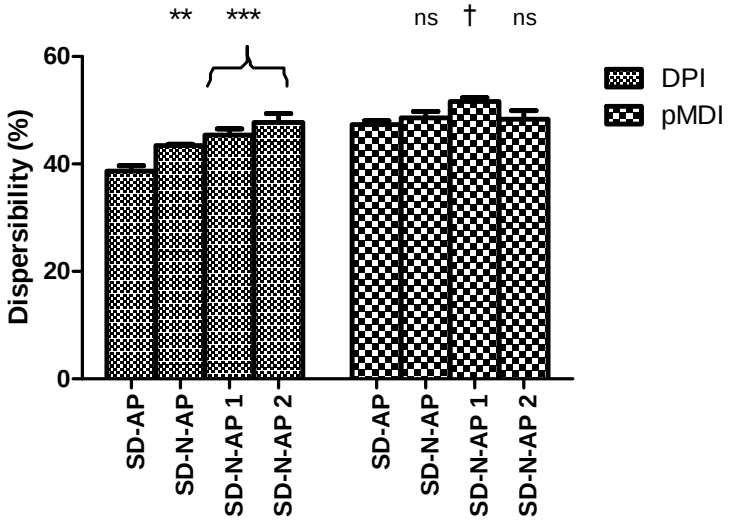


Figure 3 -8: Dispersibility of the spray-dried alkaline phosphatase and sodium carboxymethylcellulose and co-solvent modified DPI and pMDI formulations. Values are mean ±SD, n = 3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; *p* values were considered significant and the *** represents *p value* < 0.001 between SD AP and the sodium carboxymethylcellulose modified DPI formulations; ** represents *p value* < 0.01

between SD AP and the sodium carboxymethylcellulose modified formulations; †, $p < 0.01$ between SD AP and the sodium carboxymethylcellulose modified pMDI formulations. ns indicates no significance between SD AP and the sodium carboxymethylcellulose modified pMDI formulations.

The dispersibility of the various spray-dried powders aerosolised from DPIs were found to significantly ($p < 0.01$) increase with inclusion of sodium carboxymethyl cellulose as well as spray drying the sodium carboxymethylcellulose modified formulation from an increasing concentration of ethanol in the suspension feedstock. However, this was not found to be the same for the pMDI formulations as only the SD-N-AP 1 had a significantly ($p <0.05$) increased dispersibility compared to the SD AP formulation.

3.6.5.5 Mass median aerodynamic diameter of the various spray-dried powders

The mass median aerodynamic diameter (MMAD) of the powders which is the 50% mark of a plot of percent cumulative enzyme deposition versus the effective cut-off diameter of the NGI. The smaller the diameter, the better are the chances that the particle deposition would occur in the lower airways.

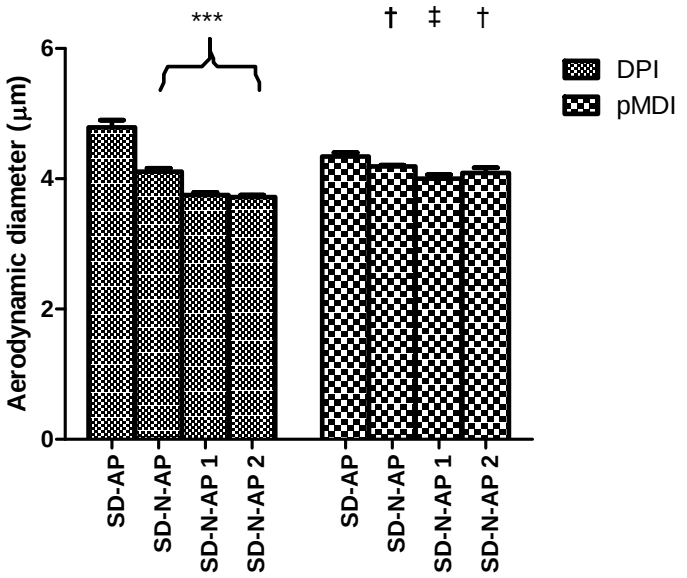


Figure 3 -9: Mass median aerodynamic diameter (MMAD) of excipient-free and sodium carboxymethylcellulose modified spray-dried alkaline phosphatase. Values are mean ±SD, n = 3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; *p* values were considered significant and the *** represents *p value* < 0.001 between SD AP and the sodium carboxymethylcellulose modified DPI formulations; †, *p* < 0.01 between SD AP and the sodium carboxymethylcellulose modified pMDI formulations. ns indicates no significance between SD AP and the sodium carboxymethylcellulose modified pMDI formulations; ‡, *p* < 0.001 between SD AP and the sodium carboxymethylcellulose modified pMDI formulations. ns indicates no significance between SD AP and the sodium carboxymethylcellulose modified pMDI formulations

The MMAD ranged between 3.72 µm and 4.79 µm for the aerosols generated by the DPI devices and between 4 µm and 4.3 µm for the aerosols generated by the pMDI devices. Statistically, the MMAD of particles formulated with sodium carboxymethylcellulose were significantly lower than for those which were excipient free.

3.6.6 Powder properties: Angle of repose

In order to predict the flowability characteristic of the various spray-dried powders, the angle of repose of the powders were determined. It is a characteristic measure of interparticulate friction, or resistance to movements between particles.The British pharmacopoeia (2013) proposes the following classification of flowing properties to the angle of repose: excellent (25 ^{o}< θ<30 ^{o}), good (31 ^{o}< θ<35 ^{o}), fair (36 ^{o}< θ<40 ^{o}), passable (41 ^{o}< θ<45 ^{o}), poor (46 ^{o}< θ<55 ^{o}), very poor (56 ^{o}< θ<65 ^{o}), and very, very poor (θ<66 ^{o}).

Table 3.5: Angle of repose values for the various formulations. Values are mean ± SD; (n = 3)

Formulation	Angle of repose (θ)	Comment
SD-AP	**	**
SD-N-AP	47 ±0.3 ^{o}	True cohesiveness
SD-N-AP 1	34 ±0.1 ^{o}	Some cohesiveness
SD-N-AP 2	28 ±0.2 ^{o}	Some cohesiveness

** not enough powder for investigation

The descending rank of powder flowability is SD-N-AP 2 (angle of repose, 28^{o}) > SD-N-AP 1 (34^{o}) > SD-N-AP (47^{o}). Significant differences were observed between all the formulations (ANOVA; $p < 0.001$),

3.6.7 The effects of spray drying on the molecular size of Alkaline phosphatase

A comparative assessment between the commercially available enzyme (positive control), a thermally destroyed enzyme (negative control) and the four spray-dried formulations were carried out samples. Polyacrylamide gel electrophoresis, which seperates proteins on the basis of charge, and size was used to estimate the molecular mass of the polypeptides that make-up the enzyme.

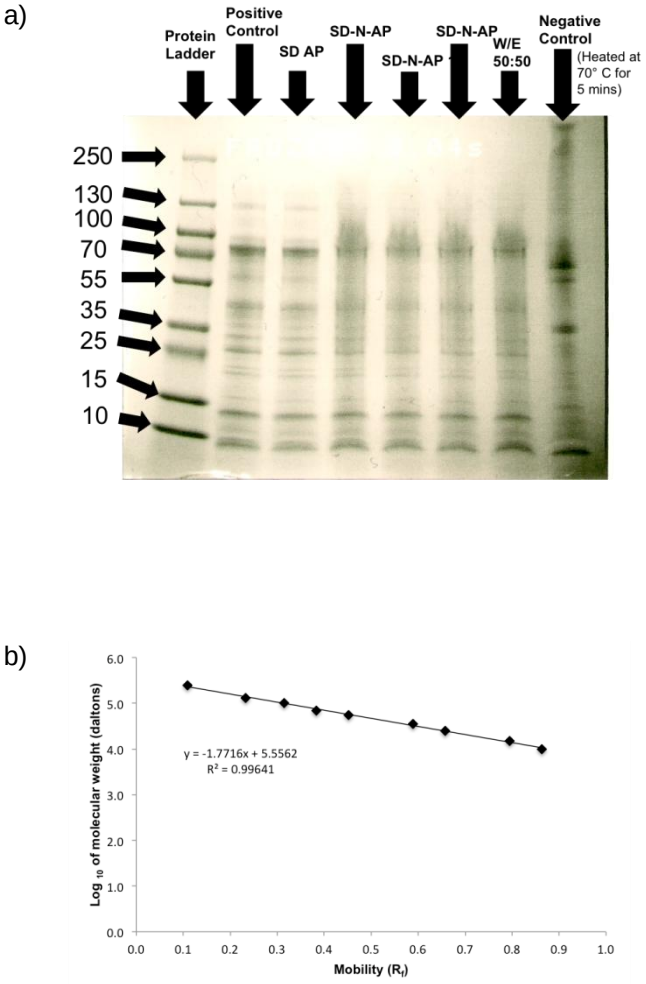


Figure 3- 10: SDS-PAGE profile of alkaline phosphatase. a) Comparison of alkaline phosphatase preparations following separation on SDS-PAGE. Positive control; 2% w/v alkaline phosphatase, SD AP; 2% w/v spray-dried alkaline phosphatase, Water only; 2% w/v (20% alkaline phosphatase and 80% sodium carboxymethylcellulose) spray-dried from water only feedstock, W/E 90:10; 2% w/v (20% alkaline phosphatase and 80% sodium carboxymethylcellulose) spray-dried from a mixture of 90% water and 10% ethanol feedstock, W/E 70:30; 2% w/v (20% alkaline phosphatase and 80% sodium carboxymethylcellulose) spray-dried from a mixture of 70% water and 30% ethanol feedstock, W/E 50:50; 2% w/v (20% alkaline phosphatase and 80% sodium carboxymethylcellulose) spray-dried from a mixture of 50% water and 50% ethanol feedstock, Negative control; 2% w/v alkaline phosphatase heated at 70°C for 5 minutes. The gel was run at a constant current of 60 mA for 22 minutes in a Bio-Rad vertical electrophoresis tank following which the gels were stained with Coomassie brilliant blue R-250 dye. (b) calibration curve of the protein standard showing log molecular weight versus mobility.

In order to enable determination of the molecular weights of the various polypeptides shown as bands on the gel, a calibration plot of log of molecular weight (daltons) against mobility (R_f) of the protein band (figure 3- 10b) was ploted and it yielded a linear relationship (y= -1.7716x + 5.5562, r^2 > 0.9964). Using the equation of the line, the molecular weights of various polypeptides was estimated to be 124,477.6, 79,604.5, 43,050.4, 26,034.8, 20,819.8, 14,888.9, 13314.5 and 9004.1 daltons. Upon heat treatment, the molecular weights of the various breakdown polypeptide products were 71,186.8, 60199.3, 34427.1, 10647.5 and 9004.1 daltons

3.6.8 Morphology of the various spray-dried formulations

Scanning electron micrographs of the various spray-dried alkaline phosphatase powders are presented in Figure 3- 11. Fig. 3- 11a shows the commercially supplied sodium carboxymethylcellulose as long cylindrical particles whilst the commercially supplied alkaline phosphatase (Fig. 3- 11b) was light and flake-like in appearance. Spray-dried alkaline phosphatase (Fig. 3- 11c) appeared spherical and heterogenous in size. Similary, co-spray dried alkaline phosphatase and sodium carboxymethylcellulose (Fig. 3-11d) also appeared spherical but with greater heterogenicity between particle sizes. Spray drying alkaline phosphatase and sodium carboxymethylcellulose from water-ethanol cosolvents (90:10 and 70:30) also produced spherical particles with heterogenous particle sizes.

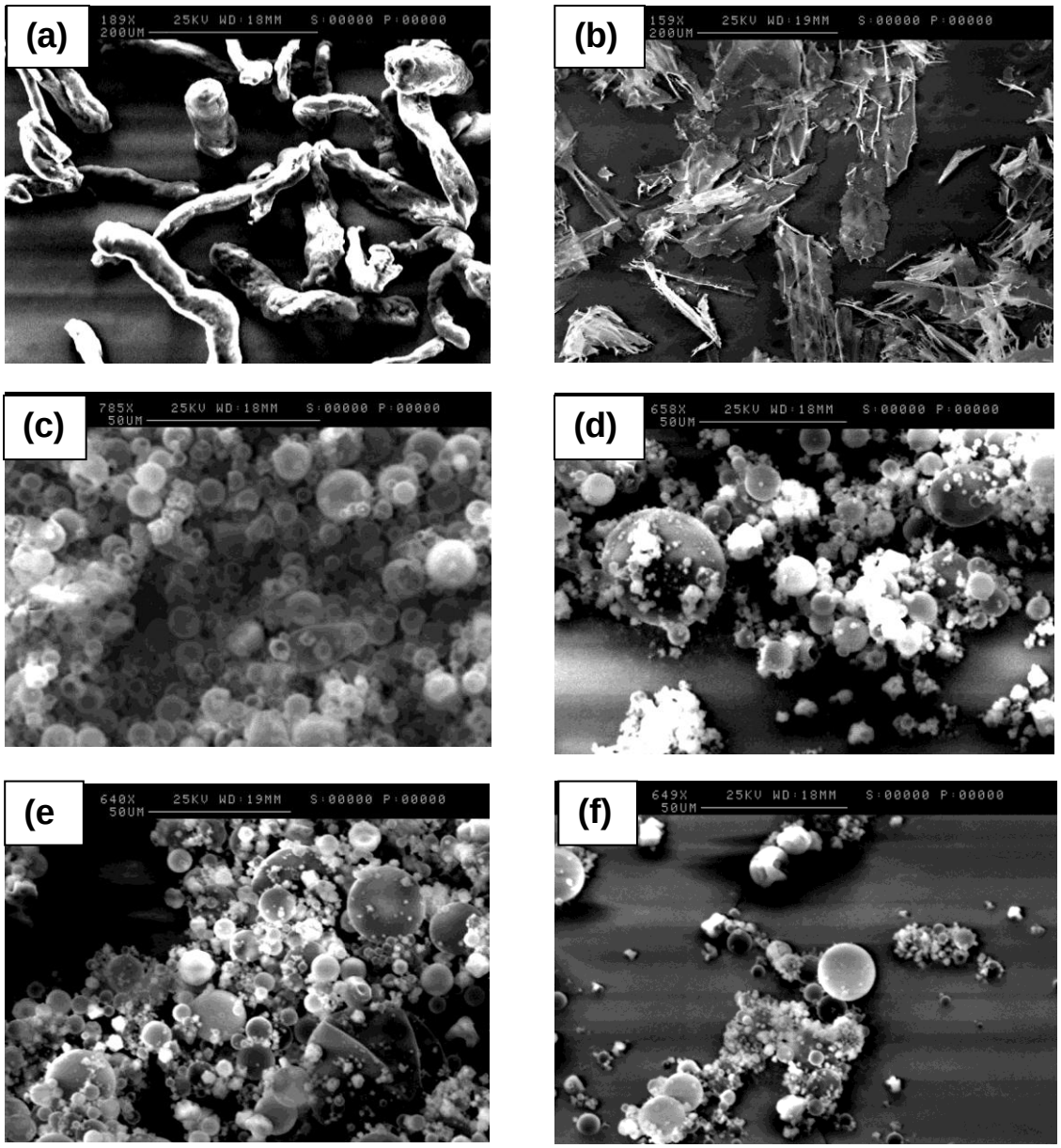


Figure 3- 11: Scanning electron micrographs of (a) commercial sodium carboxymethylcellulose (b) commercial alkaline phosphatase (c) spray-dried alkaline phosphatase (d) spray-dried sodium carboxymethylcellulose and alkaline phosphatase from water-only suspension feedstock at a mass ratio of 4:1 (e) spray-dried sodium carboxymethylcellulose and alkaline phosphatase from water (90%) and ethanol (10%) suspension feedstock at a mass ratio of 4:1 (f) spray-dried sodium carboxymethylcellulose and alkaline phosphatase from water (70%) and ethanol (30%) suspension feedstock at a mass ratio of 4:1. Magnifications are shown on the top of each micrograph along with scale bar in μm.

3.7 Discussion

Results from this study reveal that it is possible to spray dry a protein and produce respirable, more regularly shaped, spherical particles while maintaining substantial enzymatic activity of the protein. This can be achieved with or without solvents or excipients. It was also observed that the inclusion of ethanol in the suspension feedstock can be damaging to the activity of the protein even before the spray drying process (table 3.2), although water-ethanol co-solvents may help to improve the % emission and dispersibility of various spray-dried powders delivered by a Cyclohaler™ DPI device (figure 3- 7, figure 3 -8). Also, the inclusion of sodium carboxymethylcellulose were found to be useful in altering the FPF of alkaline phosphatase DPI, and spray drying the modified protein from a water-ethanol co-solvent system resulted in an even greater improvement of the FPF of the protein (figure 3-6). On the other hand, there was no major difference in the deposition profiles of excipient-free and NaCMC modified alkaline phosphatase powders delivered through a pMDI device (figure 3- 5, 3- 6 and 3- 8). The findings in this study are in agreement with a previous study by Li and Seville (2009), who found that formulating pMDI suspension containing a model protein, bovine serum albumin and NaCMC resulted in powders with statistically better aerosolisation performanace compared with NaCMC-free pMDI formulation. Li et al., (2010) also found that NaCMC-modified spray-dried powders provided improved protection to the enzymatic activity of alkaline phosphatase as well as maintaining the aerosolisation performance of the powders, when formulated as a pMDI, over a 16 week storage period. This study extends their findings by providing information on the use of co-solvents when preparing these inhalable NaCMC-modified protein formulations, and in addition, this study also demonstrated that 80% NaCMC sufficiently protected the enzymatic activity of the protein (alkaline phosphatase) from the harsh spray drying conditions (table 3.2).

Assessment of the powder flow properties of various spray-dried powders showed that spray-dried powders prepared from water-ethanol co-solvents generally had good flow properties (classified under the the British Pharmacopoeia) as the angle of repose ranged between 28° and 34° (table 3.5). The flow properties of NaCMC-modified powders spray-dried from a water only suspension feedstock were classified to be fair, however, spray-dried powders of alkaline phosphatase alone generally had poor flow properties as powder greatly adhered to the walls of storage vials (unfortunately, not enough powder was generated to enable the determination of the angle of repose). More of the SD-AP powders were retained in the DPI device as well as on the throat of the NGI. This related to powder adhesion of the various spray-dried alkaline phosphatase formulation. In summary, increases in the percent ethanol composition leads to improvement in the flow properties of spray-dried protein powders. This observation is in agreement with Rabbani and Seville (2005) who found that inclusion of ethanol as a formulation component enhanced the aerosolisation properties of β-estradiol dry powders.

With the particle size of dry powders being a cruicial factor in the prediction of DPI deposition profile, two particle sizing measurements were carried out; particle sizing by laser diffraction and MMAD determined from the 50% mark on a plot of cumulative percent deposition versus the effective cut-off diameter (table 2.5). It was found that laser diffraction measurement of between 5 mg and 10 mg of the spray-dried powder samples had a d [v,50] range of between 4 µm and 6 µm (table 3.4). However, the span of the NaCMC-modified powders indicated a significantly higher heterogenicity compared with the excipient-free alkaline phosphatase formulation. The volume mean diameter (VMD) of the formulations was found not to correlate with the MMAD, which was found to range between 3.7 µm and 4.8 µm. This maybe accounted for by the failure of the MMAD to take into consideration particles depositing on the throat of the NGI, while laser diffraction measures light scattering of an entire mass of powder.

The statistical analysis of the MMAD values (Fig. 3 -9) measured upon aerosolisation of the spray-dried powders from the different devices (DPI and pMDI) shows that there are significant (p <0.001) differences between the aerodynamic diameters of the excipient-free alkaline phosphatase powders and the NaCMC-modified (SD-N-AP, SD-N-AP 1, and SD-N-AP 2) powders, however, there is no statistical difference between both co-solvent modified formulations.

The scanning electron micrographs of the various spray-dried powders show a heterogenous particle size distribution especially among the NaCMC-modified powder formulations. This observation further supported the large span calculated from the volume distribution of the laser diffraction measurements (table 3.4). Overall, there were observable changes in the morphology of the spray-dried powders compared with the commercially supplied enzyme and excipients.

SDS-PAGE analysis of the various spray-dried powder preparation show the presence of various polypeptides which were particularly different from the heat treated alkaline phosphatase samples. Takeuchi (1990) reported the isolation of the polypeptide between 120 and 130 kDa to be responsible for the phosphatase activity of the enzyme. Similar analysis in this study showed that less of the polypeptide was visible upon spray drying NaCMC-modified preparations with co-solvents, even though the same amount of protein was loaded. This observation may account for the decreased activity of the phosphatase which was measured by the diethanolamine assay.

3.8 Conclusion

This study demonstrated that while the selection of appropriate excipients and solvent systems may enable the preparation of co-spray dried powders of model proteins and NaCMC with the requisite size, possessing low interparticulate cohesion which results in

improvements to fine particle fraction, there are challenges that may also arise from such formulations. These include heterogenous particle sizes and a significant loss in activity of the protein (i.e for protein powders prepared from co-solvent systems). On the otherhand, excipient-free spray-dried powders prepared from water only suspensions however posses the requisite size, are more homogenous (very small span) and also have the advantage of one less processing step when compared with NaCMC-modified formulations. Since the methods developed in this study will inform the subsequent development of a pulmonary delivered antimicrobial protein, dry powder inhaler devices were found to be better suited at delivering the masses of powder that would be required for the proposed therapies in one or two divided doses.

Chapter Four

Characterisation of lactoferrin

4.1 Summary

Having designed a plausible method of micronising and delivering active proteins to the airway through co-spray drying them with the excipient sodium carboxymethylcellulose (NaCMC) in the previous chapter, the next logical step was to advance the application of the method to potentially therapeutic proteins. In this chapter, we investigated the possibility of formulating the antimicrobial protein, lactoferrin, with suitable aerodynamic properties to enable its delivery directly to the airways. The fundamental aerodynamic properties required for the design of a successful inhaled therapy included; particle size, morphology, density, moisture content, and its aerosolisation performance. This chapter also examines the preparation and spray drying of iron-free lactoferrin since this compound may possess more biological activity than the iron-rich form. Micro-particles of the iron-free and iron-rich lactoferrin preparations were prepared from a suspension feedstock of 2% w/v using a small scale laboratory spray dryer. The particles generated were characterized by differential scanning calorimetry (DSC), helium gas pycnometry, scanning electron microscopy and Karl Fischer titration and *in vitro* aerosolisation properties of the micro-particles produced evaluated using the multi-stage liquid impinger. Overall, the data indicate that the spray drying parameters employed were able to generate micro-particles with requisite size, residual moisture content and suitable aerosolisation properties

4.2 Introduction

Delivering biopharmaceuticals directly to the airways as aerosols has continued to be an attractive, convenient and effective way of directly employing macromolecules as therapeutic agents for a variety of airway disorders. In particular, the direct delivery of high concentrations of antimicrobial agents (formulated as aerosols) to the various sites of infection in the airway of people with respiratory infections is advantageous over macromolecular drug delivery through other conventional routes. This delivery strategy can be employed to effectively treat respiratory infections, such as pneumonia or specific airway infections as commonly seen in diseases such as cystic fibrosis and tuberculosis (Gelperina *et al*., 2005). It is also the case that aerosolized antimicrobial therapies seem to offer an appealing and logical approach for the treatment of airway infections. This is not just because they ameliorate lung function, reduce systemic long term toxicity (due to the lower therapeutic doses required) (Adi *et al*., 2008), represent a non-invasive means of drug administration (thereby increasing patient compliance) as well as decrease hospitalizations, but also because they offer significant improvement in diseases like cystic fibrosis where their efficacy possibly should possibly make them a mainstay therapeutic concept. The efficiency of utilizing aerosolized antimicrobial particles in therapy depends on several characteristics. Of these, particle size, density, morphology, surface energy and surface roughness play a key role in determining where an aerosolized particle would most likely deposit.

In chapter three, the spray drying technique was highlighted as a popular dry powder production technique for pulmonary formulations, this is especially because it is able to produce micron sized powders suitable for inhalation in combination with a parallel optimisation of density and morphology. Spray drying could potentially be a more attractive technique for producing dry powders of therapeutic proteins suitable for

inhalation although it is however still fraught with various challenges ranging from thermal degradation of spray-dried products (Broadhead *et al*., 1994) to the production of unstable amorphous materials (Chan and Chew, 2003) (although this may also be an advantage).The drawbacks of spray drying are marked for proteins in particular, especially among those containing cysteine and methionine residues (as they are easily oxidized to sulphoxides).

4.2.1 Reasons for selecting lactoferrin as our protein of choice

Lactoferrin (formerly known as lactotransferrin) is a glycoprotein with a capacity to bind Fe^{3+} ion – and is a member of the transferrin family (Metz-Boutigue *et al.*, 1984a). Lactoferrin is the main iron-binding protein in human milk (Johanson, 1960; Montreuil *et al.*, 1960) although it is also found in specific granules of neutrophils and in most mucosal secretions including; tears, saliva, bile, vaginal secretions, seminal fluid, pancreatic juice and small intestinal secretions (Levay and Viljoen, 1995; Lönnerdal and Iyer, 1995; Baker and Baker, 2005a; Masson *et al.*, 1966; Iyer and Lonnerdal, 1993; Thompson *et al.*, 1990). Various biological functions have been ascribed to lactoferrin including antibacterial, antiviral and anti-inflammatory activities. Lactoferrin is a part of the innate immune system along with other antimicrobial peptides which makes it a potentially useful protein in the treatment of various airway infections. In this respect, aerosolized delivery of this important protein to the airway should present a suitable means of harnessing its activity against susceptible microbes present in the infectious airways. In secretions from the normal airways, lactoferrin found at between 1 to 10 µg/mL (Thompson *et al*., 1990).

4.2.2 Delivering lactoferrin to the airways

The challenges of delivering lactoferrin to the airways are enormous, therefore, a careful selection of the formulation characteristics and a method of delivering it is crucial for achieving a successful lactoferrin therapy. Furthermore, it is thought that in order to efficiently and effectively deliver lactoferrin to the airways, it is pertinent that the formulation is delivered as a dry powder (since dry powder formulations are more stable than liquid formulations). There are only two aerosol devices that can deliver dry powder formulations; the pressurized metered dose inhaler (pMDI) or the dry powder inhaler (DPI). While pMDI's can only deliver small masses of therapeutic agent, DPI's can deliver very high doses. In order to achieve the high therapeutic concentrations that are likely to be required for effective therapy, the DPI devices seem more attractive.

The DPI provides a technique for efficiently delivering proteins to the airways by utilizing the inspiratory energy of the patient. Today there are a variety of DPI devices available for clinical use. All currently available DPI systems feature several attractions including; ability to deliver high doses, they do not require a propellant to aerosolise incorporated API's and they usually do not interact with the formulations which they deliver.

Since the performance of a DPI system is not only dependent on the device employed but also on the powders aerosolized, both aspects must be considered in order to produce powders (with API's included) with favourable aerodynamic properties. This challenge is especially made difficult if the therapeutic agent is either a protein or peptide (due to their labile nature) as not many processing conditions are suited to these groups. Nevertheless, since the percentage of the emitted dose deposited in the airways is dependent on the powder's dispersibility (a determinant of the micronisation process

to be employed), the need to produce powders with suitable characteristics and which can be easily dispersed cannot be overemphasized (as efficient dispersion is key to achieving patient-reproducible delivery) (Weers, 2000b).

4.3 Aims and objectives

This chapter aims to exploit the methods used in the previous studies (which involved spray drying a model protein alkaline phosphatase) to develop microparticulate formulations of Lactoferrin. The suitability of these spray-dried formulations for inhaled therapy will then be assessed by investigating the physical aspects of various spray-dried lactoferrin formulations.

Two different lactoferrin powders will be prepared through spray drying of commercially available bovine milk lactoferrin, and a iron depleted form of this lactoferrin. These will be spray-dried in the presence and absence of sodium carboxymethylcellulose, before characterisation to determine particle size, particle density, residual moisture, thermal properties and *in vitro* aerosolisation properties. The data was then be analysed to determine the most optimal formulation for pulmonary delivery.

4.4 Materials

Lactoferrin from bovine milk was obtained as a kind donation from DMV Fonterra. sodium carboxymethylcellulose, sodium acetate, sodium dihydrogen phosphate were all of analytical grade and were purchased from Sigma aldrich (Poole, UK). Spectrosol ferric nitrate was purchased from VWR (Leicestershire, UK), BCA protein assay reagent was purchsed from Thermo Scientific (Rockford, IL USA).

4.5 Methods

4.5.1 Preparation of micro-particulate lactoferrin powders

Two grams of lactoferrin powder was dispersed in 70 mL of distilled water, stirred until a homogenous suspension resulted and then made up to a final volume of 100 mL to produce a 2 % w/v suspension at room temperature, the mixture was stirred until a fine mixture was obtained. This was spray-dried using a Buchi B290 spray dryer with the following conditions: feed rate - 3 mL/min, aspiration rate 95 %; air flow rate 606 L/hr, inlet temperature 180°C, to produce an outlet temperature of 99 – 101 °C. This procedure was repeated two more times to produce three batches of spray-dried lactoferrin powders.

4.5.2 Preparation of apo-lactoferrin

An iron deplete form of lactoferrin (aLf) was prepared from partially iron saturated lactoferrin as previously described by Newsome *et al*., (2007). Briefly, the partially iron saturated lactoferrin was resuspended at a final concentration of 2% (w/v) in deionzed water, transferred to dialysis membrane and dialyzed for 24h at 4 °C against 20 mmol/L sodium acetate, 20mmol/L sodium dihydrogen phosphate and 40mmol/L EDTA (pH 3.5).

The protein was then dialyzed against 900 mL of deionzed water with at least 2 changes and used immediately or quickly frozen at -80 °C.

4.5.3 Preparation of spray-dried apo lactoferrin

Following dialysis, the resulting protein suspension was spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; feed rate - 3 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature ranging from 100 – 102 °C.

4.5.4 Estimation of the iron content of lactoferrin preparations

A standard calibration graph of spectrosol ferric nitrate was prepared by measuring the absorbance of stock concentrations (0.05 – 5 ppm) using a Perkin Elmer AAnalyst 100 atomic absorption spectrophotometer. The amount of iron bound to the various lactoferrin preparations (Lf, SDLf, aLf and SDaLf) was then estimated from the calibration curve following iron absorption measurements.

4.5.5 Preparation of co-spray dried lactoferrin with sodium carboxymethylcellulose

A 2 % w/v suspension of lactoferrin and sodium carboxymethylcellulose was prepared by dispersing 1 g of sodium carboxymethylcellulose in 70 mL of deionised water and then stirred until a slurry resulted, before 1 g of lactoferrin was added to the slurry, stirred and the final volume made up to 100 mL with deionised water. The resulting slurry was then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; feed rate - 3 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature ranging from 97 – 99 °C. This procedure was repeated

two more times to produce three batches of co-spray dried lactoferrin with sodium carboxymethylcellulose dry powders.

4.5.6 Preparation of co-spray dried apo lactoferrin with sodium carboxymethylcellulose

One gram of sodium carboxymethylcellulose was made up to a final volume of 100 mL with apo lactoferrin (prepared as outlined in section 2.2.2.2.4) to produce a 2 % w/v suspension feedstock containing 1:1 apo lactoferrin-sodium carboxymethylcellulose suspension feedstock. The resulting slurry was then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; feed rate - 3 mL/min; aspirator rate, 95 %. These setting resulted in an outlet temperature ranging from 98 – 99 °C.

4.5.7 Karl-Fischer titration analysis

Residual moisture of the various spray-dried powders were determined (immediately following spray drying) by Karl-Fischer titration at 25 ±1°C using a C20 Coulometric Karl Fischer titrator (Mettler Toledo). Triplicate measurements of approximately 5 mg of the various powders were analysed and the percent moisture content (mean ±SD) computed for each of the powder preparations.

4.5.8 Particle size Distribution

Particle size distributions of the various spray-dried powders were determined using the same method outlined in section 3.5.5. Briefly, about 5 – 10 mg of each powder sample was analysed with a Helos H 2178 laser diffraction instrument coupled to a Rodos dry

dispersing unit at 2.0 bars using an R2 lens. The data obtained are reported as the percent of particles sized in the lower 10, middle 50, upper 10th percentile ($d[v,10]$, $d[v,50]$ and $d[v,90]$ respectively) and the volume mean diameter (VMD). Also the span of the volume distribution, a measure of the width of the distribution relative to the median diameter ($d[v,50]$), was also calculated using equation 3.1 also detailed in section 3.5.5

4.5.9 Scanning Electron Microscopy of the various lactoferrin powders

Scanning electron micrographs of the various lactoferrin powder formulation were obtained using the same method outlined in section 3.5.6. Briefly, samples were mounted on adhesive black carbon tabs (premounted on aluminium stubs) and sputter-coated (Polaron SC500, Polaron Equipment) with a thin layer of gold for 3 mins at 20 mA. The samples were subsequently studied under high magnification (which is shown on each micrograph along with a scale bar in μm).

4.5.10 Density of the various spray-dried lactoferrin powders

The true density of the various powders was determined using a helium pycnometer (Multipycnometer, Quantachrome Instruments) with a 3 cm³ sample cup at 22 °C. Prior to analysis, the helium pycnometer was calibrated against a standard steel ball. For each analytical determination the reference volume was pressurized to approximately 17 pounds per square inch (1.195 Kg/cm^2) above the ambient pressure. Measurements were performed in triplicate for each preparation.

4.5.11 X-ray powder diffraction

The crystal structure of both the single and co-spray dried powders were characterized using an x-ray powder diffractometer (Bruker D2 Phaser, UK). The system comprised of a scintillation counter and a 1-dimensional LYNXEYE detector with a CU Kα radiation source, an accelerating voltage of 30 kV and an emission current of 10 mA. Diffraction patterns were recorded between 2° and 70° of 2θ with an increment of 0.02° and a step time of 0.1 s.

4.5.12 Differential scanning calorimetry

Differential scanning calorimetry (Pyris Diamond DSC and Intracooler 2P: Perkin Elmer, Wellessey, USA) was employed to characterise the thermal behaviour of the various powder formulations. Prior to the start of the assessments, the DSC was calibrated for temperature and heat flow using standard samples of indium (melting point: 156.6 °C, ΔHm: 28.42 J/g) and Zinc (melting point: 419.5 °C, ΔHm: 108.26 J/g). Following calibration, 4 mg of each of the powders was loaded into an aluminium pan, cooled to -30 °C and then heated to 200 °C at 10 °C/min with a nitrogen purge of 10mL/min. An empty aluminium pan was used as reference for all measurements. The resulting data were analysed using a Pyris manager software. Triplicate measurements of all independently prepared sample batches were assessed.

4.5.13 Preparation of DPI formulations

Doses of 30 mg of either lactoferrin, apo lactoferrin (aLf) or spray-dried lactoferrin (SDaLf) were manually loaded into size 3 gelatin capsules. The dose loaded into the gelatin capsule was determined to be the optimum dose which can be effectively loaded

into the capsule without packing it too tightly as to impede aerosolisation as well as ensuring sufficient material to calculate a fine particle fraction.

4.5.14 Quantification of total protein in the formulations

Protein quantitation was carried out as previously described in section 2.2.8.

4.5.15 *In vitro* aerodynamic assessment

The pulmonary deposition of the dry powders were estimated *in vitro* using a Multi-stage liquid impinge (MSLI). Twenty millilitres of water was poured into each of the four stages of the impinger to wet the collection surface. A vacuum pump was attached to the MSLI and the inspiratory flow rate was calibrated (by adjusting the vacuum pump settings) and set at a flow rate of 60 L/min. Approximately 30 ±2 mg of each of the dry powders was manually weighed into a size 3 hard gelatin capsules. A capsule was placed into the capsule compartment of a Cyclocaps™ Cyclohaler™ DPI device (Pharmachemie BV, The Netherlands), pierced and actuated for 5 s. Powders depositing in the four impinger levels were recovered by agitating the apparatus to ensure complete dissolution. The powder which deposited in the throat and the back filter were also collected and the amount of protein depositing in each of these stages determined by carrying out BCA assay which is described in section 2.2.8. The assessment was carried out on three batches of dry powder with triplicate measurement for each of the protein suspensions. From these protein measurements, the recovered dose (RD, the sum of protein collected from all stages, filter and throat), the Fine Particle dose (FPD, mg of protein possessing less than 5 µm aerodynamic diameter) and the Fine Particle Fraction (FPF: the ratio between FPD and RD) were calculated. The Mass Median Aerodynamic Diameter (MMAD) was determined from a graphical plot of % cumulative undersize

against effective cut-off diameter (i.e 13.0, 6.8, 3.1 and 1.7 µm for stages 1 to 4) at a flow rate of 60 L/min. The MMAD was defined from this graph as the particle size at which the line crossed the 50 % mark.

4.5.16 Statistical analysis

Data was subjected to statistical analysis using one-way ANOVA to examine for significant difference (defined as $p > 0.05$). Significant differences between the various formulations were analysed using Dunnett post test with p values of < 0.05 being considered to be significant.

4.6 Results

4.6.1 Calibration plot of absorbance against ferric ion concentration

A calibration curve of absorbance against ferric ion (Fe^{3+}) concentration (0.05 – 5 ppm) was plotted (figure 4 -1) and it yielded a linear relationship ($y = 0.361x + 0.0013$, $r^2 > 0.999$). The equation of the line of best fit was used to estimate the ferric ion content in various lactoferrin preparations in subsequent analyses.

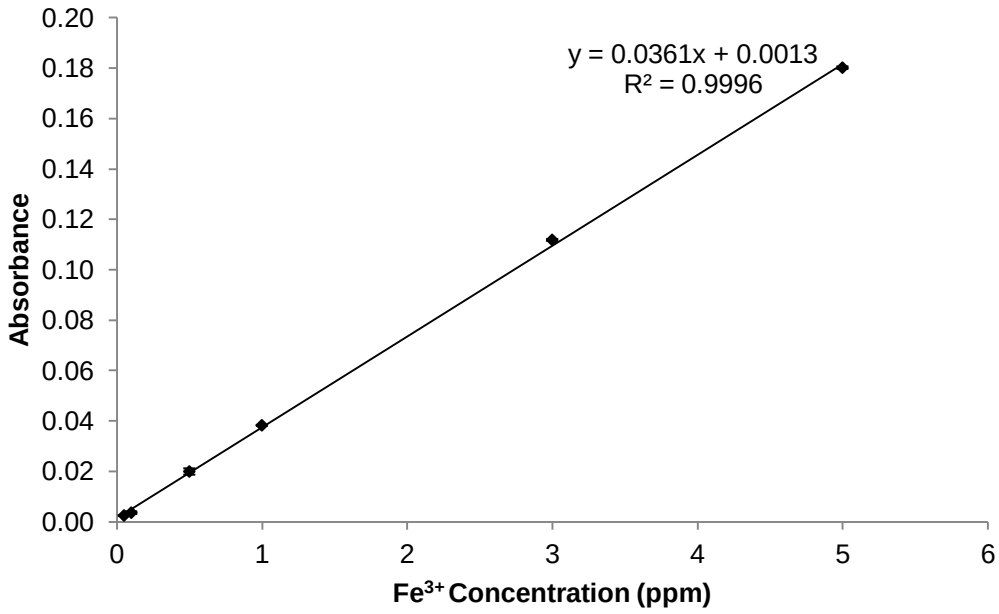


Figure 4 -1: Calibration plot of mean absorbance against standard ferric ion concentrations. The mean absorbance calculated from calibrations obtained on 3 different days are plotted against nominal ferric ion concentration values (n = 3). Error bars representing standard deviation are displayed for each point but are within the limits of the symbols.

All calibration curves obtained on different days were linear within the measured concentration range of 0.05 – 5 ppm (Day 1: y= 0.036x + 0.0012, r^2 = 0.999; Day 2: y= 0.0362x + 0.0009, r^2 > 0.999; Day 3: y= 0.036x + 0.0019, r^2 > 0.999). The assay method generally showed good reproducibility with similar inter-day results.

4.6.2 Validating the ferric ion quantitation assay

As with majority of compounds quantified using atomic absorption spectrophotometer (AAS), there is a need to validate the analytical method employed. Intra- and inter-day precisions were determined by computing the % RSD (otherwise known as coefficient of variation CV) obtained from three individual absorbance values of nominal concentrations 0.05 – 5 ppm obtained on the same day (intra-day precision) and over a three day period (inter-day precision). The lower the value, the better the assay performance. Data analysis showed that the highest intra-day % RSD calculated (an indicator of precision) was 24.74 % at ferric ion concentration of 0.05 ppm on days 1 and 2 (Table 4.1). The high % RSD is due to limitations in the sensitivity of the equipment. The inter-day assay variability was found to range between 0.28 – 14.32.

According to the assay method employed, the limit of detection (LOD) which is the lowest concentration of ferric ion that can be detected but not quantified and the limit of quantitation (LOQ) which is the lowest concentration of ferric ion that can be measured with acceptable precision by the assay method were calculated from the mean of the slope and the SD of the intercept of three calibration curves. The mathematical method for calculating LOD and LOQ are outlined in equation 2.2 and 2.3 respectively (section 2.2.1.1.4, page 80) and the values obtained are shown in table 4.1.

Table 4.1: Calculated mean absorbance, standard deviation, % RSD, R^2, LOD, LOQ and Nominal concentration of ferric ion. Linear regression measurement provided the necessary information to estimate the LODs and LOQs. Except for nominal concentrations 0.1 ppm and below, the small standard deviations of the absorbance values indicate good intra-day precision.

	Fe^{3+} Conc. (ppm)	**Mean Absorbance**	**Standard deviation**	**Relative standard deviation (%)**	**Linear regression (R^2)**	**Limit of detection (ppm)**	**Limit of quantitation (ppm)**
Day 1	0.05	0.002	0.0006	24.74	0.9996	0.0007	0.0022
	0.1	0.004	0.0006	15.75			
	0.5	0.019	0.0006	2.99			
	1	0.038	0.0006	1.51			
	3	0.111	0.0015	1.37			
	5	0.180	0.0015	0.85			
Day 2	0.05	0.002	0.0006	24.74	0.9995	0.0018	0.0054
	0.1	0.003	0.0010	17.32			
	0.5	0.019	0.0010	5.26			
	1	0.038	0.0010	2.63			
	3	0.112	0.0010	0.89			
	5	0.180	0.0010	0.56			
Day 3	0.05	0.003	0.0006	21.65	0.9995	0.0024	0.0073
	0.1	0.004	0.0010	15.75			
	0.5	0.021	0.0015	7.16			
	1	0.038	0.0015	3.98			
	3	0.112	0.0010	0.89			
	5	0.181	0.0015	0.85			

4.6.3 Ferric ion concentration in lactoferrin

The mean ferric ion concentration in the commercially supplied lactoferrin was found to be 2.81 ± 0.05 ppm (values were estimated from the calibration curve of absorbance vs ferric ion concentration), after spray drying, the ferric ion content was 2.77 ±0.04 and this did not represent any significant difference from the starting material. However dialysis of Lf against sodium acetate, sodium dihydrogen phosphate and EDTA to produce iron-free aLf resulted in a ferric ion content of 1.0 ±0.11 ppm. This was measured to be 0.92 ±0.03 ppm after spray drying.

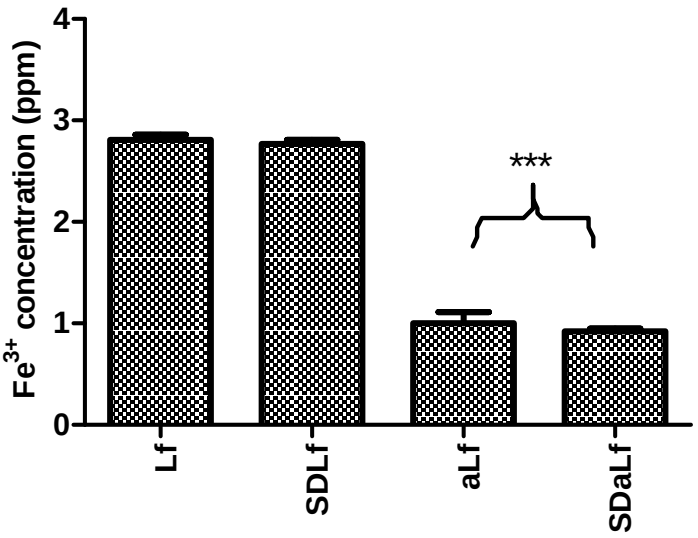


Figure 4 -2: Ferric ion concentration in parts per million of various lactoferrin preparations. The mean and standard deviation of each of the lactoferrin preparation is shown (n = 3). Error bars represent standard deviation around the mean. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; *** indicates p <0.001 compared to iron content in the commercially supplied lactoferrin (Lf).

4.6.4 Spray drying conditions, yield and moisture content

The spray drying parameters employed, yield of the dried powders and moisture content of the various lactoferrin powder formulations are summarized in table 4.2. All the formulations were spray-dried with an inlet temperature of 180 °C and airflow rate of 606 L/hr to produce various outlet temperatures ranging from 99 – 102 °C. The residual moisture in all the various spray-dried formulations was measured by Karl fischer and found to between 5 and 9 % (Table 4.2). The mean yield for the spray-dried protein only formulations were between 45 and 58 %, however, upon inclusion of NaCMC into the spray drying feedstock the percent yield improved (74 – 76 %) (Table 4.2).

Table 4.2: Spray drying parameters, yield and moisture content of spray-dried lactoferrin and spray-dried apo lactoferrin. Three different batches of the various lactoferrin powders were prepared and the mean ±SD of the yield and moisture content are shown below. Statistical analysis of the various formulations were carried out by one-way analysis of variance (ANOVA) using GraphPad prism 5. ‡ denotes $p < 0.01$ statistical difference compared to the spray-dried lactoferrin only formulation.

Formulations	Inlet temp (°C)	Outlet temp (°C)	Airflow rate (L/hr)	Mean yield (%)	Moisture content (%)
SDLf	180	99 - 101	606	45.2 ±2.7	5.9 ±0.4
SDaLf	180	100 - 102	606	57.8 ±1.8‡	5.7 ±0.2
SDLf + NaCMC	180	97 - 99	606	76.1 ±2.2‡	8.2 ±0.6‡
SDaLf + NaCMC	180	99 - 101	606	74.3 ±1.8‡	7.6 ±0.7‡

4.6.5 Particle size distribution

The volume mean particle size of lactoferrin was measured before spray drying and found to be 27.89 µm. After spray drying, the volume mean particle size decreased to 5.9 µm for SDLf and 5.7 for SDaLf. The particle size distribution (PSD) for Lf, aLf SDLf and SDaLf is summarized in table 4.3. The data is expressed in terms of the particle diameter at 10, 50 and 90% of the volume distribution ($d[v,10]$, $d[v,50]$ and $d[v,90]$ respectively). The span of the volume distribution, a measure of the width of the volume distribution relative to the median diameter ($d[v,50]$), was derived from ($d[v,90] - d[v,10]/d[v,50]$) and varied between 1.5 ±0.1 (Lf) and 3.5 ±0.4 (SDaLf + NaCMC) (Table 4.3).

Table 4.3: Particle size distribution of the various lactoferrin powder formulations determined through laser diffraction measurements. Addition of NaCMC increased the span the of the particle size distribution. The values are mean ± SD of triplicate measurements.

Formulations	Particle size distribution (µm)				
	$d[v,10]$ (µm)	$d[v,50]$ (µm)	$d[v,90]$ (µm)	Span	Volume mean diameter (µm)
Lf	8.80 ±0.3	26.24 ±0.3	48.59 ±0.5	1.52 ±0.1	27.89 ±0.4
SDLf	1.38 ±0.2	4.91 ±0.3	11.08 ±0.4	1.98 ±0.2	5.85 ±0.4
SDaLf	1.28 ±0.3	4.79 ±0.2	11.01 ±0.5	2.03 ±0.1	5.67 ±0.5
SDLf + NaCMC	1.16 ±0.6	5.23 ±0.6	14.78 ±0.8	2.63 ±0.4	7.51 ±0.5
SDaLf + NaCMC	1.29 ±0.4	5.48 ±0.6	20.56 ±0.8	3.55 ±0.4	8.55 ±0.5

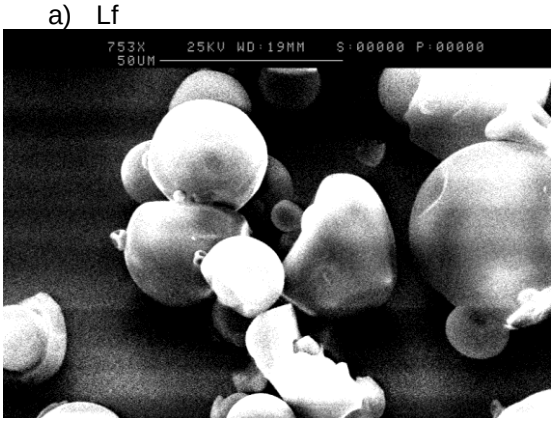
The $d[v,50]$ of the spray-dried protein particles were below 5 µm however, upon including NaCMC into the suspension feed stock, the $d[v,50]$ increased to 5.5 ±0.6 µm

while the VMD increased from < 6 µm (for the spray-dried proteins) to between 8 and 9 µm. The span of the co-spray dried formulations were also increased from about 2 (SDaLf) to 3.6 (SDaLf + NaCMC).

4.6.6 Morphology of the various spray-dried formulations

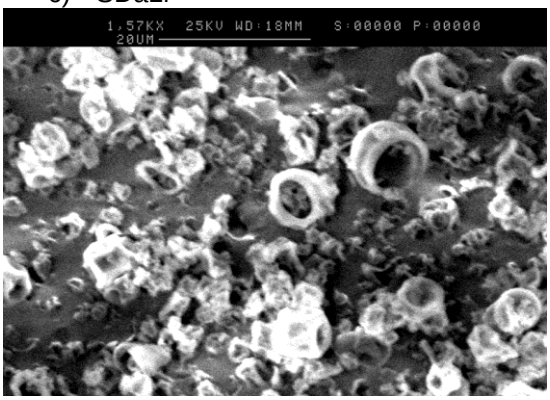
Scanning electron micrographs of native, iron-rich lactoferrin as supplied revealed that the protein was composed of spherical particles and ranged up to 40 µm (Fig 4 -3a). Upon spray drying the proteins, the particles became less smooth, less regular, formed smaller agglomerates ranging up to 10 µm (Fig 4 -3b and c). On the other hand, the co-spray dried lactoferrin-NaCMC formulations yielded particles of a heterogenous size distribution of big, compact aggromerates that ranged up to 10 µm and small irregular shaped particles of < 5 µm (Fig. 4 -3d and e)

a) Lf

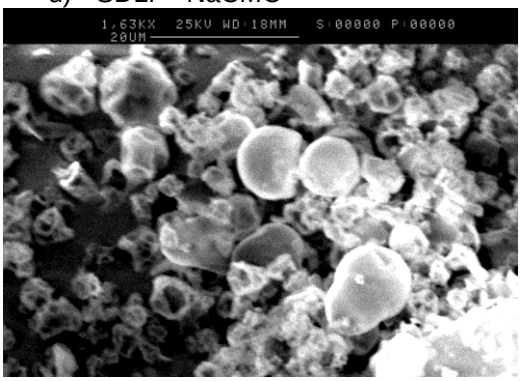


b) SDLf

c) SDaLf



d) SDLf + NaCMC



e) SDaLf + NaCMC

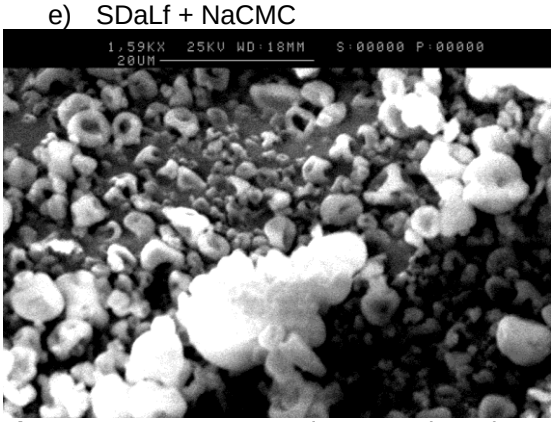


Figure 4 -3: Representative scanning electron micrographs of various lactoferrin powders (a) commercially supplied lactoferrin (b) spray-dried lactoferrin (c) spray-dried apo lactoferrin (d) co-spray dried lactoferrin and sodium carboxymethylcellulose (e) co-spray dried apo lactoferrin and sodium carboxymethylcellulose. Mostly the morphology of the protein particles were retained although there was an increase in the number of deformed particles. Magnifications are shown on the top of each micrograph. The images are a representative of three batches of powders.

SE micrographs (Fig. 4 -3) showed that the Lf (Fig. 4 -3a) powders comprised of spherical particles generally resembling broken beads. SDLf (Fig. 4 -3b) comprised of particles of an irregular shape with indentations on the surface, suggesting the

appearance of a collapsed sphere. SDaLf (Fig. 4 -3c) comprised of aggregates of irregular shaped particles with a rough surface morphology. It also comprised of very small amount of spherical particles with a hollow morphology. SDLf + NaCMC (Fig. 4 – 3d) and SDaLf + NaCMC (Fig. 4 -3e) comprised of particles with varied surface morphologies, while some particles were more rounded in appearance, others had a rough surface morphology. The addition of NaCMC to the spray drying feedstock also resulted in an apparent increase in particle size.

4.6.7 True density

The true densities of the powders were evaluated using a gas pycnometer and found to have very small inter-batch variation. However, the true densities of the various spray-dried powders were significantly higher than the true density of the commercially supplied lactoferrin powder (Lf) (Figure 4 -4). In addition, while the true density of the spray-dried iron deplete lactoferrin was significantly higher than the spray-dried protein, there were no significant difference between the particle densities of both NaCMC modified proteins.

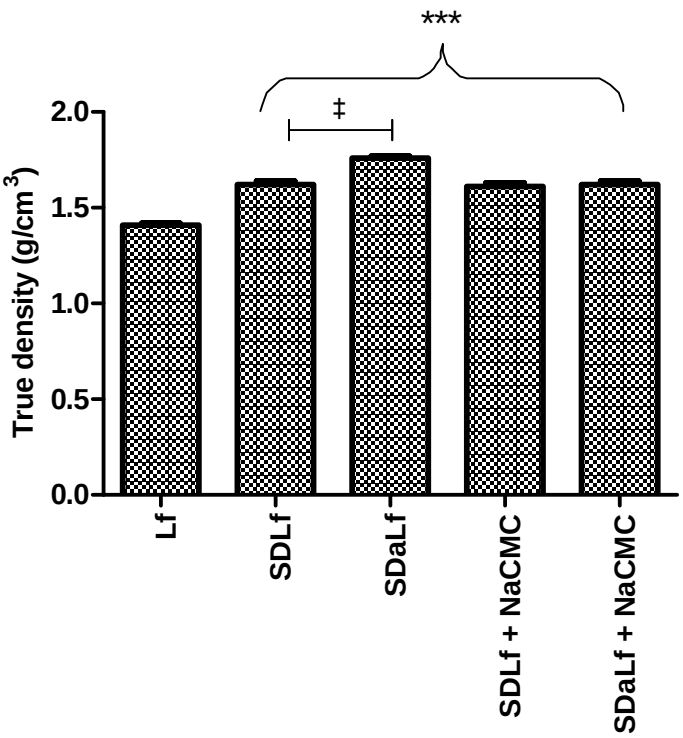
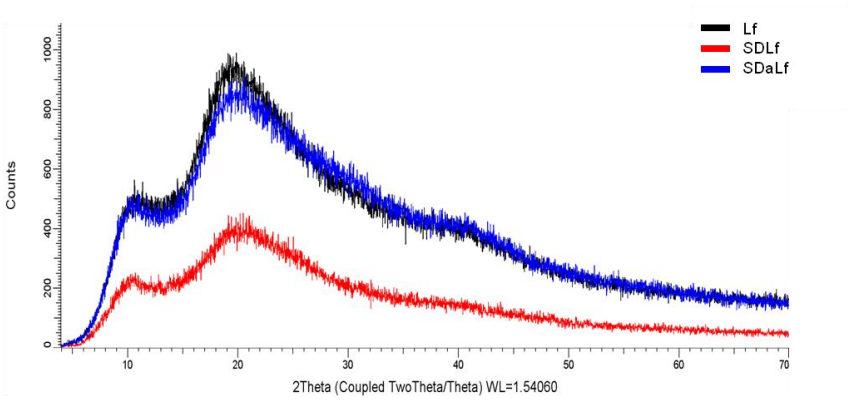


Figure 4 -4: True density of the various lactoferrin powders measured by helium gas pycnometer. The various spray-dried lactoferrin particles possessed a significantly higher true density compared to the commercially supplied lactoferrin particles, also, SDaLf possessed possessed significantly higher true density compared to SDLf. However, the particle size densities of SDLf, SDLf + NaCMC and SDaLf + NaCMC. Values are mean ±SD of triplicate measurements from three batches of powders. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; *p* values were considered significant and the *** represents *p value* < 0.001 between the various spray-dried lactoferrin powders and the commercially supplied lactoferrin powders (Lf); ‡, *p* < 0.001 between SDLf and SDaLf formulations.

4.6.7 X-ray powder diffraction (XRPD)

The X-ray powder diffraction patterns (figure 4 -5a and 4 -5b), shows that the various spray-dried powders lacked any sharp distinct peaks. An indication that all the powders were amorphous.

a)



b)

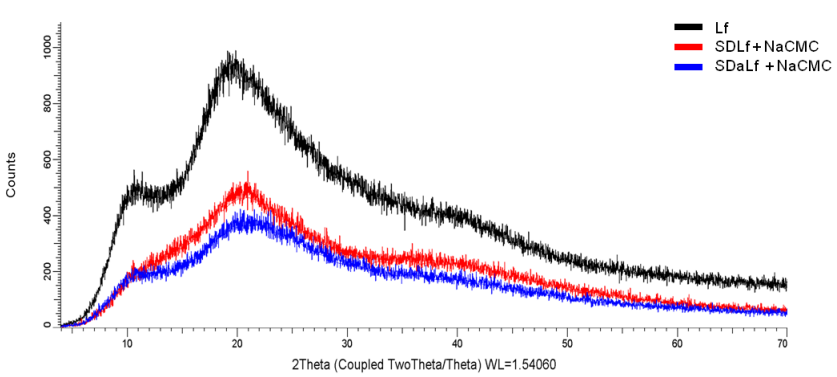


Figure 4 -5: X-ray powder diffraction patterns of various lactoferrin powder formulations. (a) shows representative XRPD patterns of the commercially supplied lactoferrin powders compared to spray-dried lactoferrin (SDLf) and spray-dried apo lactoferrin powders, (b) shows representative XRPD patterns of the commercially supplied lactoferrin powders compared to co-spray dried lactoferrin-NaCMC and co-spray dried apo lactoferrin-NaCMC powders. All the spray-dried powders have a broad halo and lack resolved reflections indicating that they are all amorphous. The results presented are a representation of repeats from three batches of powder.

All the diffractograms illustrated in Figure 4 -5 were all characterised of two broad humps between 4° 2θ and 50° 2θ and had no resolved reflection.

4.6.8 Thermal analysis

The thermal properties of the various lactoferrin formulations are shown in figure 4 -6. The DSC scans of the various spray-dried drug was characterised by abnormal heat transitions indicative of the presence of some amorphous content

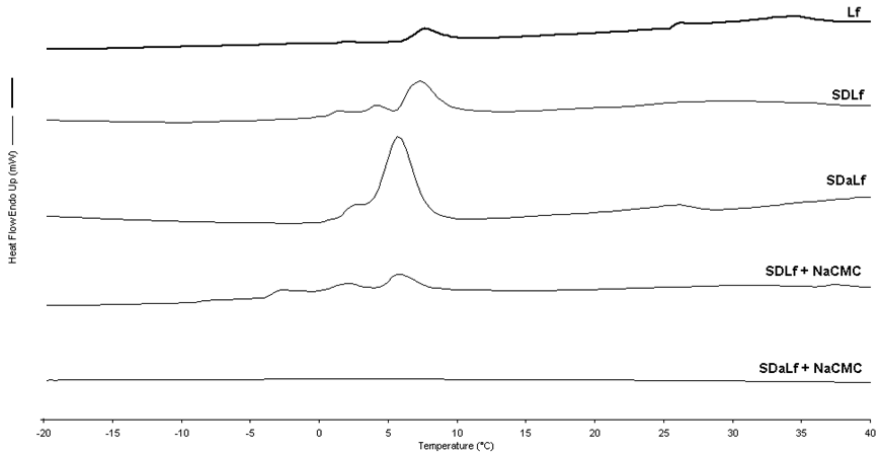


Figure 4 -6: DSC thermograms of the various protein formulations. The scans show the thermal events of the various powder samples when heated from -30 °C to 200 °C, while the thermograms were mostly uneventful, Lf, SDLf, SDaLf and SDLf + NaCMC had single or multiple broad peaks generally between -5 °C and 10 °C, SDaLf + NaCMC had no peaks at all. The absence of melting point peaks indicated that the powders were amorphous.

The thermograms obtained from DSC analysis of Lf showed that protein had no peaks except for broad peaks between 5 °C and 10 °C. Similarly, the thermograms obtained from DSC analysis of SDLf showed no melting point peaks except for three broad peaks

between 0 °C and 10 °C and the thermograms obtained from DSC analysis of SDaLf also showed no melting point peaks but had two broad superimposed peaks between 0 °C and 10 °C. Furthermore, while the thermograms obtained from DSC analysis of SDLf + NaCMC showed no melting point peaks but had three broad peaks between -5 °C and 10 °C, the thermograms obtained from DSC analysis of SDaLf + NaCMC showed no peaks at all.

4.6.10 *In vitro* aerodynamic assessment

Besides particle size, density and morphology of a drug powder, the de-agglomeration characteristics of the powder in the air stream is also a very important performance characteristic for effective aerosol deposition in the airways. This is because in order to separate particles, specific forces of interaction (for example, mechanical interlocking, capillary forces, electrostatic forces and van der Waals forces) must be overcome. Therefore it is pertinent to assess the performance of the powder formulations in order to enable the prediction of the aerodynamic properties of the various powder formulations. Results for the aerodynamic behaviour of various lactoferrin powder formulations as analyzed with the MSLI are presented in the following sub-sections.

4.6.11 Validating the protein quantitation assay

In order to ensure the validity of the assay method and the calibration graph employed in estimating protein deposition on the various stages of the MSLI following aerosolisation, some validation parameters were determined including; linearity, precision, limit of detection and limit of quantitation. The specificity of the assay to quantify proteins in the presence of sodium carboxymethylcellulose was assessed and found not to be any

different from the calibration plot in figure 4 -7. In other words, the presence of NaCMC in the formulations did not produce any significant change in the absorbance values

The calibration curves prepared on different days were linear (> 0.99) within the measured range of 5 – 2000 µg/mL. The assay method showed good reproducibility with similar inter-day results (day 1: y = 0.0011x + 0.0248; day 2: y = 0.0011x + 0.0269; day 3: y = 0.0011x + 0.0071). Figure 4 -7 shows the mean calibration plot of absorbance against concentration of triplicate values obtained on three different days. The equation of the line of best fit (y = 0.0011x + 0.0194) was used to estimate the protein concentration of the various lactoferrin formulations as well as the nominal dose depositing on each of the MSLI stages.

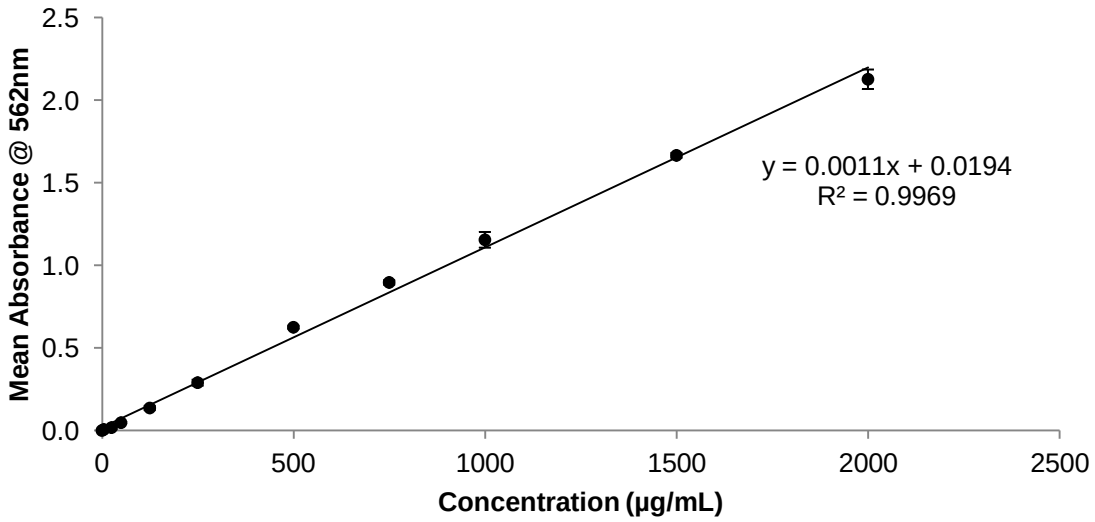


Figure 4 -7: Linear regression plot of absorbance against standard BSA concentrations. The mean absorbance calculated from calibrations obtained on 3 different days are plotted against nominal protein concentration values (n = 3). Error bars representing standard deviation are displayed for each point but are mostly within the limit of the symbols.

4.6.11.1 *In vitro* deposition properties of the various lactoferrin powders

The various powders were formulated as DPI and aerosolized at a flow rate of 60 L/min into the MSLI for 2 x 5s. The deposition profile of the various protein powders in all the stages of the MSLI is shown in figure 4 -8.

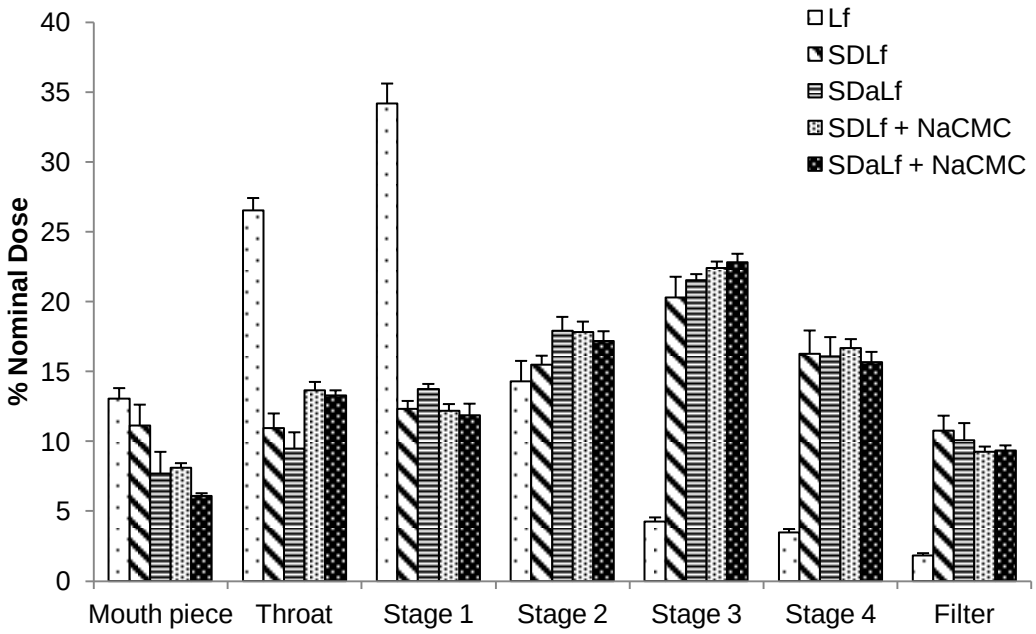


Figure 4 -8: Influence of spray drying on the aerodynamic characteristic of various lactoferrin formulations; Lf – commercially supplied lactoferrin, SD Lf – spray-dried lactoferrin, SD Apo Lf – spray-dried apo lactoferrin and co-spray dried lactoferrin with sodium carboxymethylcellulose. The nominal dose of protein depositing on each stage was assessed using a BCA protein assay. The data shown is a mean ± SD of three individual batches measured three times each.

Following aerosolisation of 30 mg of Lf from the cyclohaler, about 13 % of the protein was retained in both the capsule and the DPI device (mouth piece), ~ 27 % deposited in the throat of the MSLI, 34 % deposited on stage 1, 14 % deposited on stage 2 and about 10 % deposited on stages 3 through to the filter (particle sizes < 6.8 μm). In comparison,

Upon aerosolisation of 30 mg of SDLf, about 11 % of the protein was retained in both the capsule and the DPI device, 11 % of the protein deposited in the throat, 12 % of the protein deposited in stage 1, 16 % of the protein deposited on stages 2 and 47 % deposited on stages 3 through to the filter. Aerosolisation of 30 mg of SDaLf resulted in the retention of about 8 % of the protein in the device, while 10 % of the SDaLf protein deposited in the throat of the MSLI, 14 % deposited on stage 1, 18 % deposited on stages 2 and 48 % deposited on stages 3 through to the filter. Aerosolisation of 30 mg of the NaCMC spray-dried powders resulted in the retention of about 8% (SDLf + NaCMC) and 6 % (SDaLf + NaCMC) of the protein in both the capsule and the DPI device (mouth piece), while 14 % of the protein deposited in the throat, 12 % of the protein deposited on stage 1, about 17% of the protein deposited on stage 2 and ~ 48 % of the NaCMC modified protein deposited on stage 3 through to the filter.

4.6.11.2 Fine particle fractions of the various aerosolized powder formulations

The amount of lactoferrin depositing on all the stages of the MSLI varied for the different formulations. The commercially supplied lactoferrin powders showed poor protein mass deposition below stage 3 (effective cut-off of 6.8 µm) of the MSLI making the % $FPF_{<6.8\mu m}$ ~ 9.8 %. However, the $FPF_{<6.8\mu m}$ of the various spray-dried lactoferrin formulations (Fig 4 -9) was greater than 48 % of the total recovered protein dose. NaCMC-free formulations compared to NaCMC-modified formulations were found to possess statistically equivalent FPF.

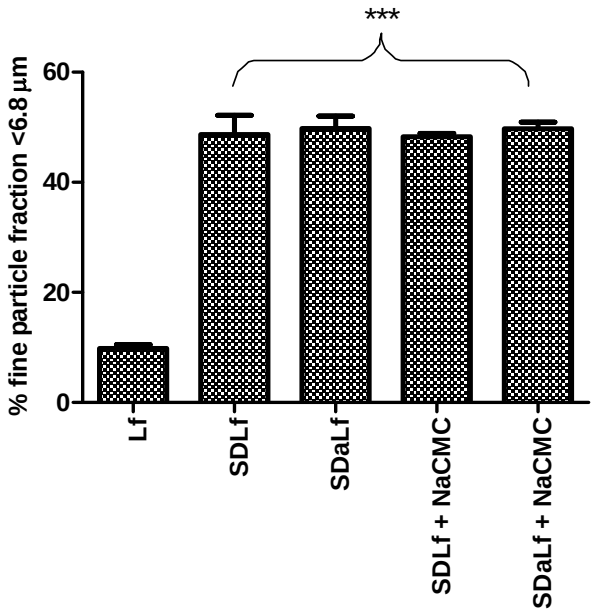


Figure 4 -9: Fine particle fraction < 6.8 µm of the various lactoferrin powders. Assessments were carried out using the MSLI and the protein content of each powder was corrected for each of the formulation and reported as a % of the nominal dose. Values are mean ± SD, n = 3. Comparisons of groups were made by one way analysis of variance using graphpad prism 5 (GraphPad Prism Software Inc., version 5.0) followed by Dunnett post-test; p values were considered significant and the *** represents p value < 0.001 between the various spray-dried formulations and the commercially supplied protein. All the spray-dried lactoferrin formulations demonstrated equivalent FPF values.

4.6.11.3 Mass median aerodynamic diameter of the various aerosolized powder formulations

The MMAD of the commercially supplied lactoferrin was found to be 33.6 µm, however upon spray drying, the MMAD of the various spray-dried powders was found to range between 4 µm and 6 µm. Statistically, while the MMAD of the spray dried formulations were significantly ($p < 0.001$) less than that of the commercially supplied lactoferrin,

there were no significant differences between the MMAD of the excipient free dry powder formulations and the sodium carboxymethylcellulose modified formulations.

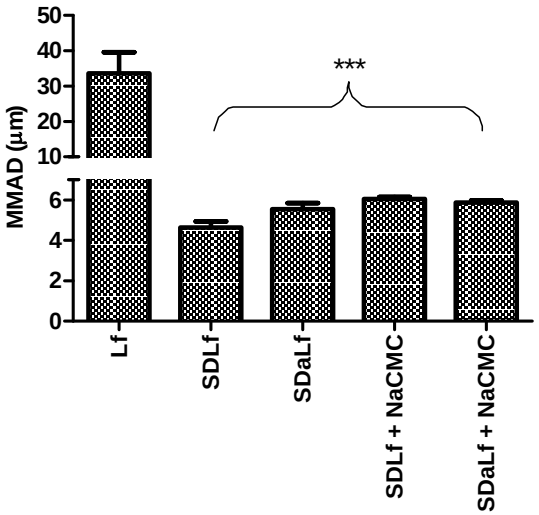


Figure 4 -10: Mass median aerodynamic diameter (MMAD) of commercially supplied (Lf), excipient-free (SDLf and SDaLf) and sodium carboxymethylcellulose modified (SDLf + NaCMC and SDaLf + NaCMC) spray-dried lactoferrin. Values are mean ±SD, n = 3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; *p* values were considered significant and the *** represents *p value* < 0.001 between Lf and the various spray dried DPI formulations. There are no significant differences between the excipient-free and the sodium carboxymethylcellulose modified spray-dried lactoferrin formulations.

4.7 Discussion

Research into employing inhaled therapeutics to manage respiratory infections usually begin with evaluating the physical characteristics of the powder formulations, not just because of the inherent advantages associated with a better understanding of the in-use performance of the formulations but also because it affords the opportunity to optimise inhaled formulations thereby increasing the delivery efficiency of the right amounts of active ingredient to the regions of the respiratory tract where they are most needed. In the present study, it was clearly demonstrated that spray drying could be a useful method that could be employed to produce powders with relatively good aerosolisation properties. To begin with, it was found that the iron content of bovine lactoferrin was significantly ($p < 0.001$) depleted by dialysis of the protein an observation that had been previously reported by Wakabayashi et al., (2009) although using a different dialysis method.

In comparison to the Wakabayashi et al., (2009) dialysis method (where apo lactoferrin was prepared through incubation of native bovine lactoferrin with in 0.1 M citric acid solution, followed by dialysis against distilled water) which was only able to reduce the iron saturation rate of native bovine lactoferrin from 8.1 % to 3.5%, atomic absorption measurements showed that the dialysis method employed in this study reduced the Fe^{3+} content from 2.8 ppm to 1 ppm (i.e. > 64% reduction). In addition, it was also found that following spray drying, the iron content of spray-dried apo lactoferrin powder formulation were not significantly different from the iron content of the apo lactoferrin preparations (Fig. 4 -3). To the best of our knowledge, this is the first time the iron content of spray-dried apo lactoferrin is being reported hence the values obtained could not be effectively compared in order to inform on the effect of the processing method employed.

Spray drying as one of the many micronisation techniques available, has proven to be a very useful method of generating micro-particles of proteins and peptides. In the present investigation, the spray drying parameters utilised were carefully chosen based on favourable results from previous pre-formulation studies (chapter three – developing a method for spray drying proteins). The yields of the process varied for the different protein formulations with NaCMC-free powders performing poorly (between 45 to 58 % yields) compared to the NaCMC-modified powders which provided much better yields of about 74%. In general incorporating NaCMC into the suspension feedstock, increased the yield of the resultant powder, this observation is in agreement with previous studies by Li and Seville, (2010) who found that co-spray dried BSA and NaCMC resulted in higher yield compared to spray drying BSA alone. The moisture content of the powders obtained from Karl Fischer showed that there was a significant ($p < 0.01$) increase in the moisture content of the spray-dried powders upon inclusion of NaCMC, this was also found to be in agreement with previous reports by Li and Seville, (2010) who reported that co-spray dried BSA powders containing 50 % (w/w) NaCMC had a moisture content of 7.8 ±0.8 %.

The particle size distributions of the various formulations obtained through laser diffraction measurements showed that the $d[v,50]$ of the powders were successfully reduced from 26 µm (commercially supplied lactoferrin powders) to < 5.5 µm, indicating that the spray drying parameters employed were duly suited for producing powders that avoid inertial impaction in the oropharyngeal cavity. Since larger particles would be expected to deposit in the upper airways due to the impaction of particles which are not able to follow the changes in the air stream, and excessively small particles are known to have a terminal velocity that is so low that they hardly deposit, the particle size of the powders produced through spray drying in this study would be expected to deposit in the lower regions of the airway due to sedimentation (i.e. over time, the velocity of the

particles would reduce to a point where they deposit under the influence of gravity). This prediction is premised upon previous reviews by Newman and Clarke (1983), Gonda (1990) and studies by Zanen *et al*.,(1994), Zanen *et al*., (1995) Zanen *et al*., (1996) who reported that optimal particle size of aerosols intended for lower airway deposition to be between 1 and 5 μm (specifically, particles with a MMAD of < 2.8 μm possessed significantly much lower airway deposition).

The span of the particle size distribution (calculated using equation 3.1) was found to be significantly higher upon co-spray drying the protein with NaCMC, this indicates that the inclusion of NaCMC must have facilitated the formation of widely heterogenous particles. This observation was found to be in agreement with previous reports by Li *et al.,* (2010) who found that NaCMC-modified powders generated through spray drying exhibited a broad particle size distribution upon measuring the particle sizes of the formulations in both wet and dry environments. In addition, investigations into the morphology of the powders using scanning electron micrographs also revealed that while the various protein powders consisted of spherical particles, these particles were of a heterogenous size distribution (Fig. 4 -3b). The particle morphology which have been known to affect particle packing in an agglomerate as well as in determining inter-particulate forces (i.e. the flowability and powder emptying from the powder inhaler) were observed to vary in the different powder formulations. In terms of roughness, the following order describes the roughness of the particles; SDaLf > SDLf > SDLf + NaCMC >> Lf.

Following aerosolisation of dry powders, out of the five possible mechanisms (inertial impaction, sedimentation, brownian diffusion, interception and electrostatic precipitation) by which particles deposit, inertial impaction and sedimentation were more likely found

to influence the deposition profile of the various protein formulations on to the different MSLI stages (this is evidence by the significantly high deposition of the powders (especially the commercially supplied Lf powders) on the throat, first and second stages of the MSLI). While impaction means that the particles are not able to follow changes of the air stream and therefore deposit on the upper stages of the MSLI (i.e. the throat, stage 1 and stage 2), sedimentation is a time dependent variable related to velocity which influenced by gravity causes particles to fall. In order words, these two variables cause large, irregular shaped particles to deposit on the upper MSLI stages representing the upper airways, whereas, smaller more spherical particles escape impaction and deposit in the lower MSLI stages representing the lower regions of the airway. FPF values that are significantly higher than 30% indicates that the powder has good aerosolisation behaviour (Heng *et al*., 2012).

In this study, *in vitro* deposition and aerosolisation studies of the various protein formulations showed that DPI formulations of the commercially supplied lactoferrin (Lf) had very poor aerodynamic properties with FPF of 9.8 % at 60L/min (an airflow rate considered to be achievable with minimal inhalation effort). In comparison, all the various spray-dried DPI formulations possessed a fairly good delivery efficiency with a FPF of approximately 50 % (Fig. 4 -9) indicating that the powders were not particularly cohesive, this observations also further validated the particle sizing data (Table 4.3, Figure 4.6.11.3) which showed the commercially supplied lactoferrin powders to possess a VMD of 27.89 ±0.4 µm and a MMAD of 33.61 ±6 µm while the spray-dried proteins had a VMD of between 5 and 9 µm and a MMAD of between 4 and 6 µm. Therefore, this study has demonstrated that spray drying using the B290 spray dryer is a useful formulation platform for facilitating the delivery of antimicrobial protein like lactoferrin to specific regions of the airway for therapeutic applications. In addition the

approach also showed the simplicity of preparing inhalable particles with a controlled size distribution.

An important drawback of current aerosol antibiotic treatments in their inability to penetrate into the deep lung, thereby setting up a concentration gradient (i.e. higher concentrations in the proximal airway but extremely low levels in the distal airway), which might lead to antibiotic resistance (Rubin, 2006). There is considerable evidence to suggest that antibiotic concentrations below the MIC, at sub-inhibitory levels, select and enrich for resistant bacteria (Gullberg *et al*., 2011). Therefore, combination therapies of antimicrobial protein like lactoferrin (which have an antibiofilm activity) and potent antibiotics produced through co-spray drying should provide a more robust antimicrobial therapy with adequate penetration potential. The FPF in this work represents a single cut-off (mass percent of particles with aerodynamic diameter < 6.8 µm) and is represented by the total amount of particles depositing from stage 3 down to the filter in the *in vitro* deposition graphs (Fig 4 -7). The FPF of a co-formulated DPI therapy prepared using the same spray drying parameters should also produce aerodynamic particle size distribution and respirable fractions similar to those observed in the present study.

4.8 Conclusion

Dry powder formulations of lactoferrin were prepared through spray drying and consisted of respirable-sized particles ($d[v,50]$ of between 4.8 and 5.5 µm) capable of achieving a FPF of approximately 50 %. In addition, a concomitant and uniform *in vitro* deposition profile could be achieved across all impaction stages when dispersed at 60 L/min. Spray-dried powders of lactoferrin may therefore offer an alternative antimicrobial therapy for control of airway infections. The next logical step to advance the current study would be to assess the *in vitro* antimicrobial activities of the spray-dried lactoferrin

formulations in order to determine the protein remained active on microorganisms following processing.

Chapter Five

Antimicrobial activity of spray-dried lactoferrin, spray-dried apo lactoferrin on planktonic and biofilms of *Pseudomonas aeruginosa*

5.1 Summary

The data presented in the previous chapter showed that spray drying lactoferrin produces microparticulates with good aerosolisation characteristics. In this chapter, we investigate the antimicrobial functionality of the various spray-dried powders with the view of determining the influence of the processing conditions (particularly heating and excipient - NaCMC) on the activity of the protein. The ability of lactoferrin to inhibit the growth of *P. aeruginosa* strain PAO1 was used as a measure of antimicrobial functionality. Using the same dry powder production techniques as in the previous result chapters, the antimicrobial activities of various lactoferrin dry powder formulations were studied. In particular their bactericidal activity on the planktonic cells and their ability to modify biofilm formation and depletion were the primary indicators used in establishing their antimicrobial functionality. Also, to better understand the mechanism by which apo-lactoferrin is able to inhibit bacterial cell growth, the effect of chemical iron chelators on the growth of both planktonic and biofilm cultures of *P. aeruginosa* was assessed. Results obtained showed that significantly ($p < 0.01$) more planktonic bacterial cells were killed by apo lactoferrin than with the commercially supplied lactoferrin. Also the various lactoferrin preparations were found to inhibit the formation of *P. aeruginosa* biofilms, although, interestingly, this property was lost upon co-spray drying the protein with sodium carboxymethylcellulose. Overall, the spray drying process was found not to significantly affect the antimicrobial activity of the proteins. The anti-biofilm activities observed with the lactoferrin preparations was thought to be related to their iron chelating abilities, however, it was not possible to replicate this using some known synthetic iron chelators, suggesting that there may be more than one mechanism of action that confers anti-biofilm activity to lactoferrin. Taken together, these data indicate that aerosolized lactoferrin and apo-lactoferrin, prepared by the spray drying method, retains antimicrobial activity against either planktonic or biofilm cultures of *P. aeruginosa*. However, this was not observed when synthetic iron chelators were

employed, indicating that this anti-biofilm activity is not purely due to the iron-chelating abilities of the protein.

5.2 Introduction

Iron binding proteins play crucial roles in the maintenance of iron homeostasis in the lung by limiting the availability of free iron in the extracellular tissue fluid (Mateos *et al.*, 1998; Reid *et al.*, 2007a). One of the many consequences of limiting the available iron in the airways is that it serves to limit the growth of microbes that rely on iron for replication and respiration under both aerobic and anaerobic conditions (Bals, 2000). Iron restriction is also known to play a crucial role in moderating phenotypic changes, such as those responsible for multiplication and pathogenicity of many bacteria. In the absence of iron restriction, unregulated growth and infectivity of these bacteria may eventually overwhelm the host's immune defence systems (Van Asbeck and Verhoef, 1983; Ward *et al.*, 1996; Griffiths and Williams, 1999; Griffiths and Chart, 1999; Crosa, 1999)

Lactoferrin is an antimicrobial polypeptide and a very important component of the innate immunity. One of the many roles of lactoferrin is to sequester essential iron from microbes thereby limiting their growth. Lactoferrin, just like the other transferrin family members (including serotransferrin or siderophilin and membrane-associated melanotransferrin) are primarily iron binding proteins found in exocrine secretions such as; tears, saliva, intestinal and cervical mucus, mucosal surfaces and within the specific granules of polymorphonuclear leukocytes (Klein *et al.*, 1988; Koch and Hoiby, 1993; Andersen *et al.*, 2001; Travis *et al.*, 2001; Adlerova *et al.*, 2008). Milk is the most abundant source of lactoferrin, especially colostrum (the early milk) which contains about 7 g/L (Masson and Heremans, 1971). There is however a great variation in the concentration of lactoferrin in various human body fluids; 1µg/mL in blood, ~10 µg/mL in airway lavage fluid and 2 mg/mL in tears (Masson and Heremans, 1971; Brogan *et al.*, 1975; Raphael *et al.*, 1989; Thompson *et al.*, 1990).

5.2.1 Structure of lactoferrin

Lactoferrin is a monomeric, bilobal glycoprotein with a molecular weight of about 78 and 80 kDa for bovine milk and human breast milk, respectively. Human and bovine lactoferrin consist of 692 and 689 amino acids, respectively, with both homologues having two sections that share over 70 % similarity in overall amino acid sequence with 100 % identity in several stretches of 10 – 15 amino acids at the C terminus (Teng, 2002). The N- and C-lobe of human and bovine lactoferrin share 66 and 73 % sequence homology (Teng, 2002). Both bovine and human lactoferrin molecules have two metal-binding sites, each of which can reversibly bind two iron atoms (as Fe^{3+}), concomitantly with two bicarbonate anions (Williams, 1982; Metz-Boutigue *et al.*, 1984b; Anderson *et al.*, 1987). Some notable features of lactoferrin include synergism between cationic and anionic binding which greatly enhances its antimicrobial actions. Its ability to tightly bind to iron (Fe^{3+}), with a binding constant of approx. 10^{22} M, means that iron is not freely available for microbial utilization. However, the lactoferrin-bound iron is still available to the host *in vivo*, through binding to specific receptors such as intelectin-1, low density lipoprotein receptor-related protein 1 and low density lipoprotein receptor-related protein 2 (Cox *et al.*, 1979; Anderson *et al.*, 1987). Furthermore, despite the striking physicochemical similarities between lactoferrin and the transferrins, lactoferrin remarkably has a higher metal binding constant (K_1) compared to the transferrins – for example, the K_1 of bovine and human lactoferrin is approximately 35 and 260 times respectively greater than human transferrin (Aisen and Leibman, 1972). Another distinguishing feature of lactoferrin from transferrin is the ability of lactoferrin to retain iron at very low pH (pH 3-4, compared with pH 5-6 for transferrins) (Mazurier and Spik, 1980; Shimazaki *et al.*, 1993).

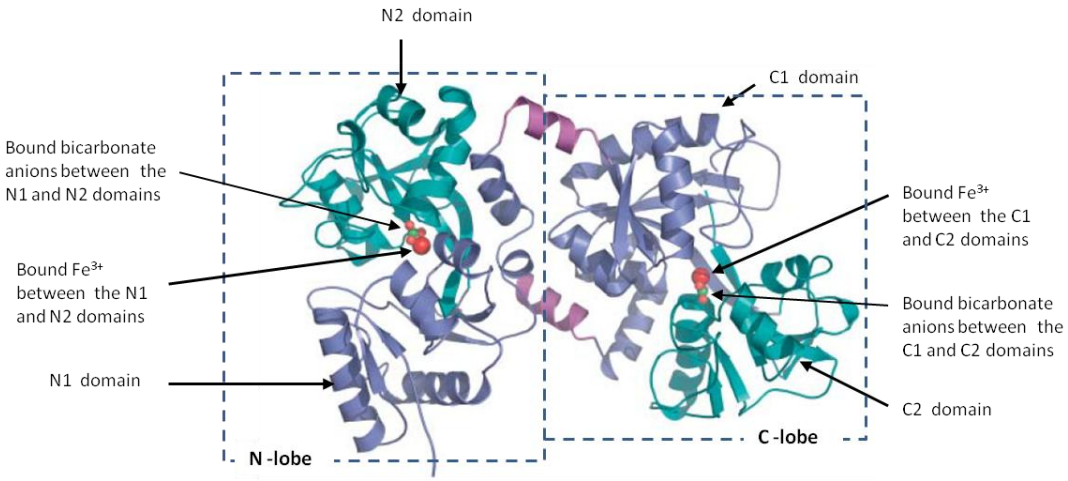


Figure 5 -1: The three dimensional structure of human lactoferrin. The two homologous lobes of the protein are shown within the dotted lines. The N-lobe is on the left, with its bound Fe^{3+} and bicarbonate anions (represented by the spherical-red coloured atoms) in the cleft between the two domains, N1 (dark blue) and N2 (teal). The C-lobe is on the right with similarly bound Fe^{3+} and bicarbonate anions depicted in between the two C domains; C1 (dark blue) and C2 (teal). The two lobes are joined by a three turn helix (magenta, top) with another helix making a final contact between the lobes at the C terminus (magenta, below). Adapted from Baker and Baker (2005b).

5.2.2 Antimicrobial activity of lactoferrin

The antimicrobial activity of lactoferrin is well established, the discovery that this activity is linked to the ability of the protein to sequester iron, thereby depriving potential pathogens of this essential nutrient, has generated a lot of interest amongst researchers (Singh *et al.*, 2002; Wakabayashi *et al.*, 2009; O'may *et al.*, 2009). Interestingly, lactoferrin has also been found to possess a secondary antimicrobial activity that is independent of iron-binding. This followed the discovery that areas of high positive charge on the N-terminal surface of lactoferrin permit binding to negatively charged bacterial membranes of bacteria (Ellison and Giehl, 1991; Leitch and Willcox, 1999a). The interaction with bacteria is favoured over the more neutral-charged host eukaryotic

membranes. Lactoferrin binding to bacterial membranes can disrupt and destabilise the regular membrane bilayer structure, thereby stopping the microbe from adhering to host cells (Vogel *et al*., 2002; Ward *et al*., 2002).

The ability of lactoferrin to inhibit bacterial growth *in vitro* was indeed one of the earliest functions described for the protein. The antimicrobial activity of lactoferrin has so far been demonstrated on a number of microbes. Apolactoferrin (i.e. the iron deplete form of lactoferrin) has been shown to be bactericidal against several different species of both gram-positive and gram negative bacteria including *Staphylococcus aureus*, *Porphyromonas gingivalis*, *Prerotella intermedia*, *P. aeruginosa, Streptococcus mutans* and *Vibrio cholerae*, but not for *Escherichia coli* (Arnold *et al.*, 1977b; Clarke and Foster, 2008; Musk and Hergenrother, 2008; Wakabayashi *et al.*, 2009). Studies have also shown that whilst lactoferrin is bactericidal in its iron-free, apolactoferrin state, iron-saturated lactoferrin also has reduced antimicrobial activity (Arnold *et al.*, 1980; Kalmar and Arnold, 1988; Yamauchi *et al.*, 1993), further implying a role for iron sequestration in the antimicrobial actions of the protein. However, apart from being able to deprive microorganisms of essential nutrient iron (Finkelstein *et al*., 1983), many studies have also suggested that lactoferrin binds to the isolated lipid A portion of lipopolysaccharide (LPS) (Appelmelk *et al*., 1994). LPS is a component of the outer membrane of Gram-negative bacteria and interactions between lactoferrin and lipid A damage the bacterial membrane and alters its permeability (Rossi *et al*., 2002). Lactoferrin has been shown to modulate the activity of known antibacterial agents such as lysozyme and antibiotics, so may work synergistically (Ellison Iii *et al.*, 1988; Ellison Iii and Giehl, 1991). Lactoferrin is also known to prevent *P. aeruginosa* biofilm formation by enhancing 'twitching motility'; a process made possible by the protein's iron chelation ability which prevents bacterial cells from forming the sessile structures that are a prerequisitie to biofilm formation (Rogan *et al*., 2004).

5.3 Aims and objectives

The aim of the current study was to better understand the value of lactoferrin as an antimicrobial agent following pharmaceutical processing (in this instance spray drying). The antimicrobial activities of lactoferrin against planktonic growth and biofilms of *P. aeruginosa* will be evaluated. In addition, in order to improve our understanding of the possible mechanism of action of lactoferrin (i.e. through iron chelation), we investigated the antimicrobial activities of a range of chemical iron chelators. The data from this study is likely to inform future searches for potent, inhalable antimicrobials that are effective against chronic *P. aeruginosa* infections and importantly that prevent or reduce biofilm formation in the airways.

5.4 Materials

Lactoferrin was kindly donated by DMV Fonterra (Netherlands), Mueller-Hinton Agar was supplied by Oxoid LTD (Basingstoke, UK), casamino acids were supplied by Becton Dickson (NJ, USA), Ferric chloride, 2,2' –dipyridyl (2DP), diethylenetriaminepentacetic acid (DTPA), ethylenediamine tetraacetic acid (EDTA), deferoxamine mesylate (DM), magnesium chloride, Kodak GBX developer, Kodak GBX fixer and Coomassie Brilliant blue R were all supplied by SIGMA-ALDRICH (Poole, UK). Immobilon® Western Enhanced Chemiluminescent substrate (ECL) were supplied by Millipore (Watford, UK), Pierce Silver stain kit were supplied by Thermo Scientific (Rockford, IL USA), PageRuler™ Plus, prestained protein ladder were supplied by Fermentas (York, UK) and primary antibodies (IG12, 4C6 and 4C3) were a Kind gift by Dr Andrew Devitt of Aston University.

Bacterial strain: *P. aeruginosa* PAO1 was kindly provided by Prof. Peter Lambert of Aston University, Birmingham, UK.

5.5 Methods

5.5.1 Medium and bacterial strains

P. aeruginosa strain PAO1 was routinely grown in 0.5% w/v casamino acid medium supplemented with 1 mM $MgCl_2$, and where specified 3 µM $FeCl_3$ and/or 0.2 % w/v glucose were also included. The various growth media were autoclaved and stored at room temperature until required. All incubations were carried out at 37 °C. Casamino acid medium with and without bacteria served as positive and negative controls, respectively.

5.5.2 Preparation of apo lactoferrin

Apo lactoferrin was prepared as previously described in section 2.2.2.2.4.

5.5.3 Spray-dried powder formulations

All batches of spray-dried lactoferrin used in this investigation were prepared as previously described in section 2.2.2.2.3 (for spray-dried lactoferrin), section 2.2.2.2.5 (for spray-dried apo lactoferrin), section 2.2.2.2.6 (for co-spray dried lactoferrin with sodium carboxymethltcellulose) and section 2.2.2.2.7 (for co-spray dried apo lactoferrin with sodium carboxymethltcellulose)

5.5.4 Minimum inhibitory concentration (MIC) determination

Susceptibility tests for each of the formulations was carried out on overnight planktonic cultures of *P. aeruginosa* according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for antimicrobial susceptibility testing. Briefly, the turbidity of an overnight bacterial suspension of *P. aeruginosa* was adjusted to that of a 0.5 McFarland standard (this should correspond to between 1 and 2 x 10^8 cfu/mL) then the suspension was diluted 1:10 in sterile distilled water. 10 µL of this inoculum was put into each well of a 96 well plate, making sure to set up positive (broth plus inoculum) and negative (broth only) control wells. 10 – 250 µg/mL of the various lactoferrin formulations were added into the appropriate wells and plates were incubated at 37°C for 18 hours. Following incubation, the MIC for each of the agents was determined as the lowest concentration that inhibited growth of the organism, this growth inhibition was apparent as a clear broth.

5.5.5 Minimum bacteriostatic concentration (MBC) determination

Whilst the MIC determines the lowest concentration of an antimicrobial that will inhibit visible growth of a microorganism, the MBC determines the lowest concentration of an antimicrobial that will prevent the growth of a microorganism (i.e. bactericidal concentration). To test for bactericidal activity of the various lactoferrin formulations against planktonic *P. aeruginosa*, a planktonic bacterial cell viability assay was performed based on the method of Xu *et al*. (2010). Briefly, 20 µL of 1:10 dilution of an overnight *P. aeruginosa* bacterial suspension was incubated at 37 °C with 180 µL of 100 µg/mL stock preparations of the various lactoferrin formulations or 180 µL of sterile distilled water (this served as a positive control). At intervals of 10 mins for up to 1 hour, 10 µL aliquots were plated out neat and serially diluted from 1:10 to $1:10^8$ in sterile, deionized water. Each dilution was plated on Mueller-Hinton agar and incubated at 37 °C for 24 hours. Following incubation, the number of colonies from each of the serial dilutions were counted and the dilutions having a colony count of < 300 colonies were used to estimate the colony forming unit per mL surviving at that time the sample was collected. The formula in section 2.2.15 was used for this determination.

5.5.6 Assessment of bacterial biofilm formation and reduction by various lactoferrin formulations

To test the ability of various lactoferrin dry powder formulations to modify biofilm formation by *P. aeruginosa* strain PAO1, a modification from methods previously described by Eckhart *et al*., (2002) and Merrit *et al*., (1998) was employed. Briefly, overnight cultures of *P. aeruginosa* were diluted 1:10 (v/v) in fresh medium containing 0.5 % w/v casamino acids, 0.2 % glucose and 1 mM $MgCl_2$ (casamino acid rich medium). 10 µL aliquots of the dilution were dispensed into a 96 well plate and 90 µL of a 1 mg/mL stock suspension of the various lactoferrin formulations were then added to

the bacterial suspension in the 96 well plate (to produce a final antimicrobial concentration of 900 µg/mL). The 96 well microplate was then covered and incubated at 37°C for 24 h, after which excess broth was removed. The biofilm, which remains adherent to the 96 well plate, was then washed and stained with 0.1% (w/v) crystal violet for 15 mins at room temperature. Following vigorous washing with water, the stained biofilms were solubilized in 30 % (v/v) acetic acid and the absorbance at 550 nm was determined using a Multiskan spectrum plate reader and 30% acetic acid in water as the blank.

To assess the effects of the various lactoferrin formulations on already formed biofilms (i.e. biofilm persistence) a slight modification was made to the above method. Briefly, 100 µL of a 1 in 10 dilution of an overnight bacterial suspension of *P. aeruginosa* was put into 96 well plates, covered with a lid and incubated at 37°C for 24 hours. Then the bacterial suspension was removed, leaving the preformed biofilm adherent to the plate, and 90 µL of a 1 mg/mL (i.e. antimicrobial concentration of 900 µg/mL) stock suspension of the various lactoferrin formulations were added and plates were incubated for a further 24 hours at 37 °C. The antimicrobial suspensions were then removed and the wells washed twice with distilled water before staining with 0.1 % w/v crystal violet solution. Detailed description of method employed in staining and quantifying biofilms is as previously described in section 2.2.17.2.

5.5.7 Assessment of bacterial biofilm formation and reduction by various synthetic chelators

Overnight cultures of *P. aeruginosa* bacterial suspension were diluted 1:10 in casamino acid rich medium supplemented with 0 or 3 µM $FeCl_3$. 10 µL aliquots of the bacterial dilution were put into 96 well micro-plate wells and 90 µL of 2 mM stock preparation of various iron chelators was added to the wells to give a final chelator concentration of 1.8

mM. The 96 well microplates were then covered and incubated at 37 °C for either 6 or 24 hours. Following incubation, the bacterial and chelator suspension were removed and the plate washed twice (to ensure complete removal of planktonic bacterial cells). Detailed description of the method employed in staining and quantifying biofilms is previously described in section 2.2.17.2.

To determine the effects of the various chelators on already formed biofilms (persistent biofilms), a slight modification was made to the method by growing the biofilm first then exposing it to the various chelators. Briefly, the biofilms were grown by incubating 100 µL of a 1:10 dilution of an overnight grown bacterial cell suspension at 37 °C for 24 hours. Then the bacterial cell suspension was removed and 90 µL of a 2 mM stock preparation of the various chelators were put into each of the wells and incubated at 37 °C for 24 hours. The method previously described in section 2.2.17.2 was employed in staining and quantifying biofilms.

5.5.8 SDS-PAGE analysis of the various lactoferrin formulations

SDS-PAGE was carried out using a 4 – 20 % precast polyacrylamide gel following the procedure described by Laemmli (1970) and detailed in section 2.2.9. The electrophoresis procedure was repeated 3 times using the Mini-Protean® TGX™ (Bio-Rad, Hercules, CA, USA). Two of the gels were used to investigate the molecular weight of the various protein preparations, while one of the detection methods used Coomassie brilliant blue R-250 (detailed method is described in section 2.2.9.1.1), the other applied the more accurate technique of silver staining (detailed method is described in section 2.2.9.1.2). The third gel was used to perform western blot analysis. A lane of prestained

molecular weight marker was included in each gel to allow determination of the apparent molecular weight of the lactoferrin in each preparation.

5.5.9 Western blot analysis of the various lactoferrin formulations

Western blot was performed as previously described in section 2.2.10. Briefly, after separation by SDS-PAGE, proteins were transferred to nitrocellulose membrane. Membranes were blocked for 1 hour using a solution of 0.3 % (v/v) Tween 20 in PBS. The blots were washed twice for 1 min with wash buffer – 0.05 % (v/v) Tween 20 in PBS, and incubated for 2 h at room temperature with a 1:500 (v/v) dilution of the primary antibody (IG12 and 4C6). After the incubation with the primary antibody, membranes were washed every 15 mins for 1 hour with 0.05 % (v/v) Tween 20 and then incubated for 2 hours at room temperature with 1:5000 dilution of secondary antibody (HRP-labelled rabbit anti-mouse). After incubation with the secondary antibody, membranes were washed again every 15 mins for 1 hour with the wash buffer. Finally, developing and detection of bound antibody was achieved with chemiluminescence (Kricka, 1996). Briefly, the membranes were incubated with Immobilon® Western enhanced chemiluminescent substrate according to the manufacturer's instructions (Millipore, Watford, UK) and exposed to Kodak film (Sigma-Aldrich, Poole, UK) for 1 minute. The films were then developed with Kodak GBX developer and fixed in Kodak GBX fixer.

5.5.10 Statistical analysis

Means and standard deviation were calculated using GraphPad prism 5. A Dunnett post-test analysis was used to examine the significant differences between the various formulations and the relevant controls.

5.6 Results

5.6.1 Minimum inhibitory concentration (MIC) of lactoferrin preparations on planktonic *P. aeruginosa*

The MIC's of Lf, aLf, SDLf, SDaLf, SDLf + NaCMC and SDaLf + NaCMC against *P. aeruginosa* were determined via the microdilution method. MIC values are summarized in Table 5.1. SDLf + NaCMC and SDaLf + NaCMC were found to have little antimicrobial effect on the bacteria at concentrations up to 2 mg/mL.

Table 5.1: Minimum inhibitory concentration (MIC) of the various lactoferrin formulations. The MIC of each drug was assessed by a microdilution method derived from clinical and laboratory standard institute (CLSI) for broth microdilution antimicrobial susceptibility testing. The MIC of the protein ranged from 1 to 2000 µg/mL. The minimum inhibitory concentration (MIC) was defined as lowest concentration which inhibited visible growth. While the the MIC of Lf and SDLf were the same, SDaLF had a reduced MIC compared to aLf.

Formulation	MIC (µg/mL)
Lf	50
SDLf	50
aLf	10
SDaLf	25
SDLf + NaCMC	>2000
SDaLf + NaCMC	>2000
NaCMC	>2000

5.6.2 Effects of Lf, aLf, SDLf, SDaLf, SDLf + NaCMC and SDaLf + NaCMC on planktonic *P. aeruginosa*

In order to determine the activity of the various lactoferrin preparations against planktonic *P. aeruginosa*, bacterial cultures were grown in the presence or absence of

the various lactoferrin dry powders. Approximately 10^8 cfu/mL of *P. aeruginosa* was incubated with 100 µg/mL of various lactoferrin preparations (prepared in casamino acid media) for 1 h, the bacterial growth was monitored by plating out a 10 µL aliquot every 10 mins. Bacterial growth in the control (no antimicrobial added) was not significant at the end of the 1 h assessment period. In contrast, incubation of the bacteria with Lf or SDLf resulted in a 2 log reduction of bacterial colonies compared to the number of colonies present at the start of the investigation. However, aLf and SDaLf reduced the *P. aeruginosa* bacterial colonies present at the beginning of the investigation by 5 log units (Fig. 5 -2).

Since the inclusion of sodium carboxymethylcellulose demonstrated an appreciable thermoprotective function on the activity of alkaline phosphatase in an earlier study (Table 3.2), we decided to co-spray dry the water soluble polymer with lactoferrin to see if this effect can be replicated with a more therapeutically relevant protein. Incubation of sodium carboxymethylcellulose with planktonic *P. aeruginosa* cells resulted in ca. 2 log unit growth in number of *P. aeruginosa* colonies after 1 h incubation compared to the number of colonies present at the start of the investigation. However, upon incubation of *P. aeruginosa* with SDaLf + NaCMC and SDLf + NaCMC, the number of bacterial colonies was suppressed by 1 log unit but this was still less efficacious compared to Lf, SDLf, aLf and SDaLF (Fig. 5 -2). A ranking order of efficacy of the of the powders against *P. aeruginosa* planktonic growth was – aLf > SDaLf > Lf> SDLf > SDLf+CMC = SDaLF+CMC > Control >> CMC.

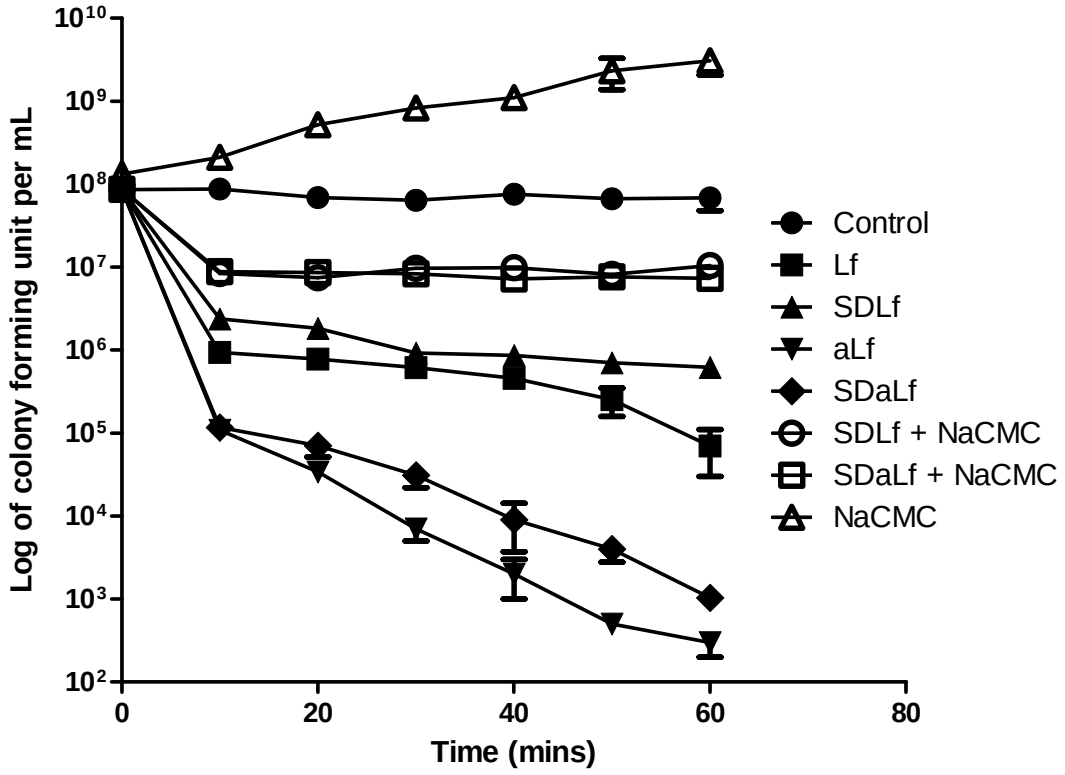


Figure 5 -2: Time-kill curves for various lactoferrin formulations. Logarithmic plot of the changes in bacterial cell viability in the presence of different lactoferrin preparations and sodium carboxymethylcellulose. The bacteria were seeded in polystyrene 96-well plates with the various antimicrobial protein preparation present at a concentration of 100 µg/mL and incubated at 37 °C. Aliquots were plated out onto MHA agar plates at 10, 20, 30, 40, 50, 60 mins. Bacterial colonies were counted after 24 hours incubation of the MHA agar plates at 37 °C and data are presented as mean ± SD; n = 3 independent evaluations of triplicate wells. While the log colony forming unit per mL of the control wells remained relatively unchanged, after 1 hour incubation, the number of viable cells began to reduce in the presence of the various antimicrobial agents. Iron depletion (aLf) increased the rate of viable cell reduction although spray drying iron deplete forms of lactoferrin (SDaLf) slightly reduces the impact of the antimicrobial protein on viable cells, in agreement with MIC data (Table 5.1). However, the inclusion of NaCMC into the various lactoferrin formulations significantly reduced the efficacy of the antimicrobial protein with bacterial colonies increasing significantly in the presence of NaCMC alone compared to the control.

Overall, these data suggests that the iron-depleted form of the protein, aLf, was the most effective antimicrobial form of lactoferrin (even after spray drying), displaying both growth inhibitory and bactericidal activity against *P. aeruginosa*. We therefore sought to examine the effects of chemical iron depletion on bacterial growth. We tested the effects of four synthetic iron chelators on the viability of planktonic bacterial cells of *P. aeruginosa* in an iron deplete environment to maximize iron sequestration from the organism. Approximately 10^8 cfu/mL of *P. aeruginosa* were incubated with the different chemical iron chelators (2 mM) in CAA media for 1 hour. Samples were plated out every 10 minutes and the cfu/mL determined following 24 hrs incubation of the plates (figure 5 -2). While the number of bacterial cells in the control (CAA media with no chelating agent added) remained fairly constant, incubation of the bacterial colonies in CAA media supplemented with EDTA resulted in a slight reduction (< 1 log unit) in the cfu/mL compared to the initial number of colonies. Incubating the bacterial cells in CAA supplemented with 2DP or DM resulted in less than 1 log reduction of the cfu/mL.

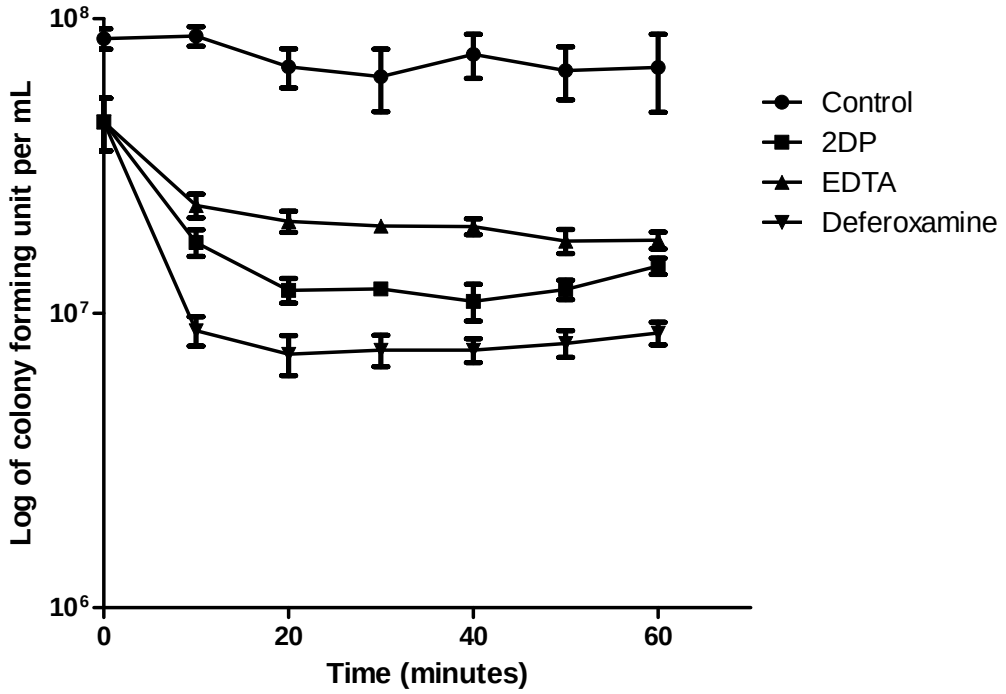


Figure 5 -3: Logarithmic plot of changes in bacterial cell viability following incubation with different synthetic iron chelators. Planktonic *P. aeruginosa* was seeded in polystyrene 96-well plates with an equal volume of the iron chelators at a final concentration of 2 mM and incubated at 37 °C for the times indicated. Aliquots were plated out onto MHA agar plates at 10, 20, 30, 40, 50, 60 mins. Bacterial colonies were counted after 24 hours at 37 °C and data are presented as mean ± SD; n = 3 independent evaluations of triplicate wells. There were no bacterial colonies to count following incubation with DTPA.

5.6.3 Inhibition of biofilm formation by Lf, SDLf, aLf, SDaLf, NaCMC, SDLF + NaCMC and SDaLf + NaCMC

We investigated whether Lf or aLf could modify the biofilm formation of *P. aeruginosa* and also, importantly, whether spray-drying the lactoferrin-related agents affected their biofilm-modifying abilities. *P. aeruginosa* cultures were incubated, in the presence or absence of 3μM Fe^{3+}, with the various Lf preparations for 24 hours then the planktonic

cells were removed and the quantity of adherent biofilm estimated by measuring the absorbance at 550 nm after crystal violet staining . Control experiments indicated the importance of free iron in the formation of the biofilm, as, significantly ($p < 0.001$) more bacterial biofilm was produced in iron-rich media, compared to iron-depleted media. This was expected as iron is known to be essential for *P. aeruginosa* biofilm formation (O'may *et al.*, 2009; Banin *et al.*, 2005; Yang *et al.*, 2007; Patriquin *et al.*, 2008). Lf significantly ($p < 0.001$) inhibited *P. aeruginosa* from forming biofilms in both the iron-rich and iron depleted environments. It is noteworthy to mention that there were no significant differences between the biofilm-inhibitory activities between Lf, aLf, SDLf and SDaLf. However, upon incubating *P. aeruginosa* with SDLf + NaCMC, there was a significant ($p < 0.05$) increase in the number of bacterial colonies in the iron-depleted media compared to the numbers seen with the control (where no agent was added). Upon incubating *P. aeruginosa* in CAA media supplemented with iron and SDLf + NaCMC, there was no significant change in the number of bacteria colonies compared with the control at the end of the 24 hr incubation period. Furthermore, there was no significant change in the number of bacterial colonies compared to the control when *P. aeruginosa* was incubated with SDaLf + NaCMC in either iron supplemented or iron free media. To better understand the reason for the reduced effectiveness of Lf in the presence of NaCMC, *P. aeruginosa* was incubated with sodium carboxymethylcellulose. Under these conditions, significantly ($p < 0.001$) more biofilm intensity was apparent in either the iron rich or iron-deplete environments compared with their respective controls (where no agent was added).

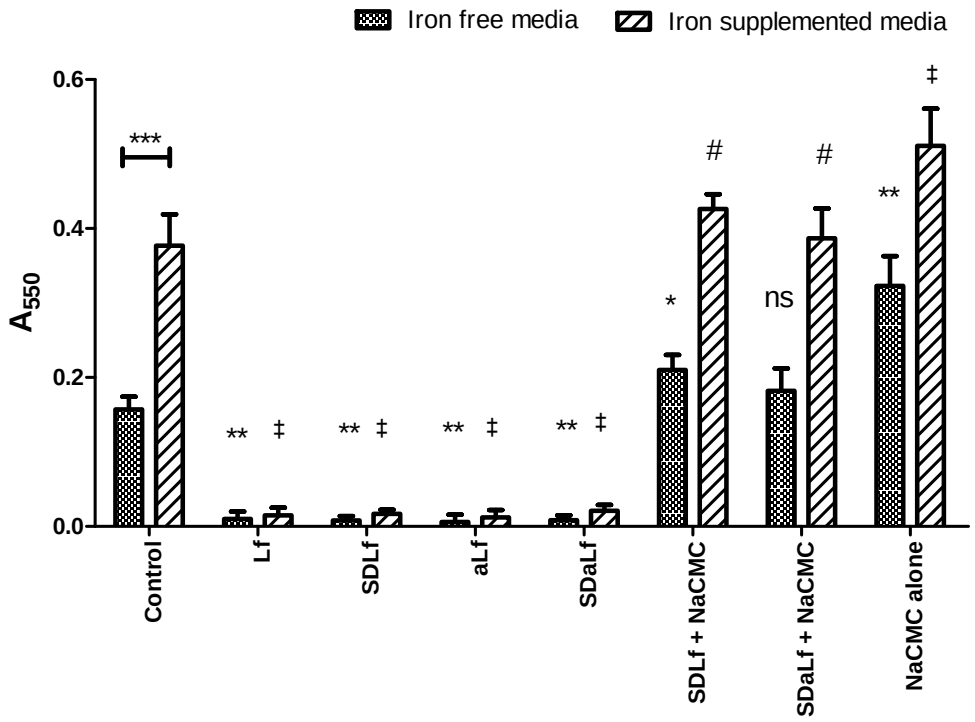


Figure 5 -4: Crystal violet staining of *P. aeruginosa* biofilms following 24 hr incubation with various lactoferrin-containing formulations, in the absence or presence of 3μm Fe^{3+}. The control had no antimicrobial agents included and indicates that Fe^{3+} significantly enhanced biofilm formation. Lf, SDLf, aLf and SDaLf retarded the formation of biofilm by the organism while the co-spray dried lactoferrin/apo lactoferrin-sodium carboxymethylcellulose formulation failed to prevent biofilm formation. Sodium carboxymethylcellulose alone encouraged more biofilm growth. Values are mean ±SD, n=3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; P values were considered significant and the *** represents *p value* < 0.01 between the iron-free and iron-supplemented media; **, P < 0.001 compared with the result for the iron-free control; *, P < 0.05 compared with the result for the iron-free control; ‡, P < 0.001 compared with the result for the iron-supplemented control; ns indicates no significance compared to the iron-free control (no antibiotic added) and # indicates no significance compared to the iron-supplemented control.

5.6.4 Reduction of preformed biofilms by Lf, SDLf, aLf, SDaLf, NaCMC, SDLF + NaCMC and SDaLf + NaCMC

We estimated the abilities of the different Lf preparations to deplete the quantities of preformed *P. aeruginosa* biofilms. These were grown in either iron-free or iron-supplemented media for 24 hours to allow development prior to the addition of the Lf compounds. Following a further 24 hour incubation with the Lf preparations, planktonic bacteria were washed away and the adherent biofilm assessed with crystal violet staining. There was no significant difference between the preformed biofilm masses in both environments. Treatment with Lf and aLf reduced the intensity of preformed *P. aeruginosa* biofilm significantly ($p < 0.05$) in both the iron rich and iron free media. Incubation of the preformed *P. aeruginosa* biofilm with SDLf resulted in a significant ($p < 0.001$ for the iron free and $p < 0.005$) reduction of the biofilm intensity. Treatment with SDaLf resulted in a significant reduction ($p < 0.05$ for the iron free and $p < 0.001$) of the biofilm intensity. However, incubation of preformed biofilms of *P. aeruginosa* with SDLf + NaCMC and SDaLf + NaCMC failed to reduce biofilm intensity in either the iron-deplete or iron-rich media but rather resulted in significantly ($p < 0.001$) increased biofilm intensity. Incubation with sodium carboxymethyl cellulose alone resulted in significant ($p < 0.001$) increases in preformed biofilm intensity in both iron rich and iron free medium.

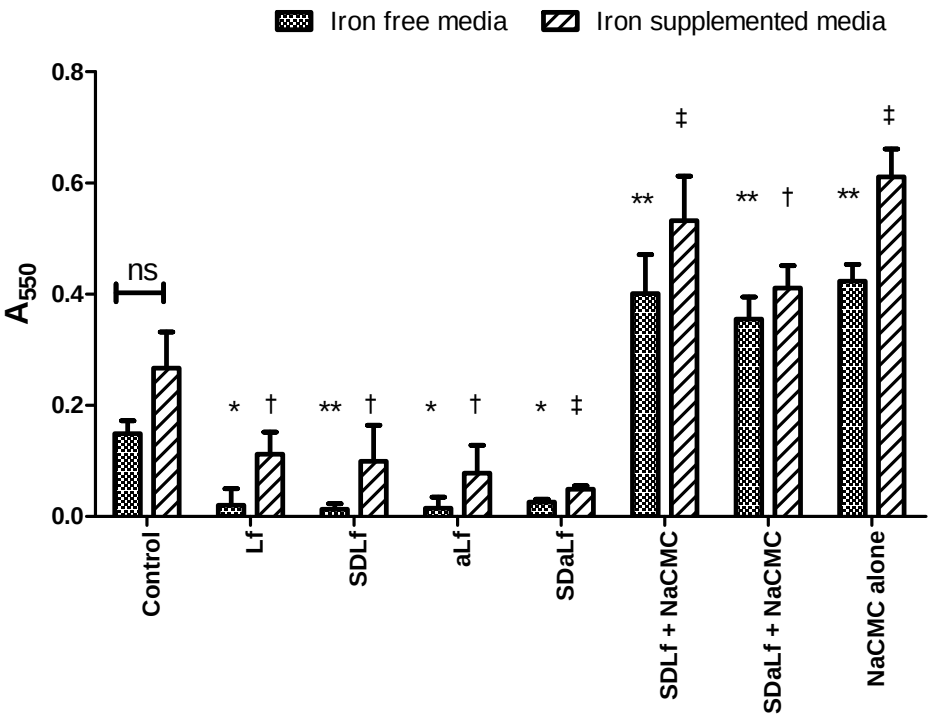


Figure 5 -5: Crystal violet staining of preformed *P. aeruginosa* biofilms following exposure of to various lactoferrin formulations. The control well had no agent added and served as a positive control. Commercially supplied lactoferrin (Lf) and spray-dried lactoferrin (SDLf), apo lactoferrin (aLf) and spray-dried apo lactoferrin (SDaLf) significantly reduced the intensity of staining of biofilm while the co-spray dried lactoferrin/apo lactoferrin-sodium carboxymethylcellulose formulation failed to reduce the intensity of preformed biofilms of *P. aeruginosa*. Sodium carboxymethylcellulose alone encouraged more biofilm growth when compared to the control. Values are mean ±SD, n=3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; 'ns' indicates no significant difference in the preformed biofilm masses under either iron-free or iron-supplemented media; **, $P < 0.001$ compared with the result for the growth of P. aeruginosa in the iron-free control; *, $P < 0.05$ compared with the result for the Iron-free control; and ‡, $P < 0.001$ compared with the result for the iron-supplemented control; †, $P < 0.05$ compared with the result for the iron-supplemented control.

5.6.5 Biofilm formation in the presence of iron chelators

In order to examine the contributions of iron chelation in preventing biofilm formation by *P.aeruginosa*, we incubated the organism with various iron chelators (at a chelator concentration of 2 mM) in an iron free media. Crystal violet staining was used to measure the intensity of biofilm formed in the presence of the various chelators after 6 hrs and 24 hrs (Figure 5 -6). Upon incubation of *P. aeruginosa* with either 2DP, DTPA or EDTA, there were no significant changes in terms of biofilm intensity compared with the control (no agent added) by 6 h post incubation, however, deferoxamine caused a significant ($p < 0.001$) increase in *P. aeruginosa* biofilm intensity. Although, at the end of the assay (24 h post incubation with *P. aeruginosa*), all the chelators had reduced biofilm formation to levels that were significantly ($p < 0.001$) less than the control, they were still not as effective as Lf, aLf, SDLf and SDaLf in reducing biofilm intensity (fig 5 -5).

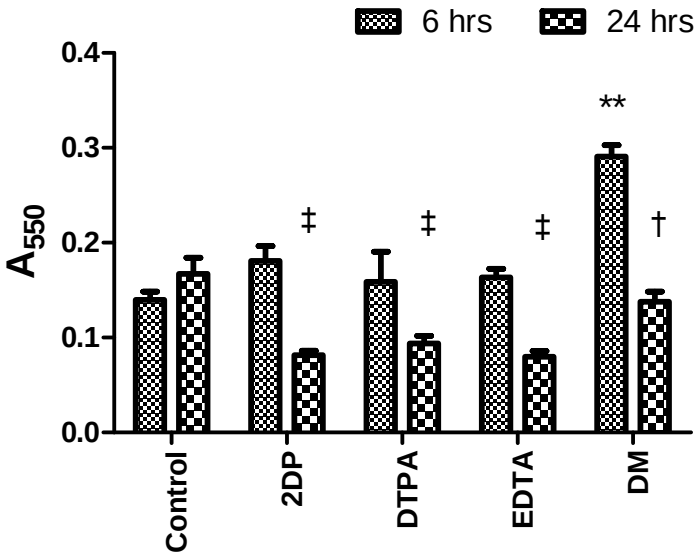


Figure 5 –6: Crystal violet staining of biofilms of PAO1 following 6 and 24 hr incubation with various synthetic iron chelators (chelator concentration of 2 mM prepared in iron-free CAA media). The control contained bacteria in the absence of any chelator. 2,2' –dipyridyl (2DP), diethylenetriaminepentacetic acid (DTPA), ethylenediamine tetraacetic acid (EDTA), and failed to retard biofilm formation following 6 hrs of incubation whilst deferoxamine mesylate DM significantly ($P < 0.001$) increased the biofilm mass following 6 hrs incubation. However, there was significant reduction in biofilm intensity after 2h compared with the control. This was true for all the iron chelators. Values are mean ±SD, n=3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; **, $P < 0.001$ compared with the result for the control 6 h post incubation; †, $P < 0.05$ compared with the result for the control 24 h post incubation and ‡, $P < 0.001$ compared with the result for the control 24 h post incubation.

Since the iron chelators were able to alter biofilm formation after 24 hours incubation in iron-deplete medium, we sought to investigate whether this effect could be mirrored in iron-supplemented medium. *P. aeruginosa* was incubated with various iron chelators (at a chelator concentration of 2 mM) in an iron supplemented media containing 3 µM

$FeCl_3$. The extent of biofilm formation was determined by measuring crystal violet staining intensity after 24 h. The values for the growth of *P. aeruginosa* in the iron-free media are the same as the 24 h data in the previous experiment and has only been duplicated in figure 5 -6 for comparison purposes. Incubation of *P. aeruginosa* in CAA medium supplemented with 3 µM $FeCl_3$ and 2DP resulted in a significant ($p < 0.001$) decrease in biofilm mass compared to the iron supplemented control. Additionally, incubation of the gram negative bacteria with iron-supplemented medium and either DTPA or EDTA still resulted in a significant ($p < 0.05$) decrease in biofilm mass compared to the iron supplemented control. There was however no significant change in the biofilm mass post incubation of *P. aeruginosa* with CAA medium supplemented with 3 µM $FeCl_3$ and DM.

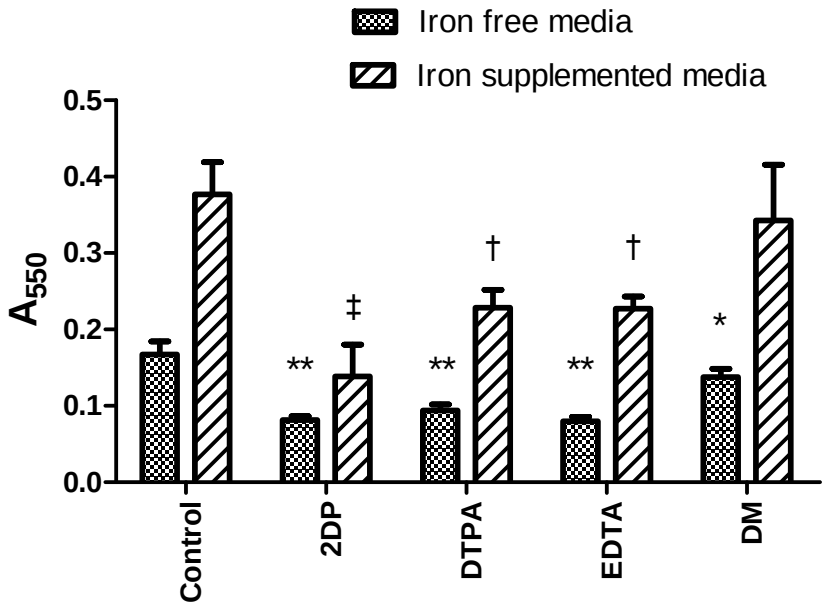


Figure 5 -7: *P. aeruginosa* biofilm formation in CAA medium supplemented with 3 µM $FeCl_3$ and 2 mM of various chemical iron chelators. The A_{550} values for growth of *P. aeruginosa* in iron-free medium are the same as the A_{550} values for the 24 h data in figure 5 -6. Whilst CAA medium supplemented with 3 µM $FeCl_3$ and 2DP showed the most significant reduction in biofilm intensity, DTPA and EDTA also significantly ($p < 0.05$), reduced biofilm intensity, whereas CAA medium supplemented with 3 µM $FeCl_3$ treated with DM resulted in no significant change in biofilm intensity compared to the control. Values are mean ±SD, n=3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; **, $P < 0.001$ compared with the result for *P. aeruginosa* growth in the iron-free control, †, $P < 0.05$ compared to the result for the iron-supplemented control; and ‡, $P < 0.001$ compared with the result for the iron-supplemented control.

5.6.6 Effect of chemical iron chelators on preformed *P. aeruginosa* biofilms in the presence of Fe^{3+}

The ability of four chemical chelators to disrupt preformed *P. aeruginosa* biofilms formed in iron-supplemented medium was investigated. These studies were carried out in order to better understand the contribution of iron sequestration in the ability of apo-lactoferrin (aLf) to disrupt preformed biofilms. To directly measure the contributions of chemical iron chelation on preformed *P. aeruginosa* biofilms, bacterial biofilms were grown in 96 well microtitre plates under iron restricted and iron supplemented conditions. There was no significant difference between the quantities of biofilms pre-formed in the iron-free media (CAA alone) and quantities of biofilms pre-formed in iron supplemented media (3 µM). When preformed biofilms produced under iron-supplementation, were exposed to the various chemical iron chelators, only EDTA produced a significant ($p < 0.05$) reduction in the quantity of biofilm compared to the control (i.e. where no chelator agent was added), DTPA and DM both produced significant ($p < 0.05$ and $p < 0.01$ respectively) increases in the amount of biofilm quantified when compared to the control while 2DP produced no significant change compared to the amount of pre-formed biofilms that were produced in the control wells. None of the iron chelators had any significant effects on preformed biofilms formed in iron-supplemented media compared to the relevant iron-supplemented control.

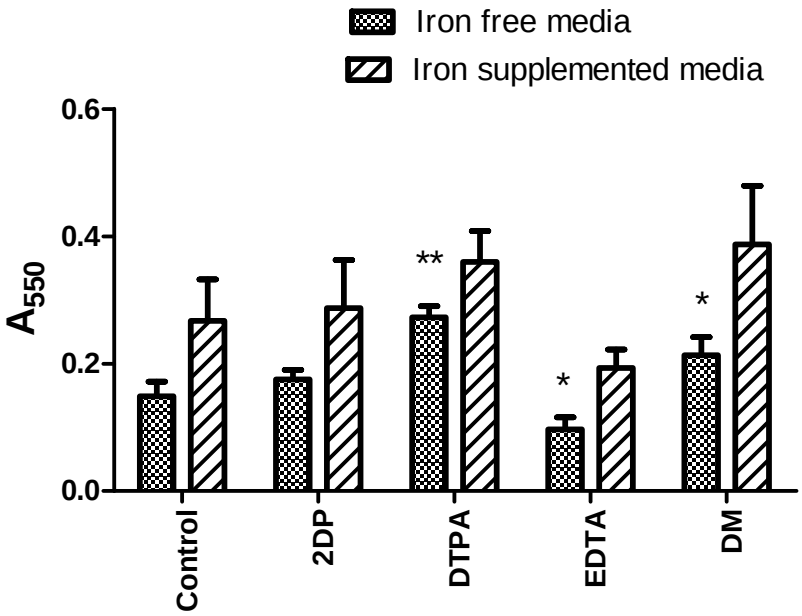


Figure 5 -8: Crystal violet staining of *P. aeruginosa* biofilms following exposure of preformed biofilms, produced in the presence or absence of Fe^{3+}, to various chemical chelators. The control wells had no agent added and served as a positive control. EDTA was the only chelator to significantly reduce the quantities of preformed biofilms grown from the iron-free media while there was no change in biofilm quantities when preformed *P. aeruginosa* biofilms were incubated with 2DP. DTPA and DM significantly increased biofilm quantities. When preformed biofilms grown from iron supplemented media were exposed to the various chemical iron chelators, there were no significant changes in the quantities of the preformed biofilms compared to the control. Values are mean ±SD, n=3. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; P values were considered significant and the symbol; **, $P < 0.001$ compared to preformed biofilm quantities obtained in the control wells of the iron-free media; and *, $P < 0.$ compared to preformed biofilm quantities obtained in the control wells of the iron-free media.

5.6.7 SDS-PAGE and Western blot analysis for confirmation of the identity of Lf, SDLf, aLf and SDaLf.

Fig 5 -9a shows the SDS-PAGE patterns of Lf, SDLf, aLf and SDaLf when the gels were stained with Coomassie Brilliant Blue. One major band was observed for all four preparations, and the molecular weight which was estimated from a linear plot of log of molecular weight against mobility of the protein band ($y = -0.1812x + 5.6499$, $r^2 > 0.9968$) was found to be 80 kDa - equivalent to the reported molecular weight of bovine lactoferrin (Wakabayashi *et al*., 2006). Fig 5 -9b shows the SDS-PAGE patterns of Lf, SDLf, aLf and SDaLf when the gels were silver stained. The molecular weight bands on the silver stained gels were more sensitive and were generally characterized by three molecular weight bands which were mostly observable in all four preparations; the first band which is the most intensive staining was estimated from a linear plot of log molecular weight against mobility ($y = -0.1822x + 5.6166$, $r2 > 0.9979$) to be 78 ±4 kDa (n = 3), the second band which was less broad is estimated to be 52 ±4 kDa (n = 3) and the third band which is even less broad (and almost absent in apo lactoferrin) is estimated to be 28 ±1 kDa.

Fig 5 -9c shows a representative example of Western blot analysis of Lf, SDLf, aLf and SDaLf. Lactoferrin bands correspond to the 80 kDa bands on the gel and are recognized by 4C6 and IG12 anti-bovine lactoferrin antibody.

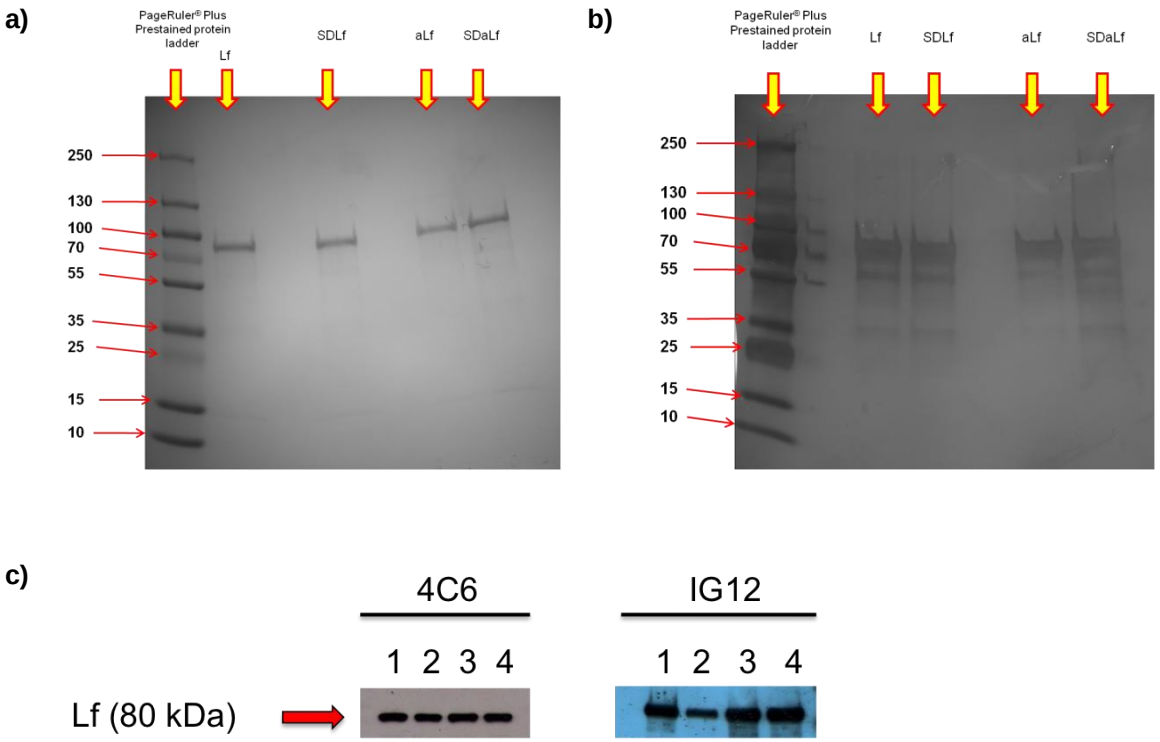


Figure 5 -9: Analysis of the apparent molecular weight of various lactoferrin formulations, before and after spray drying using SDS-PAGE (a and b) and Western blotting (c). 5 -9a- shows a representative image of a Coomassie-blue stained SDS gel. prestained protein ladder (lane 1), lactoferrin (lane 2), spray-dried lactoferrin (lane 3), apo lactoferrin (lane 4) and spray-dried apo lactoferrin (lane 5). 5 -9b shows a representative image of a Silver stained SDS gel. prestained protein ladder (lane 1), lactoferrin (lane 2), spray-dried lactoferrin (lane 3), apo lactoferrin (lane 4) and spray-dried apo lactoferrin (lane 5). For both gels, approximately 375 ng of each of denatured protein was loaded into each well and a constant current of 60 mA applied for 22 minutes. The molecular weight of the different proteins in the marker are shown in the left of the images. 5.9c shows a representative image of the specific detection of lactoferrin through Western blot analysis Lf (1), aLf (2), SDLf (3) and SDaLf (4).

5.7 Discussion

This study is the first analysis to demonstrate that spray-dried lactoferrin retains its antimicrobial activity against planktonic and biofilms of *P. aeruginosa*. It was found that following spray drying of the protein, the MIC remained unchanged, however, following spray drying of the iron-deplete form of the protein (aLf) the MICs of the protein was increased from 10 µg/mL to 25 µg/mL (Table 5.1). This indicates that spray drying the protein in its iron-free form (apo lactoferrin), reported by Anderson *et al.,* (1990) to exist in an open conformation compared to the closed conformation of the iron-bound form, may account for the observed detrimental effects to the antimicrobial activities of the protein. This observation however requires further investigation as there is currently no other evidence that shows that the three-dimensional structure of spray-dried apo lactoferrin is in any way different to the three-dimensional structure of apo lactoferrin.

The different time-kill studies showed that 100 µg/mL of Lf and aLf suppressed the growth of planktonic cultures of *P. aeruginosa*. However, findings in this study suggest that iron depletion of lactoferrin is essential to achieve high efficacy in bacterial killing. This observation was found to correspond with previous reports by Arnold *et al*., (1980) who previously reported that while 2.2×10^7 cfu/mL of *P. aeruginosa* survived incubation with 83 µM iron saturated lactoferrin, only 8.6×10^3 cfu/mL of *P. aeruginosa* survived incubation with 4.2 µM apo lactoferrin with no detectable growth at 42 µM and 83 µM. Most importantly, regardless of the iron content of the proteins, it was observed that the proteins remained active to a considerable degree following spray drying; a clear indication that the particle production technique was well suited for producing micronized antimicrobial proteins.

Furthermore, although lactoferrin and spray-dried lactoferrin elicited bactericidal activity against *P. aeruginosa* at MICs of between 10 – 50 µg/mL, upon co-spray lactoferrin/apo lactoferrin with NaCMC, the MICs were found to be > 2 mg/mL, therefore indicating that the antimicrobial activities of the protein on planktonic cultures of *P. aeruginosa* were subdued in the combination. This may probably have occurred due to protein-polymer interactions which although functioning to stabilize the protein, resulted in the significant reduction in the efficacy of the protein. Another important antimicrobial property of lactoferrin which was investigated is its antibiofilm activity against *P. aeruginosa.* Since the protein had been previously reported by Singh *et al*., (2002) to prevent *P. aeruginosa* biofilm formation by increasing the twitching motility of the bacteria, we therefore sought to assess the antibiofilm activity of the spray-dried proteins compared to the non spray-dried proteins. Results obtained indicate that the antibiofilm activities of SDLf and SDaLf were comparable to the antibiofilm activities of Lf and aLf respectively.

Furthermore, upon incubating the co-spray dried lactoferrin/apo lactoferrin with NaCMC formulations with *P. aeruginosa*, more biofilms were formed in both the iron-free and iron supplemented media, indicating that the antibiofilm effects of lactoferrin were lost. A possible explanation for this observation is that sodium carboxymethylcellulose provided an enabling environment for bacterial growth. A similar observation was also reported by Valappil *et al*., (2013) who found that following 24hrs of incubation *P. aeruginosa* and NaCMC had increased numbers of colony forming units (1.5 log units), but upon substituting the Na^{+} for Ga^{3+} in carboxymethylcellulose salts (of various molecular weights) there was a reduction in *P. aeruginosa* biofilm growth by 0.85 log units compared to the growth of the bacteria in NaCMC over the same 24 hour period.

Another antimicrobial property of the various lactoferrin formulations which was also assessed was the ability of the proteins to deplete already established biofilms. Tests were carried out on two different types of biofilms; structured biofilms (iron replete media) and flat biofilm (iron limited media) as previously shown by Banin *et al*., (2005) and Klausen *et al*., (2003). The results in this study showed that Lf, SDLf, aLf and SDaLf were more efficient than co-spray dried lactoferrin/apo lactoferrin with sodium carboxymethylcellulose and the various synthetic iron chelators at depleting both types of biofilms (figure 5 -5). The relevance of this finding is that biofilms developed by *P. aeruginosa* can be particulary susceptible to Lf and more importantly in this case spray-dried lactoferrin which have been optimized for lower airway delivery, where the presence of *P. aeruginosa* biofilms usually result in recurrent infections.

SDS-PAGE analysis which is a useful tool for identifying and differentiating proteins, showed that there was a very prominent band at about 80 kDa (Fig. 5 -11a and b, values were estimated from a calibration plot of log molecular weight against mobility of the individual protein bands), this was a verification that the spray drying process did not have detrimental effects on the molecular structure of the protein, therefore, making spray drying a viable option for preparing lactoferrin dry powders which are well suited for inhalation. Furthermore, the various staining procedures (Coomassie and silver staining) employed during SDS-PAGE analysis allowed for detailed comparisons of the molecular integrity of the different lactoferrin preparations (Lf and aLf) following spray drying. It was observed that the protein bands from the various lactoferrin formulations presented similar patterns before and after spray drying, therefore, indicating that the protein (lactoferrin) was not destroyed during spray drying. Western blot analysis confirmed the identity of the protein band at 80 kDa to be lactoferrin as this particular band was recognized by IG12 and 4C6 anti-bovine lactoferrin antibody for both spray-dried and non-spray-dried sources.

5.8 Conclusions

In this study, assessments of the antimicrobial activity of the various protein formulations revealed that spray-dried lactoferrin/apo lactoferrin formulations remained active against; planktonic *P. aeruginosa*, preventing formation of biofilms as well as reducing the quantities of matured (preformed) biofilms by *P. aeruginosa*. The antimicrobial activity of spray-dried protein was also found to possess greater efficacy when compared to synthetic iron chelators. Although the lactoferrin employed in this pilot study was from a bovine source (as human lactoferrin is strategically more difficult and expensive to obtain) and may elicit some immunological responses if delivered into humans, it basically should be seen as a proof of concept study and with prospect of delivering recombinant human lactoferrin-derived peptide (hLF1-11) which has a similar antimicrobial activity to lactoferrin. It has also been observed in this study that although NaCMC may have been a good thermoprotectant for the enzyme (alkaline phosphatase), it was however not the appropriate excipient of choice for formulating lactoferrin dry powders which are required to retain their antimicrobial activities following processing. Consequently, NaCMC would be excluded from subsequent studies and relevant antibiotics which improve the antimicrobial activities of the protein on *P. aeruginosa* through a combination therapy would be pursued. In the end, this study generally suggest that the delivery of active antimicrobial proteins to the airway of CF patients is possible and may potentially constitute a new strategy for managing *P. aeruginosa* infections of the airways.

Chapter Six

Co-formulation of lactoferrin with antibiotics

6.1 Summary

In the previous chapter the antimicrobial activities of various lactoferrin formulations against *P. aeruginosa* were studied. One of the key findings in that chapter was that, SD lactoferrin and apo lactoferrin gave a 2 log reduction in the number of surviving bacterial colonies after 60 minutes, despite a tendency for spray drying to reduce the activity of the antimicrobial proteins. In addition, the spray-dried preparations were effective at reducing the intensity of bacterial biofilms and the effects of iron-free apolactoferrin could not be mimicked by iron chelation alone. These data demonstrated that lactoferrin exhibits antimicrobial effects on *P. aeruginosa*. In this chapter, the effects of formulating a combination therapy of lactoferrin with currently prescribed antibiotics were studied. The selected antibiotics of choice for this assessment were tobramycin and gentamicin. These are both members of the aminoglycoside class of antibiotics and have activity against *P. aeruginosa*. Although the antimicrobial action of these two antibiotics have been extensively studied, to our knowledge, no study has investigated the potential of delivering a combination therapy of lactoferrin or the iron depleted lactoferrin (apo lactoferrin) with tobramycin/gentamicin to the airways for the purpose of preventing or eradicating *P. aeruginosa* especially in the context of managing this pathogen in cystic fibrosis. This chapter therefore focuses on co-spray drying each antibiotic with lactoferrin or apo-lactoferrin, investigating the physical characteristic of the spray-dried powders, assessing the antibacterial properties of the combinations on both planktonic cultures and biofilms of *P. aeruginosa* and measuring the penetration of spray-dried formulations through PAO1 biofilms. Results obtained indicate that antibacterial potency of tobramycin was significantly ($p < 0.01$) increased by co-formulating with lactoferrin or apo lactoferrin, and also the synergy obtained on combining apo lactoferrin with tobramycin was not replicable by co-formulating tobramycin with known chemical iron chelators. In conclusion, the spray drying process process produced amorphous, micron

sized dry powders suitable for lower airway deposition, as well as an active antimicrobial powder containing combinations of lactoferrin and aminoglycosides (tobramycin/gentamicin) which were observed to have a better activity than that of either lactoferrin or tobramycin/gentamicin alone on both planktonic and biofilm cultures of *P. aeruginosa*.

6.2 Introduction

Bacterial biofilms result when sessile unicellular organisms coalesce to form a surface attached community usually surrounded by an exopolysaccharide matrix – a major distinguishing feature between planktonic cells and biofilms (Costerton *et al.,* 1994). This sessile mode of growth is also called the biofilm mode of growth. Studies have found that the biofilm mode of growth is responsible for the exceptional ability of most bacteria to resist destruction by their host immune cells and antimicrobial. Cystic fibrosis is a classic example where the persistence of bacterial biofilms leads to respiratory failure, lung transplantation or death (Høiby and Pressler, 2006; Govan and Deretic, 1996).

In the United Kingdom, *P. aeruginosa* is the most frequent cause of chronic pulmonary infections – accounting for about 69 % of all bacterial infections in people with CF. The age specific prevalence of *P. aeruginosa* in pre-school children is 2.9 %, rising to 65.3 % for 28 to 31 year olds. Overall, available data suggests that chronic *P. aeruginosa* infections are more prevalent among adults (55.8 %) compared to children (10.9 %) due to improved paediatric care (Cystic Fibrosis Trust, 2013). In contrast, the United States has a higher incident rate of P. aerugionsa infections across all age groups (Cystic Fibrosis Foundation Patient Registry, 2011)

When *P. aeruginosa* exist in biofilms they not only become resistant to both innate and adaptive immune responses but also to antimicrobial therapy. In fact, several studies have suggested that mucoid bacterial cells of *P. aeruginosa* are 10 – 1000 times more resistant to the effects of antimicrobial agents than their nonattached (non mucoid) individual planktonic counterparts (Rogan *et al.,* 2004; Smith, 2005; Stewart and William Costerton, 2001; Mehrotra, 2007; Nickel *et al.,* 1985).

To date, aggressive antibiotic therapy continues to be one of the most important components of cystic fibrosis management especially since this successively increases the median survival age, improves the quality of life and determines the overall cost of care of the cystic fibrosis patient (Langton Hewer and Smyth, 2009). However, the overall outcome of antimicrobial therapies is controversial, with some studies suggesting that the currently available antimicrobial agents are virtually ineffective at eradicating chronic *P. aeruginosa* infections (Doring *et al.,* 2000; Hoiby, 2000; Koch, 2002; The Uk Cystic Fibrosis Trust Infection Control Group, 2004; Frederiksen *et al.,* 1997). This is thought generally to be due to the difficulty of achieving effective concentrations of antimicrobials in the CF airways, partly as a consequence of the viscous nature of desiccated mucus lining the CF airway as well as the production of an mucoid exopolysaccharide matrix (biofilm) or alginate which surrounds *P. aeruginosa* (Costerton, 1977; Govan and Fyfe, 1978; Slack and Nichols, 1981; Tannenbaum *et al.,* 1984) . However, other studies like the ones by Li *et al.,* (2005) and McPherson *et al.,* (2010) suggest otherwise, generally positing that it is possible to eradicate or suppress early mucoid *P. aeruginosa* by aggressive anti-*P. aeruginosa* treatment.

Several antibiotic strategies currently exist to treat early *P. aeruginosa* lung infections in CF patients. Some of these strategies are based in part on a variety of factors including patient's age, disease severity, patient history and *P. aeruginosa* susceptibility tests. However, the UK cystic fibrosis trust antibiotic working group (2009) standard of care guidelines recommends three approaches to effectively manage infections in patient with CF: the first approach is prophylactic antibiotic therapy which serves to reduce infection and inflammation in the developing lung (hence reduced prevalence) as well as prevent secondary bacterial infection and colonization, however, prophylactic antibiotics use are not without consequences as they may sometimes present other challenges

following administration such as adverse effects like oral candidiasis and diarrhea (Smyth and Walters, 2012); the second approach generally deals with treating acute pulmonary infectious exacerbations by the use of various combinations of oral quinolones such as ciprofloxacin (Taccetti *et al.,* 2005), inhaled antibiotics such as colistin and tobramycin (Ratjen *et al.,* 2001) and intravenous antibiotics usually consisting of combination of tobramycin and a beta-lactam (Douglas *et al.,* 2009); the third approach generally deals with inhaled and intravenous antibiotic administration to control infections as well as to slow the progression of chronic lung disease (Ryan *et al.,* 2011; Ballmann *et al.,* 2011)

Unfortunately, notwithstanding all the strategies currently in place to delay or prevent chronic *P. aeruginosa* infections, the infection still persists, becoming progressively resistant to conventional antibiotic therapy especially those involving the aminoglycosides in particular (Emerson *et al.,* 2010). There is a clear need for new antimicrobial agents and novel approaches including combination of currently existing antimicrobial agents that possess activity against the increasingly resistant respiratory pathogen; *P. aeruginosa*.

Antimicrobial peptides/proteins which are components of the innate defence system are known to possess an unusually broad activity spectra against microorganisms resistant to conventional antibiotics as well as being relatively nonimmunogenic. These are a particularly attractive candidate for pharmaceutical development. One of such antimicrobial peptide which does have applications in the pharmacotherapy of airway infections is Lactoferrin. Lactoferrin and apo lactoferrin have been shown to kill or inhibit bacterial growth of a variety of organisms; *Porphyromonas gingivalis* (Dashper *et al.,*

2012); *V. cholera, E. coli* and *S. thyphimurium* (Ellison and Giehl, 1991); *Streptococcus mutans, Streptococcus pneumonia, P. aeruginosa* and *Candida albicans* (Arnold *et al.,* 1980). Additionally, the presence of lactoferrin has been shown to enhance the susceptibility of various bacteria to a range of different antibiotics (Alkawash *et al.,* 1999; Qamruddin *et al.,* 2005; Ellison *et al.,* 1990; Naidu and Arnold, 1994; Singh *et al.,* 2000). More importantly, lactoferrin has been shown to inhibit the formation of *P. aeruginosa* biofilms (Singh *et al.,* 2002).

Tobramycin, an aminoglycoside, is currently one of the most commonly used aerosolized antibiotics in the management of airway infections resulting from *P. aeruginosa*. It is one of several components of nebramycin – a complex of antibiotic substances produced by *Streptomyces tenebrarius* (Higgins and Kastner, 1967; Preston and Wick, 1970). It is currently recommended for use in patients with CF who are six years of age and older with moderate to severe lung disease and persistent *P. aeruginosa* infection (Flume *et al.,* 2007).

Tobramycin and gentamicin both have similar spectra of activity and both antibiotics function by irreversibly inhibiting protein synthesis in susceptible bacteria by binding to the 30S ribosomal subunit (Lambert, 2002). They however have a poor lipid solubility and so do not readily cross biologic membranes. To improve upon the activity of the aminoglycoside in treating airway infections it has become a common practice to combine an aminoglycoside with a second agent to achieve a synergistic effect as well as to minimize the risk of developing bacterial resistance to the aminoglycosides (Hodson *et al.,* 1981; Hodson *et al.,* 1983; Downs *et al.,* 1986; Tré-Hardy *et al.,* 2008; Moreau-Marquis *et al.,* 2009; Cai *et al.,* 2009; Macleod *et al.,* 2009; Mccaughey *et al.,* 2012). Tobramycin and gentamicin both show a concentration dependent tendency in

causing nephrotoxicity and ototoxicity. The risks of these side effects developing is based on the duration of therapy. A delivery system that reduces the drugs' drug toxicity while increasing their therapeutic index is of great interest and a dry powder formulation containing combinations of aminoglycosides and an antimicrobial protein (lactoferrin/apo lactoferrin) would be expected to provide these benefits.

6.3 Aims and objectives

The purpose of this study was threefold: (i) to develop dry powder co-formulations of an antimicrobial peptide and aminoglycoside antibiotics (tobramycin/gentamicin) and evaluate their physicochemical properties in order to establish that the dry powders produced were well suited for delivery to the lower regions of the respiratory tract. (ii) to determine the activity of the co-formulations against both planktonic and biofilm cultures of *P. aeruginosa* and also determine whether synergism was present between the various co-formulations against *P. aeruginosa*, and (iii) to determine the ability of the co-formulations to penetrate bacterial biofilms.

6.4 Materials

Tobramycin and gentamicin were supplied by Fagron (Newcastle upon Tyne, UK), Mueller-Hinton Agar, 30 µg tobramycin and 200 µg gentamicin antibiotic disks were supplied by Oxoid LTD (Basingstoke, UK), LIVE/DEAD® cell viability assay kit was supplied by Invitrogen Molecular Probes (Life Technologies Ltd, Paisley, UK), Formaldehyde solution 37% by weight in water was supplied by Sigma-Aldrich (Poole, UK), Nucleopore track-etched Membranes (13 mm diameter and 25 mm diameter) with pore size 0.2 µm were supplied by Whatman Int. Ltd (Kent, UK)

6.5 Methods

6.5.1 Preparation of spray-dried tobramycin/gentamicin

Spray-dried tobramycin or gentamicin were prepared according to the method previously outlined in section 2.2.2.2.8. Briefly, 2 g of either tobramycin or gentamicin were dissolved in 50 mL of deionised water, upon complete dissolution of the antibiotic, the final volume of the solution were made up to 100 mL. The resulting solution were then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 10 %; aspirator rate, 95 %. These settings resulted in an outlet temperature of 102 – 104 °C (for spray-dried tobramycin) and 99 – 102 (for spray-dried gentamicin). This procedure was repeated two more times to produce three different batches of spray-dried antibiotic powders.

6.5.2 Preparation of co-spray dried lactoferrin/apo lactoferrin with tobramycin/gentamicin

Co-spray dried powder formulations of lactoferrin/apo lactoferrin and tobramycin/gentamicin were prepared according to the method describe in sections 2.2.2.2.9 and 2.2.2.2.10. Briefly, to prepare co-spray dried lactoferrin and tobramycin/gentamicin powder formulations, 1 g of lactoferrin was mixed with 1 g of either tobramycin or gentamicin in 50 mL deionised water, upon complete dissolution of the powders, the final volume was made up to 100 mL with deionised water. The resulting suspension was spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 10 %; aspirator rate, 95 %. These setting resulted in an outlet temperature of 104 – 106 °C (for co-spray dried lactoferrin with tobramycin) and 102 – 104 (for co-spray dried lactoferrin with gentamicin). This procedure was repeated two more times to produce a total of three batches of co-spray dried lactoferrin with tobramycin/gentamicin dry powders

To prepare co-spray dried apo lactoferrin and tobramycin/gentamicin dry powders, 1g of either tobramycin or gentamicin were dissolved in 50 mL of apo lactoferrin prepared according to the method outlined in section 2.2.2.2.4. Upon complete dissolution of the antibiotic, the final volume of the solution was made up to 100 mL with apo lactoferrin solution. The resulting solution was then spray-dried with the following parameters; Inlet temperature, 180 °C; spray flow rate, 606 L/hr; pump setting, 10 %; aspirator rate, 95 %. These setting resulted in an outlet temperature ranging from 103 – 104 °C (for co-spray dried apo lactoferrin with tobramycin) and 93 – 103 °C (for co-spray dried apo lactoferrin with gentamicin). This procedure was repeated two more times to produce a total of three batches of co-spray dried apo lactoferrin with sodium carboxymethylcellulose dry powders.

6.5.3 Measurement of moisture content

Moisture content was determined using the method previously described in section 2.4.2

6.5.4 Measurement of spray-dried yield

Spray-dried yield was calculated using the following equation:

$$\text{Yield} = \frac{\text{Total mass of spray} - \text{dried powder}}{\text{Mass of powder prior to spray drying}} \text{ x } 100 \qquad \text{(Equation 6.1)}$$

6.5.5 Zeta potential analysis

The zeta potential of suspensions of the various tobramycin/gentamicin preparations was determined using a Zetaplus (Brookhaven Instruments) at 25 °C. Deionised water was used as dispersion medium and 2 mL volumes were assessed. Triplicate measurements were conducted on each of the three batches of tobramycin, spray-dried tobramycin, co-spray dried lactoferrin-tobramycin, co-spray dried apolactoferrin-tobramycin, co-spray dried lactoferrin-gentamicin, co-spray dried lactoferrin-gentamicin.

6.5.6 Density of the various spray-dried powders

The true densities of the various powders were determined as previously outlined in section 4.5.10. Briefly, 3 cm^3 volume of dry powders were put into the sample cup and pressurised to approximately 17 pounds per square inch (1.195 Kg/cm^2) above the ambient pressure. Recordings were made and the true density of the powders calculated in MS Excel by determining the the pore volume (i.e. the difference between

the total volume and the solid volume as determined by the helium pycnometer) and dividing the weight of the powder sample by the total sample volume.

6.5.7 Particle size analysis

Particle size distribution of the various spray-dried tobramycin/gentamicin and co-spray dried powders were determined as previously described in section 2.2.5

6.5.8 Differential scanning calorimetry

Differential scanning calorimetric determinations were carried out as previously described in section 4.5.12. Briefly, 4 mg of the powder was loaded into an aluminium pan, cooled to -30 °C and then heated to 200 °C at 10 °C/min with a nitrogen purge of 10 mL/min. An empty aluminium pan was used as reference for all measurements. The resulting data were analysed by Pyris manager software. Triplicate measurements of all independently prepared sample batches were assessed.

6.5.9 X-ray powder diffraction

X-ray powder diffraction measurements were carried out as previously described in section 4.5.11. Briefly, the sample holder was filled with the spray-dried powders, and diffraction patterns of the powders recorded between 2° and 70° of 2θ with an increment of 0.02° and a step time of 0.1 s.

6.5.10 Determination of minimal Inhibitory concentration and minimal bactericidal concentration (MBC)

The MIC of each antimicrobial agent against *P. aeruginosa* was determined by the microdilution technique (100 µL volume) as described by NCCLS guidelines for antimicrobial susceptibility testing. The inoculum was prepared from an overnight culture of *P. aeruginosa* and diluted to yield $1x10^6$ CFU/ml. Plate counts were performed to determine the exact count. The MIC was the lowest concentration of drug allowing no visible growth after 18 hr of incubation at 37°C. From each well where there was no visible growth, 10 µL was plated on a Mueller-Hinton agar plate, incubated at 37 °C for 24, and counted. The MBC was defined as the lowest concentration of drug that killed 99.9% of the original inoculum.

6.5.11 Determination of fractional inhibitory concentration

The fractional inhibitory concentration were calculated by the formula of Berenbaum (1978) as follows:

$$FIC = \frac{MIC\ of\ antimicrobial\ combination}{MIC\ of\ antibiotics\ alone} \qquad \text{(Equation 6.2)}$$

An FIC index of ≤ 0.5 was considered synergism, 0.5 to < 1 was considered weak synergism, 1 was considered additive, and >1 was considered antagonism.

6.5.12 Time-kill curve of the various protein-antibiotic co-formulations

20 µL of ~ 6.6 x 10^7 CFU/ml *P. aeruginosa* PAO1 was incubated at 37 °C with 180 µL of a 100 µg/mL stock preparations of the various protein/antibiotic co-formulations or 180 µL of sterile distilled water (as positive control) or 180 µL of 500 µg/mL gentamicin (as negative control). At intervals of 10 mins for up to 1 hour, 10 µL aliquots were plated out neat as well as serially diluted with deionized water before plating out on Mueller-Hinton agar. The Muller-Hinton agar plates were then incubated at 37 °C for 24 hours. Following incubation, the number of colonies from each of the serial dilutions plated out was counted and the dilution having a colony count of < 300 colonies was used to estimate the colony forming unit per mL surviving at that time the sample was collected. The colony forming unit per mL was estimated by the equation previously stated in section 2.2.15

6.5.13 Assessment of the ability of tobramycin/gentamicin alone or in combination with lactoferrin/apo lactoferrin to inhibit biofilm formation

To test the ability of *P. aeruginosa* to form biofilms (on the surface of microtitre dishes) in the presence of the various tobramycin/gentamicin dry powder formulations, a method previously described by Eckhart *et al.,* (2002) and Merrit *et al.,* (1998) was employed. Briefly, overnight cultures of *P. aeruginosa* were diluted 1:10 (v/v) in fresh medium containing 0.5 % w/v casamino acids, 0.2 % glucose and 1 mM $MgCl_2$ (casamino acid rich medium). 10 µL aliquots of the dilution were dispensed into a 96 well plate and 90 µL of a 1 mg/mL (i.e. 900 µg/mL of the antimicrobial agent) stock suspension of the various antimicrobial formulations were then added to the bacterial suspension in the 96

well plate. The 96 well micro-plate was then covered and incubated at 37°C for 24 h, after which, excess broth was removed and the adherent biofilms to the microtitre plates stained. Detailed description of the method employed in staining and quantifying biofilms is as previously described in section 2.2.17.2.

6.5.14 Assessment of the ability of tobramycin/gentamicin alone or in combination with lactoferrin/apo lactoferrin to reduce preformed biofilms

To assess the effects of the various tobramycin/gentamicin formulations on already formed biofilms (i.e. biofilm persistence) a slight modification was made to the above method. Briefly, 100 µL of a 1 in 10 dilution of an overnight grown bacterial suspension of *P. aeruginosa* was put into 96 well plate, covered with a lid and incubated at 37°C for 24 hours. Then the bacterial suspension was removed, leaving the adherent biofilm in the well, then, 100 µL of a 1 mg/mL (i.e. 100 µg/mL of the antimicrobial agent) stock suspension of the various tobramycin/gentamicin formulations was added and incubated for a further 24 hours at 37 °C. The various formulations were removed and the wells washed twice before staining with the method previously described in section 2.2.17.2

6.5.15 Assessment of the ability of combinations of tobramycin/gentamicin with iron chelators to inhibit biofilm formation

Overnight cultures of bacterial suspension were diluted 1:10 in casamino acid rich medium supplemented with 3 µM $FeCl_3$. Then, 2 mM of various iron chelators (2DP, DTPA, EDTA and DM) was prepared using a 1 mg/mL solution of tobramycin/gentamicin

to produce various combinations of tobramycin/gentamicin with the iron chelators. 10 µL aliquots of the bacterial dilution were put into 96 well microplate and 90 µL of the stock preparation of the combinations of tobramycin/gentamicin with iron chelators was added to the wells. The 96 well microplates were then covered and incubated at 37 °C for either 6 or 24 hours. Following incubation, the procedure previously described in section 2.2.17.2 was employed to stain and quantify the biofilms that formed.

6.5.16 Preparation of antibiotic disks

Antibiotic susceptibility disks containing combinations of tobramycin/gentamicin with lactoferrin/apo lactoferrin were all prepared locally by impregnating a sterile blank filter paper disk (diameter 6.3 mm) with 0.02 mL of 1mg/mL standard antibiotic solutions (to prepare a 20 µg antibiotic disc). The procedure previously described in section 2.2.19.1 was employed to prepare the antibiotic disks.

6.5.17 Antibiotic penetration through *P. aeruginosa* biofilms

The penetration of the tobramycin and gentamicin either alone or in combination with lactoferrin preparations through the biofilms was determined using a method previously described by Singh *et al.,* (2010) with some modifications. Sterile track-etched ploycarbonate membranes were inoculated with 5 µL of *P.aeruginosa* bacterial suspensions and incubated on Muller-Hinton agar plates at 37 °C for 48 hours with the membrane-supported biofilms transferred to a new agar plate every 18 hours. The polycarbonate membranes supporting biofilms growth were transferred to MHA plates inoculated with a quality control organism *Escherichia coli* NCTC 10418 whose growth was adjusted to the density of a 0.5 McFarland standard to give a confluent lawn of

grown. A 6 mm nitrocellulose membrane was then placed on top of each biofilm together with the antibiotic disc and moistened with 20 µL of sterile distilled water so as to prevent capillary action in the movement of the antibiotics. Controls, which comprised of sterile polycarbonate membranes and antibiotic disc without biofilms were also setup. All plates were incubated at 37°C for 24 hrs and the clear zones visible on the plates indicating the zone of inhibition of growth by the antibiotic (tobramycin or gentamicin) in both the control and test were measured. A calibration plot of standard zone diameter measurements against log concentration (for antibiotic discs containing 1 – 10,000 µg) was used to estimate the concentration of the antibiotic that passed through the biofilms. The diameter of the zones of inhibition in the controls were taken to signify 100% penetration and used to determine the percent penetration and percent retardation of the antibiotics through the *Pseudomonas* biofilms. All experiments were performed in triplicates and a two-tailed, paired t-test was used for statistical analysis. In other to avoid false negative results arising from side diffusion from the antibiotic disc precautions were taken to ensure that the biofilms obtained after incubation was <13 mm in diameter so as to prevent overgrowth of the biofilms unto the control organism.

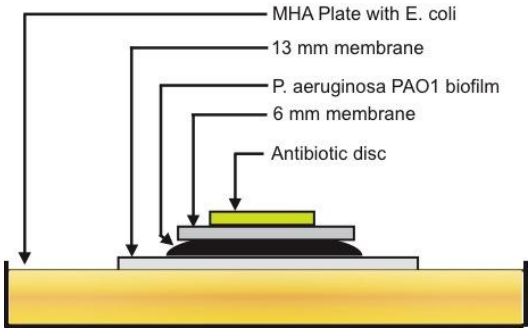


Figure 6 -1: Schematic depiction of experimental setup used to track the penetration of antibiotics through *Pseudomonas aeruginosa* biofilms. The Mueller-Hinton agar plate is covered with a confluent lawn of the control strain (*E. coli* NCTC 10418). A sterile track-etched polycarbonate membrane (0.2 µm pore size) that supports biofilm growth is placed on MHA with a smaller diameter polycarbonate membrane and a moistened paper disk containing the test compound place on the biofilm.

6.5.18 Confocal microscopy to investigate co-formulation-dependent changes in the viability of biofilms of *P. aeruginosa* grown on glass

Bacterial colonies of *P. aeruginosa* grown on Mueller-Hinton agar were suspended in a media rich in casamino acids and incubated for 24 hours at 37°C. An aliquot of 1mL of bacterial cell suspension (~ 10^7 cfu/mL) was added into each of the chambers created on a glass slide using a silicon gasket and incubated at 37°C. The media was removed and replaced every 16 hours. Following 72 hours growth of the biofilms on the coverslip, were carefully washed twice with phosphate-buffered saline (PBS) to remove planktonic bacteria while leaving behind the biofilm adherent to the glass.

The biofilms were then treated for 24 hours with 100 µL per chamber of a 1mg/mL solution of the various antimicrobial formulations. Positive controls contained 100 µL of the CAA rich media without any antibiotic included, whilst the negative control contained

100 µL of 100 % isopropanol. The entire chamber containing the antimicrobial agents were incubated at 37°C.

Following treatment, the slides were washed again with PBS and stained with a LIVE/DEAD® bacterial viability kit using a mixture of 3 µL of syto 9 (green fluorescent) + 3 µL of propidium iodide (red fluorescent) per mL of saline. Both syto 9 and propidium iodide are nucleic acid stains. While, syto 9 stains all nuclei acid in a population (both those with intact and damaged membranes), propidium iode on the other hand stains only bacteria with damaged membranes and when used together with syto 9 causes a reduction in the green fluorescence of the latter.

Visualisation of biofilms was carried out using a Leica TCS SP5 II (Leica Microsystems, Milton Keynes, UK) confocal laser scanning microscope (CLSM). For detection of Syto 9 (green channel), the 488 nm line of the argon laser with a detection bandwith of 495 – 515 nm was used, while, for detection of propidium iodide (red channel), the 561 nm line with a detection bandwidth of 615 – 600 nm was used. Image analysis was carried out using the Leica software (LAS AF Version 2.2.1 build 4842).

6.5.19 Statistical analysis

Data was subjected to statistical analysis using one-way ANOVA to examine for significant differences (defined as $p > 0.05$). Significant differences between the various formulations were analysed using Dunnett post test with p values of < 0.05 being considered to be significant.

6.6 Results

6.6.1 Physical characteristics of various spray-dried powders

The spray drying yield and moisture content of 2 % (w/v) tobramycin/gentamicin formulations are summarized in table 6.1. All the formulations were spray-dried with an inlet temperature of 180 °C and airflow rate of 606 L/hr to produce various outlet temperatures ranging from 99 – 102 °C. The lactoferrin-tobramycin/gentamicin formulations had the highest mean percent spray drying yield while the apo lactoferrin-tobramycin/gentamicin formulations had the lowest mean spray drying yield. The moisture content, as determined by Karl Fischer titration of all the various spray-dried formulations, was generally below 5 %. Zeta potential measurements indicate that both tobramycin and gentamicin had a net negative charge while the combinations of protein:aminoglycoside had a net positive charge although the lactoferrin-tobramycin/gentamicin combinations were slightly more positive than the apo lactoferrin-tobramycin/gentamicin. The average true density measurements of the various powders was found to be 1.7 g/cm^3 for the co-spray dried formulations while it was less for the spray-dried tobramycin and gentamicin only powder formulations.

Table 6.1: Percent spray drying yield, percent moisture content, zeta potential and true density of spray-dried tobramycin/gentamicin and co-spray dried tobramycin/gentamicin with lactoferrin. Three different batches of the various tobramycin/gentamicin powders were prepared and the mean values for the measurements are shown below.

Formulation	Mean yield (%)	Moisture content (%)	Zeta Potential (mV)	True Density (g/cm^3)
SD Tobi	82.1 ±2.2	3.2 ±0.4	-3.34 ±3.1	1.52 ±0.01
SDLf + Tobi	87.5 ±1.4	3.7 ±0.2	2.67 ±0.4	1.68 ±0.01
SDaLf + Tobi	76.3 ±2.4	3.3 ±0.5	1.14 ±0.9	1.66 ±0.01
SD Genta	85.4 ±1.3	4.0 ±0.2	-2.99 ±2.0	1.61 ±0.01
SDLf + Genta	87.3 ±2.1	3.9 ±0.3	2.29 ±0.7	1.67 ±0.01
SDaLf + Genta	80.1±1.9	3.4 ±0.4	0.97 ±0.8	1.67 ±0.01

6.6.2 Particle size analysis of spray-dried tobramycin/gentamicin and co-spray dried lactoferrin-tobramycin/gentamicin

The particle size distribution of the various co-spray dried formulations is summarized in table 6.2. Particle size analysis reveals that all the spray-dried powders were within the respirable size range, with a $d[v,50]$ from 4.90 ±0.54 to 6.23 ±0.30 µm.

Table 6.2: Particle size distribution represented as volume fractions 10 % *d*[*v,*10], 50 % *d*[*v,*10], 90 % *d*[*v,*10], their respective span and volume mean diameter (VMD). The values are mean ± SD.

Formulations	*d*[*v,*10] (µm)	*d*[*v,*50] (µm)	*d*[*v,*90] (µm)	Span	VMD (µm)
SD Tobi	1.25 ±0.45	4.90 ±0.54	11.29 ±0.70	2.07 ±0.28	6.00 ±0.6
SDLf + Tobi	1.17 ±0.20	5.27 ±0.30	15.23 ±0.51	2.68 ±0.21	7.51 ±0.4
SDaLf + Tobi	1.02 ±0.39	5.06 ±0.40	14.31 ±0.45	2.63 ±0.22	7.07 ±0.5
SD Genta	1.40 ±0.55	6.06 ±0.65	14.38 ±0.83	2.16 ±0.28	7.61 ±0.5
SDLf + Genta	1.47 ±0.23	6.23 ±0.30	14.41 ±0.57	2.08 ±0.16	7.72 ±0.4
SDaLf + Genta	1.47 ±0.32	5.15 ±0.33	11.53 ±0.55	1.96 ±0.17	6.11 ±0.3

6.6.3 Thermal properties of spray-dried tobramycin/gentamicin and co-spray dried lactoferrin-tobramycin/gentamicin

The thermal properties of six spray-dried powders are shown in Figure 6 -2. The absence of endothermic peaks on the DSC scans for spray-dried tobramycin and gentamicin powder formulation is indicative that the powders were amorphous. The DSC scan for the various co-spray dried powder formulations was characterized by broad endothermic peaks usually between 2 and 7 °C.

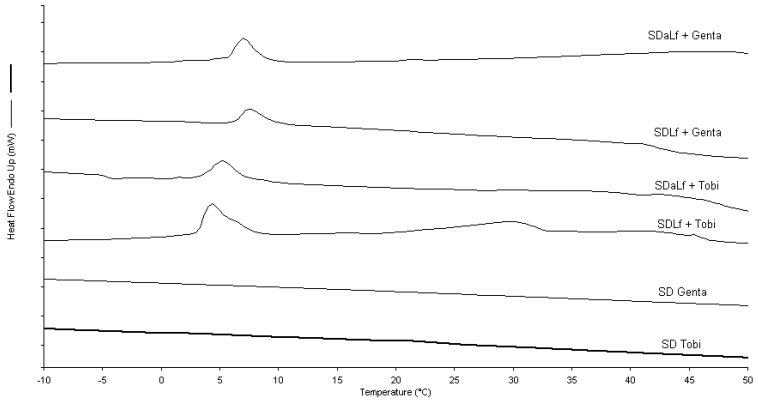
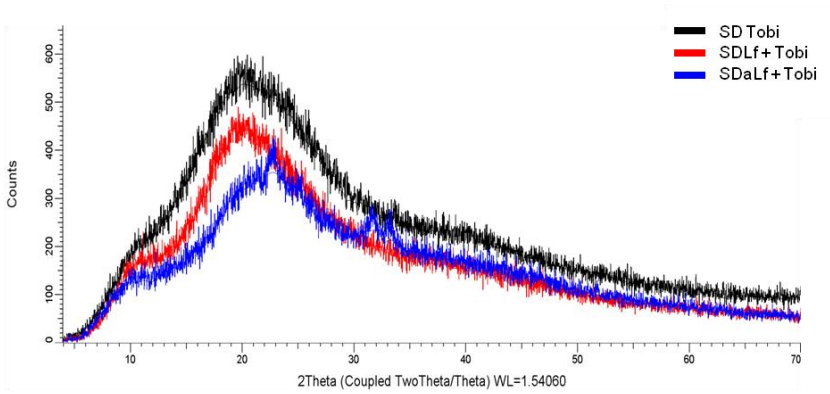


Figure 6 -2: DSC scans showing a comparison of the thermal behaviour of spray-dried tobramycin (SD Tobi), spray-dried gentamicin (SD Genta), co-spray dried lactoferrin + tobramycin (SDLf + Tobi), co-spray dried apo lactoferrin + tobramycin (SDaLf + Tobi), co-spray dried lactoferrin + gentamicin (SDLf + Genta) and co-spray dried apo lactoferrin + gentamicin (SDLf + Genta)

6.6.4 X-ray powder diffraction (XRPD)

X-ray diffraction analysis of the various spray-dried powder formulations revealed that the were no distinct peaks for any of the different formulations indicating that most of the formulations were amorphous following spray drying (Figure 6- 3a and 6- 3b)

a)



b)

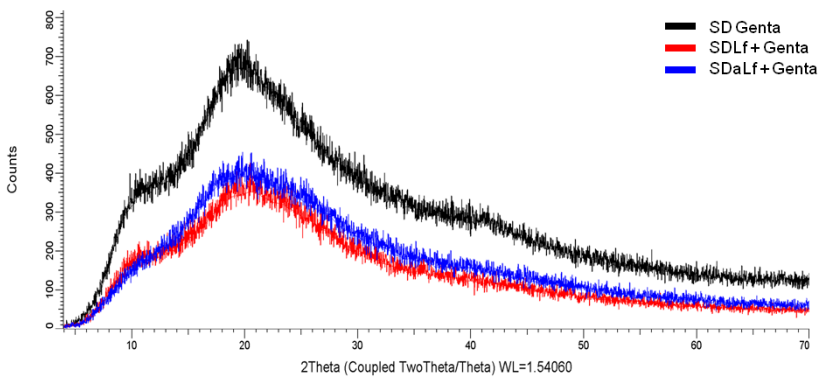


Figure 6 -3: X-ray powder diffraction patterns of various lactoferrin-tobramycin/gentamicin spray-dried powder formulations. (a) shows the XRPD patterns for the tobramycin containing formulations; spray-dried tobramycin (SD Tobi), co-spray dried lactoferrin-tobramycin (SDLf + Tobi) and co-spray dried apo lactoferrin-tobramycin (SDaLf + Tobi), (b) shows the XRPD patterns for the gentamicin containing formulations; spray-dried gentamicin (SD Genta), co-spray dried lactoferrin- gentamicin (SDLf + Genta) and co-spray dried apo lactoferrin- gentamicin (SDaLf + Genta). All the spray-dried powders have a broad halo and lack resolved reflections indicating that they are all amorphous. The results presented are a representation of three repeats.

6.6.5 MIC, MBC and FIC of the various antimicrobials

The MIC's and MBC's and the FIC index (compared to the single therapy) of SD Tobi, SDLf + Tobi, SDaLf + Tobi, SD Genta, SDLf + Genta and SDaLf + Genta against *P. aeruginosa* are summarized in Table 6.3. FIC calculations were made using equation 6.2 and show SDaLf + Tobi, SDLf + Genta and SDaLf + Genta to be synergistic while SDLf + Tobi was weakly synergistic.

Table 6.3: MIC, MBC and FIC of the various lactoferrin formulations. The MIC of each antimicrobial was assessed by a microdilution method derived from clinical and laboratory standard institute (CLSI) for broth microdilution antimicrobial susceptibility testing and defined as

the lowest concentration which inhibited visible growth. The MBC of the various antimicrobial was determined by plating out the wells with no visible growth on Mueller-Hinton agar. The FIC were calculated by dividing the MIC of the various combinations by the MIC of the antibiotic component of the combination

Formulation	MIC (µg/mL)	MBC (µg/mL)	FIC Index
SD Tobi	1.17 ±0.1	2.08 ±0.1	
SDLf + Tobi	0.67 ±0.1	1.17 ±0.1	0.57 ±0.06
SDaLf + Tobi	0.58 ±0.1	1.08 ±0.1	0.50 ±0.10
SD Genta	2.33 ±0.1	4.00 ±0.0	
SDLf + Genta	1.17 ±0.1	2.00 ±0.3	0.50 ±0.06
SDaLf + Genta	0.83 ±0.1	1.83 ±0.1	0.36 ±0.08

6.6.6 Antimicrobial activity of the various powder formulations against planktonic bacteria

The antimicrobial activity of the various spray-dried powders against planktonic *P. aeruginosa* was determined quantitatively through time-kill studies. *P. aeruginosa* cultures were grown in the presence or absence (control) of the various protein-antibiotic dry powders. Over a 1 hr period the number of colonies in the control remained fairly constant, spray-dried tobramycin reduced bacteria count by 2- log units within 1 hour, this was enhanced to 3- log units when tobramycin was spray-dried with lactoferrin. However, combinations of apo lactoferrin and tobramycin were even more effective, reducing viable counts to 5- log units within the same 1 hour period

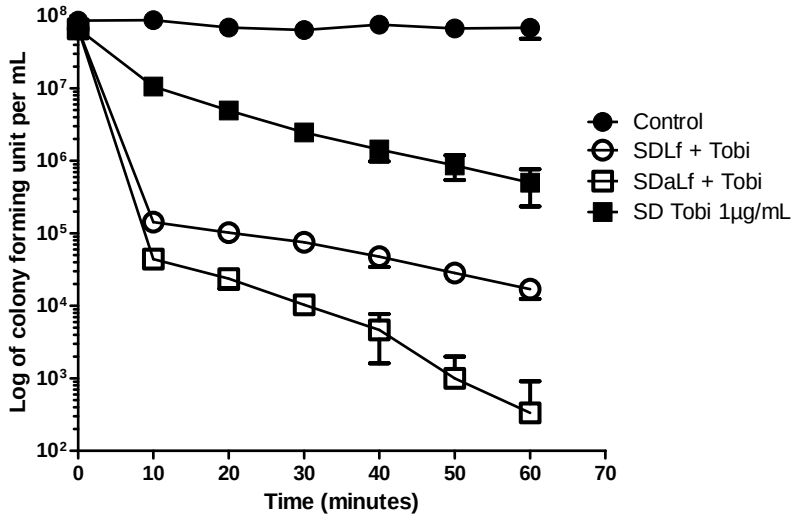


Figure 6 -4: Logarithmic plot of bacterial cell viability in the presence of different combinations of lactoferrin:tobramycin preparations. The bacteria were seeded in polystyrene 96-well plates with equal volume of the antimicrobial preparation present at a concentration of 1 µg/mL of the antibiotics (tobramycin) and incubated at 37 °C for the times indicated. Aliquots were plated out onto MHA agar plates at 10, 20, 30, 40, 50, 60 mins. Bacterial colonies were counted after 24 hours at 37 °C and data are presented as mean ± SD; n = 3 independent evaluations of triplicate wells. While the log colony forming unit per mL of the control wells remained relatively unchanged, after 1 hour incubation, the number of viable cells began to reduce in the presence of the various antimicrobial agents. 1 µg/mL of SD tobramycin-only showed the least impact on the population of viable cells. Iron depletion increased viable cell reduction.

For the gentamicin containing formulations, the number of colonies in the control (no antibiotic added) were fairly constant at about 10^8 colonies over a 1 hr period (figure 6 - 5). Spray-dried gentamicin only formulations decreased the bacterial colonies by 2 log units compared to the control. Co-spray dried lactoferrin-gentamicin (SDLf + Genta) combinations were more effective than gentamicin alone in eliminating bacteria colonies by a 4 log unit reduction within 1 hr. However, co-spray dried apo lactoferrin-gentamicin (SDaLf + Genta) were able to completely eliminate all the bacterial colonies within 40 minutes.

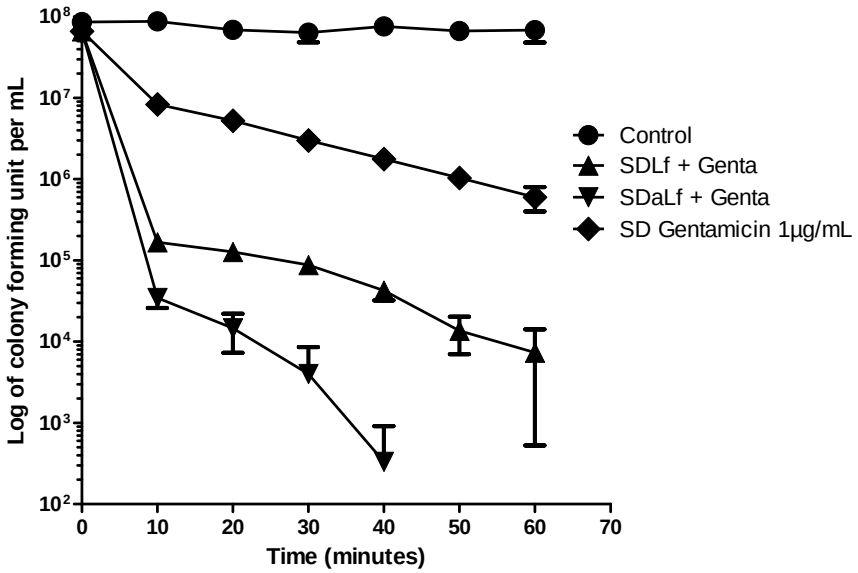


Figure 6 -5: Logarithmic plot of bacterial cell viability in the presence of different combinations of lactoferrin:gentamicin preparations. The bacteria were seeded in polystyrene 96-well plates with equal volumes of the antimicrobial preparation present at a concentration of 1 µg/mL of the antibiotics (gentamicin) and incubated at 37 °C for the times indicated. Aliquots were plated out onto MHA agar plates at 10, 20, 30, 40, 50, 60 mins. Bacterial colonies were counted after 24 hours at 37 °C and data are presented as mean ± SD; n = 3 independent evaluations of triplicate wells. While the log colony forming unit per mL of the control wells remained relatively

unchanged, after 10 min incubation, the number of viable cells began to reduce in the presence of the various antimicrobial agents. 1 µg/mL of SD gentamicin showed the least impact on the population of the viable cell.

6.4.6 Combination activity against biofilms

In order to assess the effect of the various formulations on *P. aeruginosa* biofilm formation, a control (i.e. no antibiotic added) was setup which involved incubating the bacteria in two media; casamino acid only (CAA; iron free) and casamino acid supplemented with 3 µM $FeCl_3$ medium. Absorbance values show that more *P. aeruginosa* biofilm formed in the iron rich medium within a 24 hr period (figure 6 -6). Spray-dried tobramycin only formulations were able to reduce biofilm formation in both the iron free and iron rich medium (figure 6 -6). However, co-spray dried lactoferrin-tobramycin (SDLf + Tobi) and co-spray dried apo lactoferrin-tobramycin (SDaLf + Tobi) further significantly (p value < 0.001) reduced biofilm formation compared to the spray-dried tobramycin only formulations. Similarly spray-dried gentamicin was also able to reduce biofilm formation in both the iron free and iron rich medium figure 6 -6 with co-spray dried lactoferrin-gentamicin (SDLf + Genta) and co-spray dried apo lactoferrin-gentamicin (SDaLf + Genta) further significantly reducing biofilm formation.

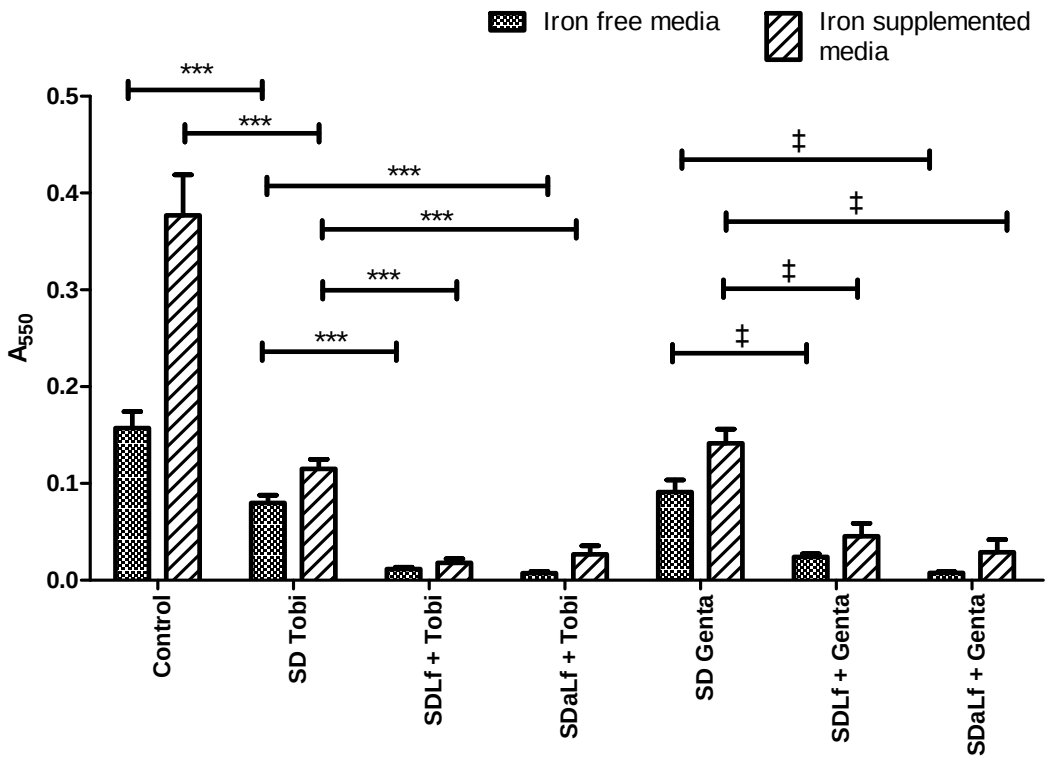


Figure 6 -6: *P. aeruginosa* biofilm formation in the presence and absence of various antimicrobial agents. Biofilm formation was assessed in both an iron free medium (CAA alone) and iron supplemented medium (with 3 µM $FeCl_3$). The concentration of all the antibiotics was 1 mg/mL. Co-spray dried protein:antibiotic combinations were more effective at preventing biofilm formation than aminoglycosides alone. The values are mean ±SD of triplicate measurements. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Prism Software Inc., Version 5.0) followed by Dunnett post-test; P values were considered significant and the symbol *** is used to represent $p < 0.001$ of the lactoferrin/apo lactoferrin – tobramycin combinations compared with spray-dried tobramycin; ‡ is used to represent $p < 0.001$ of the lactoferrin/apo lactoferrin – gentamicin combinations compared with spray-dried gentamicin

The ability of the various antimicrobial formulations to reduce or modify pre-formed biofilms were also assessed. Here, a control (i.e. no antibiotic added) was setup in the iron-free and iron-supplemented media and *P. aeruginosa* was allowed to form biofilms over a 24-hour period. The various antimicrobial agents were added to 96 well microtitre plate (bacterial growth medium was added to the control wells) and incubated at 37°C for a further 24 hours. The absorbance values show that more *P. aeruginosa* biofilm was formed in the iron-rich medium within the 48 hr period growth compared with the iron free media. There was however no significant difference between the quantity of biofilm measured when a 24-hr biofilm was exposed to spray-dried tobramycin only formulations (SD Tobi) in both the iron free (CAA alone) and iron rich (casamino acid supplemented with 3 μM $FeCl_3$) media following a further 24-hr incubation. In comparison, the quantity of biofilm measured when pre-formed biofilms were exposed to SDLf + Tobi and SDaLf + Tobi formulations for a further 24-hrs were significant ($p < 0.05$ and $p < 0.001$ respectively) in both the iron free or iron rich media (figure 6 -7).

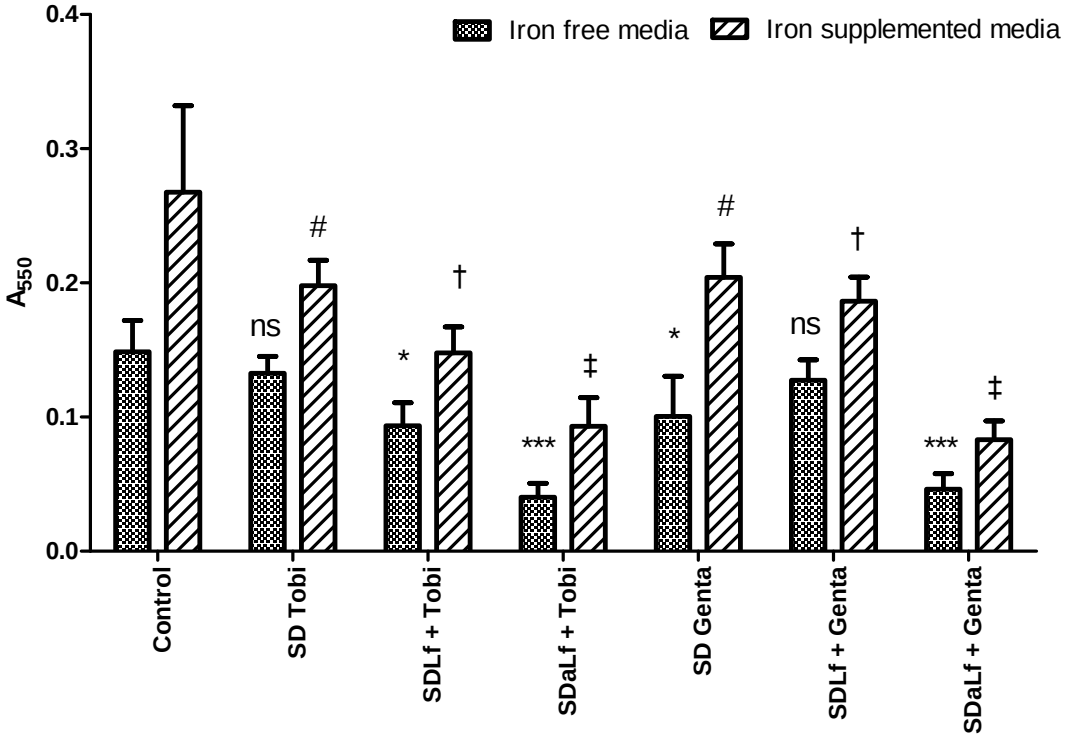


Figure 6 -7: Effect of antimicrobial agents on pre-formed *P. aeruginosa* biofilms. Antimicrobial effect was assessed in both in an iron free medium (CAA alone) and iron supplemented medium (with 3 µM $FeCl_3$). The concentration of all the antibiotics was 1 mg/mL. Co-spray dried apo lactoferrin-antibiotic combination showed the most pronounced antimicrobial effect on pre-formed biofilms of *P. aeruginosa,* this is evidenced by the low absorbance units. The values are mean ± SD of triplicate measurements. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; ns indicates no significance compared to the CAA alone control (no antibiotic added), # indicates no significance compared to the 3 µM $FeCl_3$ supplemented control, *** indicates $p < 0.01$ compared to the CAA alone control, * indicates $p < 0.05$ compared to the CAA alone control, ‡ indicates $p < 0.01$ compared to the 3 µM $FeCl_3$ supplemented control and † indicates $p < 0.05$ compared to the 3 µM $FeCl_3$ supplemented control.

While spray-dried gentamicin did not have any effect on the quantity of biofilm measured in the iron rich medium ($p > 0.05$), in the iron free medium it slightly reduced the quantity of biofilm measured compared to the respective controls (figure 6 -7). Upon incubating the 24 hr pre-formed biofilms with co-spray dried lactoferrin-gentamicin formulations there was no significant reduction in the pre-formed biofilm quantified in the iron free medium although there were significant ($p < 0.05$) reduction in the amount of biofilm quantified in the iron rich medium compared to the respective controls (figure 6 -7). The co-spray dried apo lactoferrin-gentamicin formulations significantly ($p < 0.01$) reduced the pre-formed biofilm quantified in both the iron free and iron rich media compared to the respective controls (figure 6 -7).

6.6.7 Activity of combinations of iron chelators with aminoglycoside against biofilms of *P. aeruginosa*

The activities of combinations of tobramycin and iron chelators on *P. aeruginosa* biofilms were evaluated at 6 and 24 hr post incubation in iron supplemented media. Results obtained shows that there was significantly more biofilm formed 6hrs post incubation of the control compared to 24 hr post incubation (figure 6 -8). Tobramycin was also found to significantly ($p < 0.01$) prevent biofilm formation for up to 24 hrs post incubation with this inhibition being more pronounced 6 hrs post incubation, however the combination of tobramycin and 2DP was most effective combination at preventing biofilm formation at both 6 and 24 hr post incubation. Combinations of tobramycin and DTPA and tobramycin and EDTA had similar biofilm prevention profiles; both being very effective at preventing biofilm formation 6 hrs after incubation and less effective at 24 hr post

incubation. The tobramycin-deferoxamine combination also significantly prevented biofilm formation up to 24 hrs post incubation.

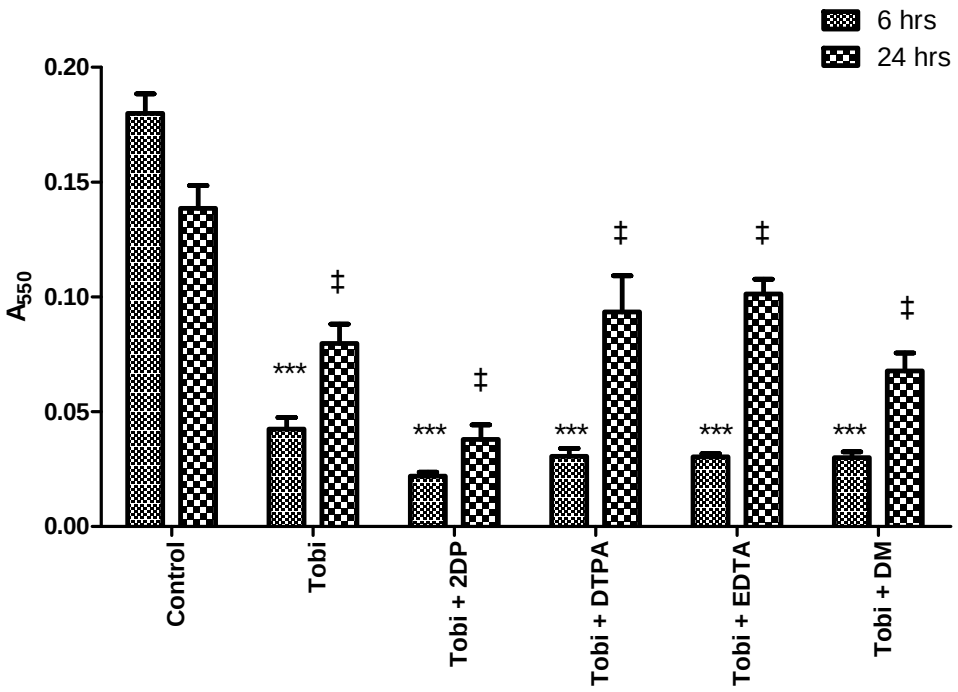


Figure 6 -8: *P. aeruginosa* biofilm formation following 6 and 24 hr incubation with 1 mg/mL tobramycin in the absence and presence of various iron chelators at 2mM. The entire experiment was carried out in casamino acid (iron free) medium. Values are mean ±SD of triplicate measurements for three batches of the combinations. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; *** indicates $p < 0.01$ compared to the 6 hr biofilm growth control (no antibiotic or iron chelator added) and ‡ indicates $p < 0.01$ compared to the 24 hr biofilm growth control

Similarly, the activity of gentamicin and combinations of gentamicin and various iron chelators on biofilm formation was assessed at 6 and 24 hrs after incubation with *P. aeruginosa*. While gentamicin alone was able to significantly prevent biofilm formation up to 24 hrs post incubation, the gentamicin-2DP combination had the best anti-biofilm

activity of all the combinations following 6 or 24 hr incubation with *P. aeruginosa*. Gentamicin-DTPA combination was no different than gentamicin alone, again being more effective at 6 hrs post incubation compared to 24 hrs post incubation. Finally, even though Gentamicin-EDTA and gentamicin-DM combinations were able to significantly ($p < 0.01$) prevent biofilm formation in the first 6 hrs post incubation they failed to sustain this activity 24 hr post incubation.

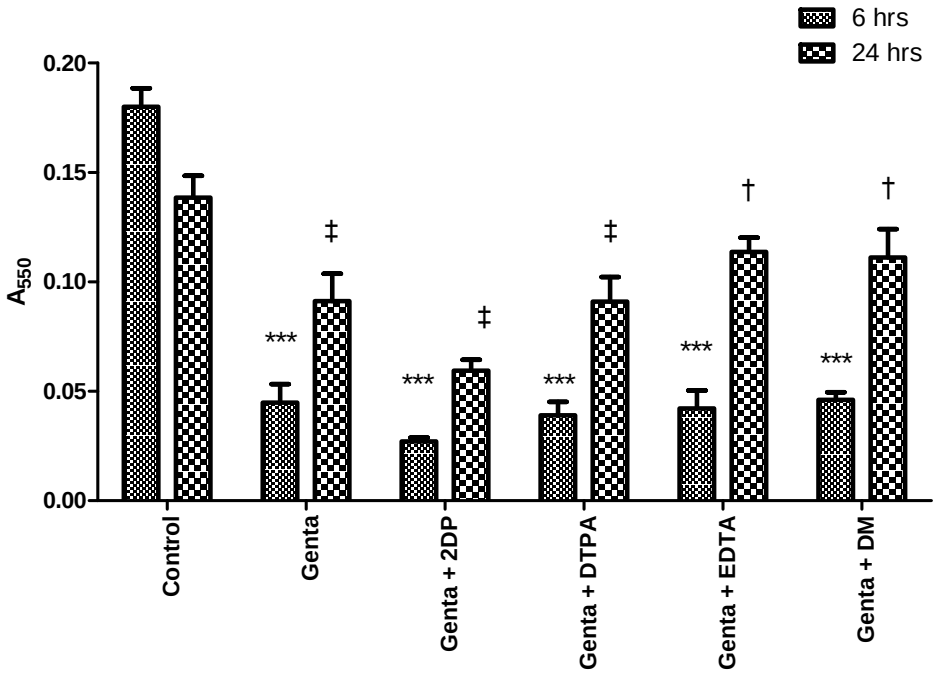


Figure 6 -9: *P. aeruginosa* biofilm formation following 6 and 24 hr incubation with gentamicin (Genta) in the absence and presence of various different iron chelators at 2mM. The entire experiment was carried out in casamino acid (iron free) medium. Values are mean ±SD of triplicate measurements for three batches of the combinations. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; *** indicates $p < 0.01$ compared to the 6 hr biofilm growth control (no antibiotic or iron chelator added), ‡ indicates $p < 0.01$ compared to the 24 hr biofilm growth control and † indicates $p < 0.05$ compared to the 24 hr biofilm growth control.

6.6.8 Penetration of antibiotics only and protein-antibiotic co-formulations through *P. aeruginosa* biofilms

Antibiotic penetration through biofilms is commonly used to determine how much antibiotic is able to pass through a biofilm. The penetration of the various antimicrobials through *P. aeruginosa* biofilms was evaluated to determine their suitability for treating chronic *P. aeruginosa* infections. The results presented here provide some mechanistic insight into the effectiveness of the various antimicrobial formulations during use.

6.6.8.1 Calibration curves

A calibration curve was set-up to allow the conversion of the zone of inhibition obtained, to effective concentration of drug that got through the biofilm. The penetration of tobramycin or gentamicin through *P. aeruginosa* biofilms was assessed from a calibration plot of zone diameter against log concentration of aminoglycoside. Representative images from three separate repeats showing changing zone diameter with increasing concentrations of tobramycin or gentamicin are shown in Figure 6- 10.

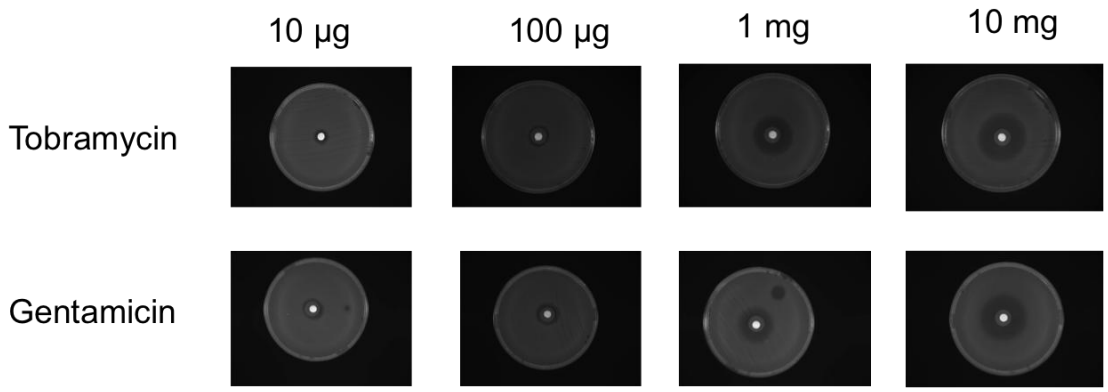


Figure 6 -10: Representative photographs showing the zone of inhibition apparent following the placement of antibiotic disc containing the various concentrations of tobramycin/gentamicin on an agar plate seeded with *E. coli*. These zones were measured and used to prepare a standard calibration curve relating aminoglycoside concentration to zone of inhibition. Subsequently, the calibration curve was used to evaluate antibiotics penetration through *P. aeruginosa* strain PAO1 biofilms grown on track-etched polycarbonate membranes. The images show agar plates seeded with the control organism – *Escherichia coli* NCTC 10418. A sterile polycarbonate membrane and an antibiotic disk containing 10, 100, 1000, and 10,000 µg of tobramycin (for the top row) and 10, 100, 1000, and 10,000 µg of gentamicin (for the bottom row) were place at the centre of each plate and incubated at 37 °C for 24 h. Following incubation, the zone of inhibition was measured and used in the preparation of a calibration plot of mean zone diameter against log concentration. The calibration plot was subsequently used to assess the concentration of combinations formulations penetrating through the biofilms.

All calibration curves obtained on different days for zone of inhibition of tobramycin on the control organism were linear within the assessed concentration range of 10 – 10,000 µg ($y = 5.0667x + 17.6$, $R^2 > 0.99$) Figure 6 -11 shows the mean calibration curve for mean zone diameter against log concentration of tobramycin.

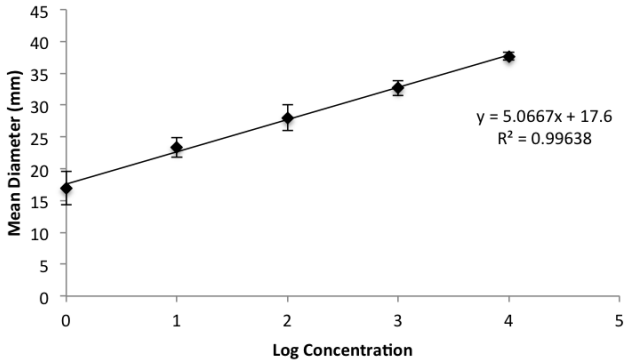


Figure 6 -11: Calibration curve showing a plot of mean diameter versus log concentration for tobramycin. The mean diameter calculated from calibrations obtained on three different days are plotted against $\log_{10}$ of concentration values (n = 3). Error bars representing standard deviation are displayed for each point.

All calibration curves obtained on different days for zone of inhibition of gentamicin on the control organism were linear within the assessed concentration range of 10 – 10,000 µg (y = 5.0667x + 17.6, R^2 > 0.99) Figure 6 -12 shows the mean calibration curve for mean zone diameter against log concentration of gentamicin.

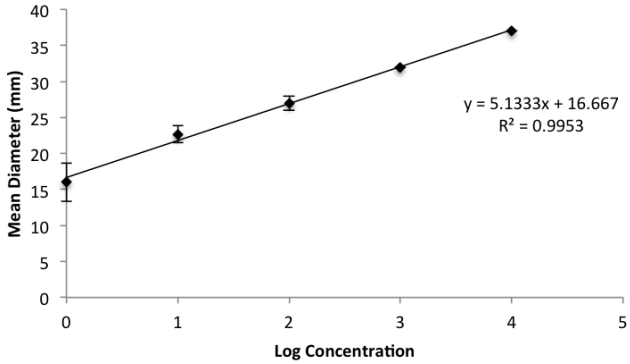


Figure 6 -12: Calibration curve showing a plot of mean diameter versus log concentration for gentamicin. The mean diameter calculated from calibrations obtained on three different days are plotted against $\log_{10}$ of concentration values (n = 3). Error bars representing standard deviation are displayed for each point.

6.6.8.2 Penetration of antimicrobial agents through *P. aeruginosa* biofilms

The assembly was set up as shown in figure 6 -1. A control assembly was set-up and contained no biofilm. An antimicrobial disc containing ~30 µg either tobramycin was placed on the nitrocellulose membrane and 20 µL of distilled water added and the entire assembly was incubated at 37°C for 24 hours.

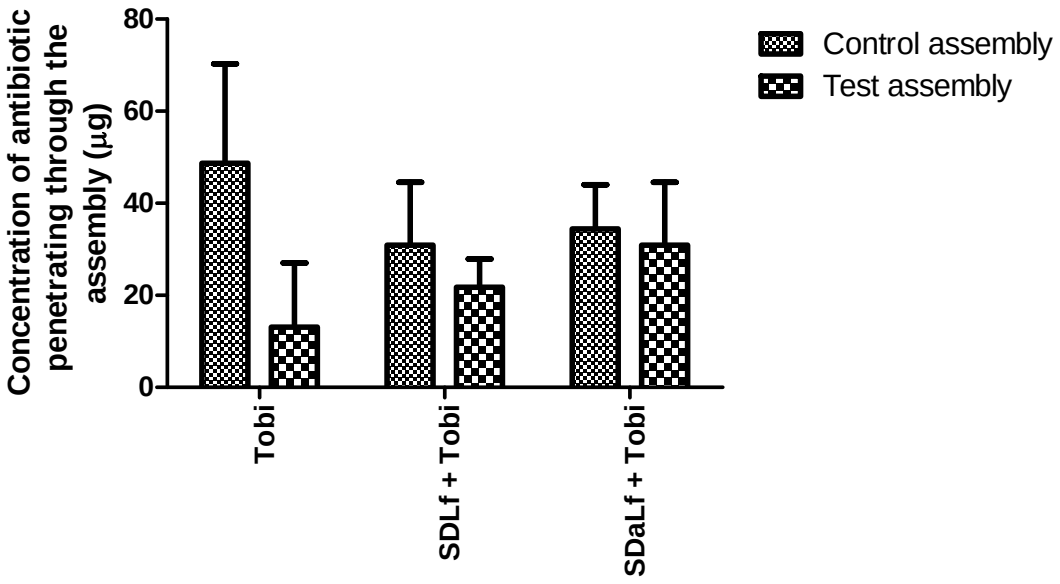


Figure 6- 13: Penetration of various tobramycin formulations through *P. aeruginosa* biofilms. While the control assembly contained no biofilm, the test assembly contained a biofilm. The concentration of tobramycin penetrating through the biofilm was estimated from a calibration plot of zone diameter against log of concentration. No significant difference was found between the concentration penetrating through the control assembly compared with the concentration penetrating through the test assembly. Values are mean ± SD.

The penetration of various antimicrobials formulations through *P. aeruginosa* biofilm showed that while 48.7 ±21.6, 30.9 ±13.7 and 34.4 ±9.6 µg of Tobi, SDLf + Tobi and SDaLf + Tobi were able to penetrate through the control assembly (i.e. no biofilm present) respectively, only 13.1 ±13.9, 21.8 ±6.1 and 30.9 ±13.7 µg of Tobi, SDLf + Tobi and SDaLf + Tobi were able to penetrate through the test assembly (i.e. in the presence of *P. aeruginosa* biofilm) thereby indicating that the mean percent penetration of tobramycin alone through the biofilm was 24.14 ±16.9 %. In comparison, 80.1 ±34.5 and 87.8 ±21.1 of co-spray dried lactoferrin-tobramycin and co-spray dried apo lactoferrin-tobramycin penetrated through the *P. aeruginosa* biofilm.

A similar setup was also used to determine the penetration of gentamicin through *P. aeruginosa* biofilms. The amount of gentamicin, co-spray dried lactoferrin-gentamicin and co-spray dried apo lactoferrin-gentamicin passing through the control assembly (i.e. no biofilm present) were determined to be 172.4 ±75.4 µg as estimated from the calibration curve.

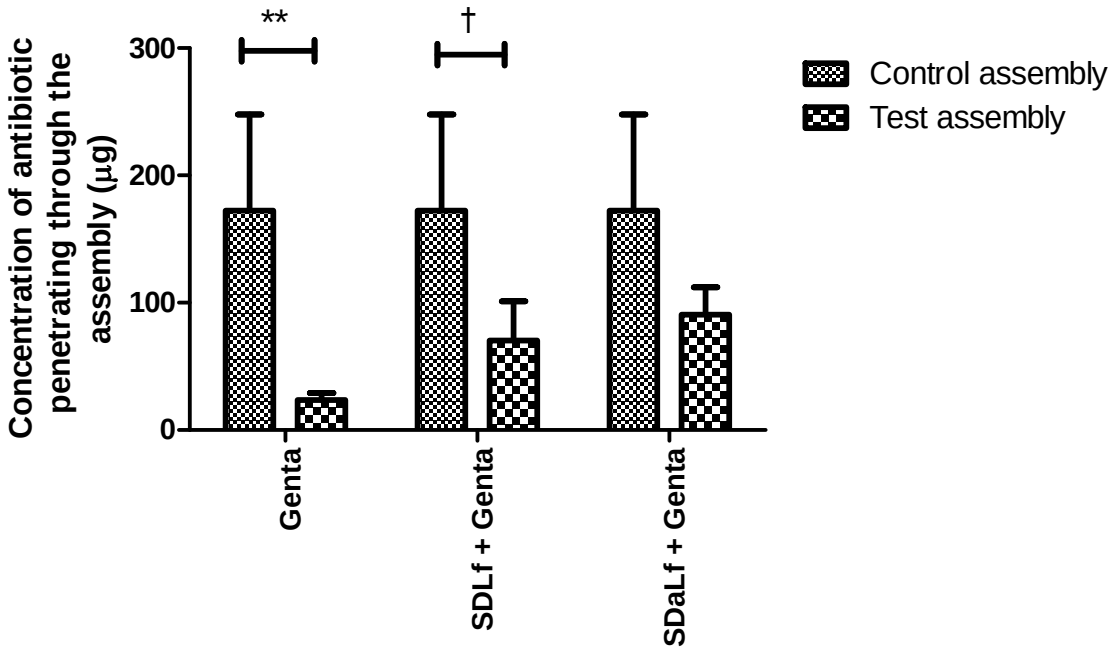


Figure 6- 14: Penetration of various gentamicin formulations through *P. aeruginosa* biofilms. While the control assembly contained no biofilm, the test assembly contained a biofilm. The concentration of gentamicin penetrating through the biofilm was estimated from a calibration plot of zone diameter against log of concentration. Values are mean ± SD. Comparisons of groups were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; ** indicates $p < 0.01$ compared to the control assembly of gentamicin and † indicates $p < 0.05$ compared to the control assembly of the SDLf + Genta.

In the test assembly, i.e in the presence of *P. aeruginosa* biofilms 23.6 ±5.6, 70.3 ±30.8 and 90.6 ±21.5 µg of gentamicin, co-spray dried lactoferrin-gentamicin and co-spray dried apo lactoferrin-gentamicin respectively was able to penetrate through the biofilm. Indicating that about 14.6 ±3.5, 40.8 ±0 and 56.2 ±13.3% of gentamicin were able to penetrate through the biofilm matrix. Statistical analysis showed that significant amounts

of gentamicin ($p < 0.01$) and SDLf + Genta ($p < 0.05$) were retarded by the *P. aeruginosa* biofilms.

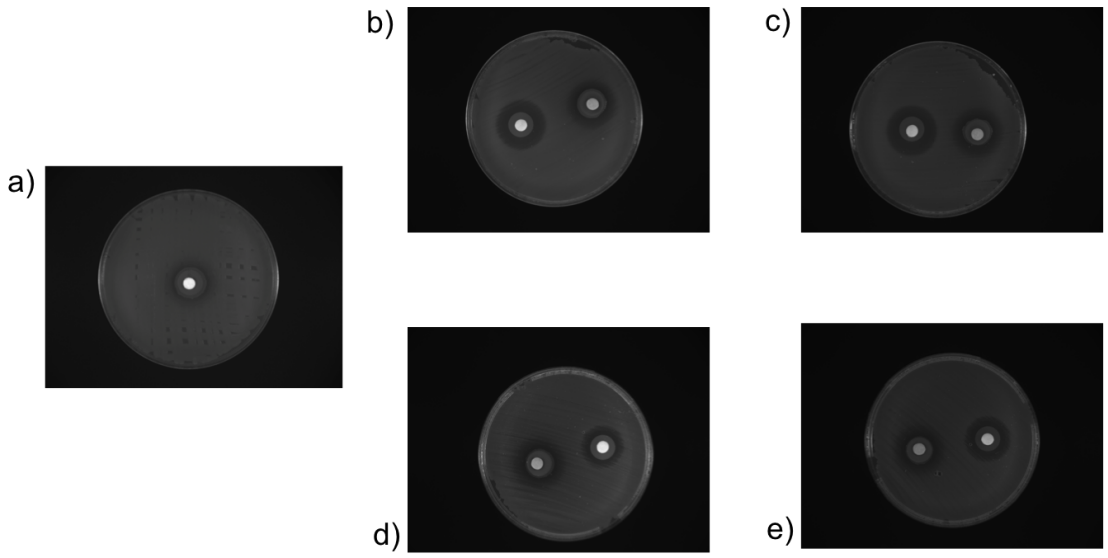


Figure 6 -15: Zone of inhibition studies showing antibiotic penetration through *P. aeruginosa* strain PAO1 biofilms grown on track-etched polycarbonate membranes. All plates were seeded with the control organism – *Escherichia coli* NCTC 10418. A track-etched polycarbonate membrane on which a 48 hr biofilm of *P. aeruginosa* was grown or a sterile track-etched polycarbonate membrane without biofilm (serving as a control) were placed on the seeded agar plates and the various tobramycin and gentamicin antibiotic disk containing a 30 µg of tobramycin or 200 µg of gentamicin or combinations were placed at the centre of each plate and incubated at 37 °C for 24 h. The zone of inhibition was measured and the concentration of antibiotic penetrating through the biofilm matrix estimated from a calibration curve.

6.6.9 Confocal laser scanning microscopy of biofilms exposed to various antimicrobial agents.

To further characterize the penetration of the formulations through *P. aeruginosa* biofilms grown on cover slip. The biofilms were exposed to the various agents or in the case of the control, remained untreated for 24 hours following which propidium iodide was used to stain dead cells and syto-9 was used to stain bacteria on the coverslip. Confocal microscopy of untreated *P. aeruginosa* biofilms shows very little propidium iodide stain (red fluorescence, indicating dead cells) although more syto-9 (green fluorescence, indicating live cells) was observed (fig. 6 -14a). Confocal microscopy of biofilms of *P. aeruginosa* treated with tobramycin alone shows the relatively low penetration (indicated by the low intensity of the red fluorescence- propidium iodide showing limited cell death) of the antibiotic into the biofilm (Figure 6 -14b). Upon treating the biofilm with SDLf + Tobi and SDaLf + Tobi, there was deeper penetration (indicated by the high intensity of the red fluorescence that indicates propidium iodide uptake into dead bacterial cells) (Figure 6 -14c -d). The penetration of gentamicin into the biofilms was also very low (Figure 6 -14e) while the penetration of the combinations of lactoferrin and gentamicin shows relatively deeper penetration of the antimicrobial agents (Figure 6 -14f -g). Overall, the co-spray dried apo lactoferrin-tobramycin/gentamicin formulations reduced viability the most, evidenced by the intense yellow colouration of the composite image.

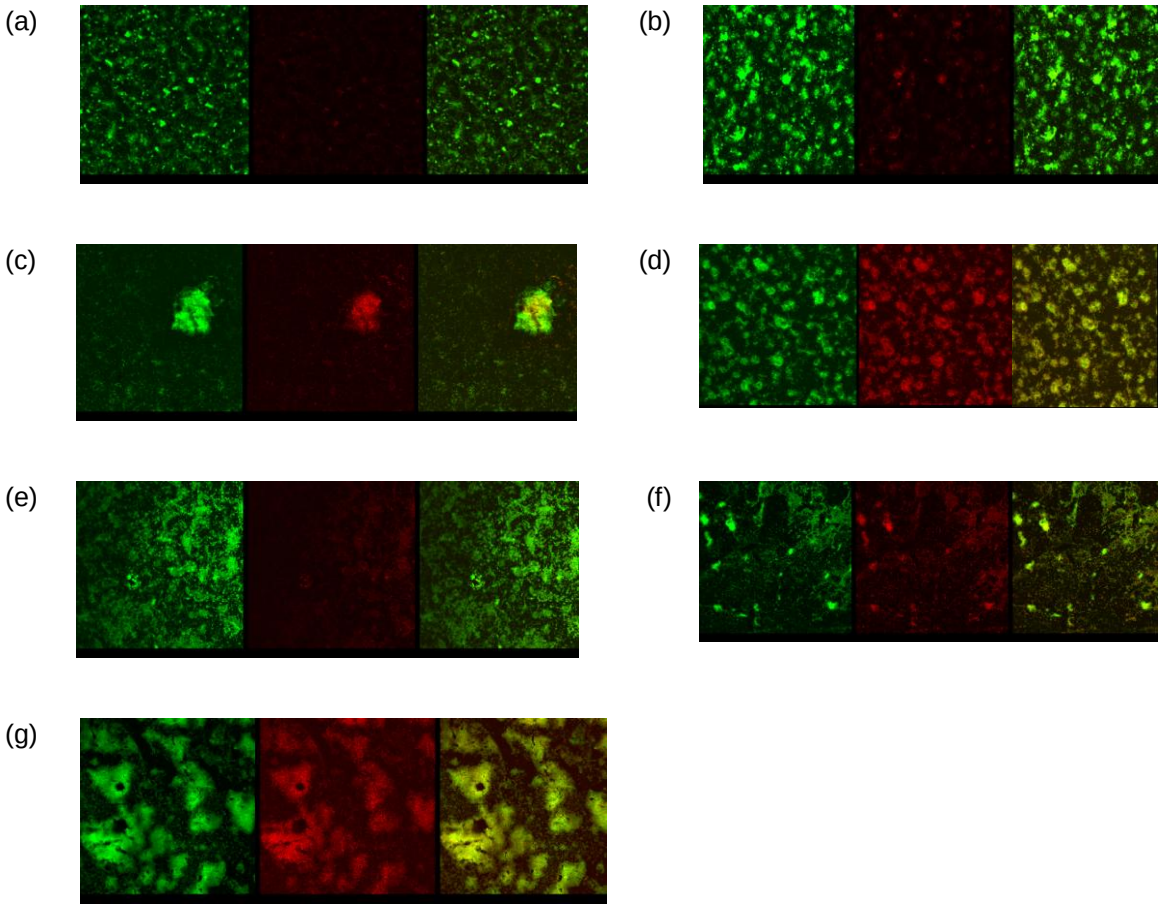


Figure 6 -16: Confocal microscopic images of *P. aeruginosa* biofilms formed after 72 h growth on glass slide. The biofilms were stained using the LIVE/DEAD® staining assay and visualised using confocal laser scanning microscopy. The green-fluorescent SYTO 9 staining (left-most images) is indicative of live cells while the red-fluorescent propidium iodide staining (middle image) is indicative of dead cells. The yellow image on the right shows the overlay to indicate increased number of dead cells which are stained by propidium iodide (a) untreated biofilms; (b) biofilms treated with 1mg/mL spray-dried tobramycin for 24 h; (c) biofilms treated with 1mg/mL co-spray dried lactoferrin-tobramycin for 24 h; (d) biofilms treated with 1mg/mL co-spray dried apo lactoferrin-tobramycin for 24 h; (e) biofilms treated with 1mg/mL spray-dried gentamicin for 24 h; (f) biofilms treated with 1mg/mL co-spray dried lactoferrin-gentamicin for 24 h; (g) biofilms treated with 1mg/mL co-spray dried apo lactoferrin-gentamicin for 24 h. Control shows little cell death (very low red fluorescence), while biofilms treated with the various antibiotics showed different degrees of cell death (increased red fluorescence compared with the control). The images are representative of two independent experiments.

6.7 Discussion

The idea of combining various antibiotics to increase their efficacy has been around for decades. In the present study, both the powder properties and antibacterial activities of a novel inhaled antibiotic combination consisting of a 1:1 spray-dried combinations of lactoferrin and tobramycin/gentamicin were investigated.

It was found that upon spray drying the various preparations, the spray-dried yield of the various powders varied, depending on aminoglycoside composition. Co-spray drying lactoferrin with tobramycin/gentamicin was found to produce a high percent yield of over 87.3 ±2.1% compared to co-spray drying apo lactoferrin with tobramycin/gentamicin or even spray drying tobramycin or gentamicin alone (Table 6.1). As this was the first time such a combination has been prepared, there were no prior data to compare the results with, however compared with the typical yield of spray-dried powders which is usually between 20% and 50% (Labrude *et al.,* 1989) the percent yield of the spray-dried powders obtained in this study were well above average.

A residual moisture content of between 3.2 ±0.4% and 4.0 ±0.2% was obtained indicating that the drying process was efficient in evaporating most of the moisture. The residual moisture content of spray-dried powders have been shown to be a particular important factor in determining the long term stability of dry powders especially those containing proteins. Typically, residual moisture contents of between 2% and 8% (Tzannis and Prestrelski, 1999b; Maa *et al.*, 1998) have been previously reported upon spray drying proteins alone or in combination with excipients. In this study however, a residual moisture content of between 3 and 4 % was obtained and consequently, the dry powders produced would be expected to exhibit better long term protein biochemical

stability.

The zeta potential measurement of the various formulations showed that the net charge of spray-dried tobramycin was -3.34 ±3.1 while the net charge of gentamicin was -2.99 ±2.0 (table 6.1). Upon co-spray drying lactoferrin with the tobramycin/gentamicin, a net positive charge of 2.67 ±0.4 (SDLf + Tobi), 1.14 ±0.9 (SDaLf + Tobi), 2.29 ±0.7 (SDLf + Genta) and 0.97 ±0.8 (SDaLf + Genta) were obtained indicating that the co-spray dried formulations were cationic and would therefore have greater antimicrobial effect on Gram-negative bacteria (like *P. aeruginosa* which have anionic membranes) (Gottenbos *et al.,* 2003) compared to spray-dried tobramycin or spray-dried gentamicin only formulations. Probably, the net positive charge of the co-formulation may account for the greater antimicrobial activity of the various combinations on both planktonic and biofilms of *P. aeruginosa* compared to the monotherapies.

Analysis of the particle size distribution of the various spray-dried powders show that the *d*[*v,*50] of the powders ranged between 4.90 ±0.54 and 6.23 ±0.30 µm. Particles within this size range will be expected to deposit in the lower regions of the airway according to the prediction models by Martonen and Yang, (1996) and will therefore provide distinctive benefits such as; the required amount of drug at the site where it is most needed and a cost effective medication due to minimization of waste. The span of particles (table 6.2), which was found to range between 1.96 ±0.17 and 2.68 ±0.21 only further, corroborate deposition of the drug at the desired location. The similarity in particle size distribution was expected, as all the powders were prepared under similar particle engineering condition.

XRPD studies showed that all the spray-dried formulations had a broad halo and lacked resolved reflections (figure 6 -3a and 6 -3b) this is clearly indicative that all the spray-dried powders were predominantly amorphous as the powders showed no evidence of crystallinity. Hence indicating that while the powder formulations would be expected to have a good solubility in biological fluids they may however present with stability issues during long-term storage. DSC scans for the various co-spray dried powders revealed a broad endothermic peaks usually between 2 and 7 °C, indicative of moisture loss and no crystalline melting temperature a further indication of the amorphous nature of the spray-dried powders.

The activity of the various antimicrobial agents against planktonic and biofilms of *P. aeruginosa* were evaluated. SDaLf + Tobi was found to be the most potent with MIC values of 0.58 ±0.1 µg/mL and MBC values of 1.08 ±0.1 µg/mL (Table 6.3). The FIC index shows that all the various protein-antibiotic combinations showed synergism (i.e. the combined effect of the combinations were larger than the effect predicted from the sum of each agent alone). The highest degree of synergy was obtained with the SDaLf + Genta formulations (Table 6.3). The indications of this finding is that by combining tobramycin/gentamicin with apo lactoferrin, the minimum bactericidal concentration of tobramycin and gentamicin against a, *P. aeruginosa* can be significantly reduced. To our knowledge, this is the first study to show that the activity of an antibiotic currently indicated for the management of infections in cystic fibrosis has increased activity against *P. aeruginosa* when combined with lactoferrin. Previously, Singh *et al.,* (2000) reported that combined actions of lysozyme and tobramycin as assessed by a checkerboard assay produced an additive interaction (FIC index 1.04) in the killing of *Escherichia coli* DH5α whereas lysozyme in combination with lactoferrin showed synergistic interaction (FIC index 0.23) in the killing of the same *E. coli* strain. As their

choice of test organism is not relevant CF and the stability of the combinations were not considered, the antimicrobial activities of the powder formulations in this study was deemed to be relatively good and fit for purpose.

Time-kill experiments were also carried out to evaluate the effects of the individual agents as well as the combinations over time. The time-kill curves are presented in Figures 6 -4 and 6 -5. A bactericidal effect as defined by the Clinical and Laboratory Standards Institute (1999) is a 3 $\log_{10}$ reduction in cfu/mL or a 99.9 % kill over a specified time period (in this case it was 1 hr).

Results show that while spray-dried tobramycin and gentamicin were poorly active against planktonic *P. aeruginosa* at 1 µg/mL within 60 mins, co-spray dried combinations of lactoferrin and tobramycin/gentamicin produced significant and amplified bactericidal effect at the same concentration within the same time period. This amplified bactericidal effect resulted in the overall reduction of the cfu/mL by 3 $\log_{10}$ units. The co-spray died apo lactoferrin-tobramycin/gentamicin combinations performed even better than other formulations (since it reduced the cfu/mL by over 5 $\log_{10}$ units) presumably this was due to synergy. The significance of this is that by combining lactoferrin with aminoglycoside antibiotic, less of the aminoglycoside combination would be required to kill more *P. aeruginosa* bacterial cells, hence there is a chance that such a combination may probably improve compliance (due to possible reduction in the adverse effects of aminoglycoside).

Given that chronic *P. aeruginosa* infection in the airway usually results from biofilm

formation, we sought to assess the antimicrobial activity of the various formulations on *P. aeruginosa* biofilms grown on abiotic (polystyrene) surfaces. This evaluation was carried out in both iron free (i.e. casamino acid only) and iron rich environments so as to determine the efficacy of the various formulations on biofilms of *P. aeruginosa* in both extremes of iron concentrations especially since increased levels of iron in the airway surface liquid has been cited as the possible reason for increased persistence of *P. aeruginosa* (Stites *et al.*, 1998; Reid *et al.*, 2007b). Results obtained in this study indicate that by supplementing the growth media with low levels of iron, the amount of biofilm quantified increased significantly (figures 6 -6, and 6 -7) this observation is in agreement with previous studies by Yang *et al.,* (2007) and Banin *et al.,* (2005) who have all reported low iron concentrations to be essential for *P. aeruginosa* biofilm formation. While Yang *et al.,* relate this increased biofilm development to the up-regulation of *pqs* genes (a quorum sensing system) and the increased formation of extracellular DNA, Banin *et al.,* relate this occurrence to the ability of the ferric uptake regulator, Fur, (a protein which controls expression of iron responsive genes in many bacteria including *P. aeruginosa*) of the bacteria to promote biofilm formation. Any or all of these reasons may probably account for the high amount of biofilm quantified when *P. aeruginosa* was grown in the iron supplemented media, however, upon incubating the bacteria with the various formulations, spray-dried tobramycin and spray-dried gentamicin exhibited some biofilm prevention activity in both iron rich and iron free environments, the inclusion of lactoferrin into the various formulations (especially apo lactoferrin) significantly restricted the development of *P. aeruginosa* biofilms even in the iron supplemented environment (Figure 6 -6). A possible explanation for this observation may reside in a previous report by Singh et al (2002) who found that exposing *P. aeruginosa* to lactoferrin resulted in increased incessant twitching motility of the bacteria, a phenomenon which usually means that the *P. aeruginosa* cells do no attach to a surface (a necessary step involved in the formation of microcolonies).

Upon exposing already existing biofilms to the various formulations, spray-dried tobramycin and gentamicin were found not to have any significant effect on biofilms formed in both iron free and iron supplemented environments. These observations were found to be in agreement with those of Tré-Hardy *et al.,* (2008) and Moreau-Marquis *et al.,* (2009) who have previously reported that tobramycin failed to disrupt established biofilms. The reason for these occurrences may reside in the mechanism of action of the aminoglycoside. Aminoglycosides generally inhibit the growth of bacteria cells, but to achieve this they need to be in contact with the bacteria cell, however, as they have poor penetration through bacterial biofilms due to the high degree to which they bind to the alginate matrix (Gordon *et al.,* 1988) which is related to their overall charge (found to be net negative, table 6.1), thereby rendering their antimicrobial activity ineffective. On the other hand, co-spray dried apo lactoferrin and tobramycin/gentamicin powder formulations were found to significantly ($p < 0.01$) disrupt preformed biofilms (Figure 6 - 7) this may also have some relationship with their overall charge which was found to be net positive (table 6.1) and hence would mean that the negatively charged biofilm membrane formed by the bacteria are more readily disrupted. The effects of co-spray dried apo lactoferrin-tobramycin/gentamicin powder formulations on preformed biofilms have not been previously reported. But unlike the previous report by McCaughey *et al.,* (2012) which found that 5.120 mg/mL of a 4:1 (w/w) combination of fosfomycin:tobramycin was required to effectively disrupt preformed biofilms, results from our study showed that 1 mg/mL of a co-spray dried apo lactoferrin-tobramycin/gentamicin was enough to significantly disrupt preformed *P. aeruginosa* biofilms.

The increased susceptibility of *P. aeruginosa* to antibiotics in the presence of lactoferrin was thought to be as a result of iron chelation as previously indicated (Singh *et al.,* 2002; Banin *et al.,* 2005; Banin *et al.,* 2006; Singh, 2004). We decided to investigate the degree to which iron chelation contributes to observed activities of our lactoferrin-tobramycin/gentamicin combinations in preventing or disrupting *P. aeruginosa* biofilms. Four iron chelators were chosen and combined with tobramycin. The combinations of tobramycin and the various iron chelators were exposed to *P. aeruginosa* growing in an iron free medium and it was observed that the iron chelators were not as effective as the co-spray dried apo lactoferrin-tobramycin preparation in preventing biofilm formation. Iron deprivation however, is not the sole mechanism by which lactoferrin/apo lactoferrin is able to increase the susceptibility of *P. aeruginosa* to antibiotics. Some other additional mechanism of action have been described by Ellison *et al.,* (1990) who showed that lactoferrin and transferrin alter the outer membrane of Gram-negative bacteria thereby sensitizing the bacteria to both a hydrophobic antibiotic and a surface-active agent. A study by Leitch and Wilcox (1999a) have also similarly reported that the antibacterial synergy of lactoferrin and lysozyme against Gram-negative bacteria is dependent on the ability of the protein to disrupt the outer-membrane of the bacteria or the biofilm. Particularly, they found that incubating *Staphylococcus epidermidis* with artificial tear fluid containing serum (ATFS) and 1.8 mg/mL of lactoferrin for 6 hours at 37°C and then adding 5.4 mg/mL of lysozyme and incubating for a further 6 hours resulted in a significant reduction of the number of colonies per mL due to disruptions to the outer membrane of the bacteria.

Understanding that one of the underlying factors of antibiotic resistance is the inability of the antibiotic to penetrate to all areas of a biofilm and effect killing of bacterial cells within the biofilm elicited investigations into how a co-spray dried powder formulation of

lactoferrin and aminoglycoside would perform. A method previously described by Singh *et al.,* (2010) was employed albeit with some modifications. Briefly, antibiotic discs containing either tobramycin or gentamicin with/without lactoferrin or apo lactoferrin were placed on *P. aeruginosa* biofilms grown on track-etched polycarbonate membranes (the setup for the experiment is shown in figure 6- 1). The zone of inhibition of *E. coli* growing on Mueller-Hinton agar plates were measured and the quantities of tobramycin or gentamicin penetrating through the biofilms assessed (fig 6- 13 and 6-14). Results obtained showed that > 80% of tobramycin and > 40 % of gentamicin penetrated through the *P. aeruginosa* biofilms when the antibiotics were combined with either lactoferrin or apo lactoferrin compared to the approximately, 24% penetration of tobramycin or approximately, 15% penetration of gentamicin when the antibiotic disk contained one agent alone. While our results extend previous reports that aminoglycosides have reduced penetration through *P. aeruginosa* biofilms, a phenomenon typically referred to as impermeability resistance (Bryan *et al.,* 1976; Maloney *et al.,* 1989; Turnidge, 2003), it also informs on a probable mechanism that explains why the various combinations significantly reduced preformed biofilms as quantified by the crystal violet assay. The reason for this occurrence is probably linked to the net cationic charge of the combination which destabilizes the negatively charged biofilm membrane thereby enabling the penetration of the antibiotic into the biofilm to effect its antimicrobial actions.

Confocal microscopy images of the P. aeruginosa biofilms grown on cover slip and treated with/without various antimicrobial agents shows varying degrees of dead cells (indicated by the red fluorescence of propidium iodide). While the untreated biofilm showed very little propidium iodided stain (fig 6 -16a), SDaLf + Tobi, SDLf + Genta and SDaLf + Genta caused the most damage to the biofilms and indicates that the antimicrobial agents were able to penetrate into the biofilm visually confirming previous

observations in the antimicrobial penetration experiments. As this is the first time these images are being made reported, there are no data to compare them against.

6.8 Conclusion

The present study is novel as it is the first to report the physical characteristics and antimicrobial activities (in both iron supplemented and iron deplete environments) of lactoferrin and tobramycin/gentamicin combinations. Overall, results from the present study show that the spray-dried combinations of aminoglycosides and lactoferrin appear to have a superior antimicrobial activity on *P. aeruginosa* PAO1 biofilms compared to monotherapy. The delivery of spray-dried tobramycin-apo lactoferrin combination to lower airway structures could be a potentially new therapeutic strategy for combating biofilm associated infections. However, before the various combinations can be used for such a therapeutic application, there is a need to investigate their *in vitro* activities.

Chapter Seven

Characterisation of *in vitro* activity of co-formulations

7.1 Summary

Several potential antimicrobial formulations have been evaluated for physicochemical properties and their activity against planktonic and biofilm cultures of *P. aeruginosa* evaluated. For an inhaled therapy to be accepted by the "host", it was important to confirm that there were no deleterious effects on the host. The work presented in this chapter used an *in vitro* cellular model to investigate the potential cytotoxic and pro-inflammatory effects of individual formulations on human bronchial epithelial cells. This work has assessed the effectiveness of the antimicrobial formulations at reducing bacterial cell viability when administered through biofilms cultured at the air-liquid interface. Four immortalised human bronchial epithelial cell lines were employed; CALU-3 (expressing high levels of wild-type CFTR), BEAS-2B (expressing wild-type CFTR), IB3-1 (cystic fibrosis cell line possessing mutant CFTR) and C38 (mutation corrected IB3-1 cells transfected with wild-type CFTR). All cell lines were grown in submerged cell culture at an air-liquid interface (ALI; a condition which promotes cellular differentiation). Confluent submerged cells were exposed to formulations for 24 hours prior to determination of cell viability (using CellTiter-Blue® (CTB)) and quantification of chemokine interleukin-8 (IL-8) (measured using an enzyme linked immunosorbent assay) this would allow discussion of the metabolic activity of the epithelial cells. A co-culture model whereby a *P. aeruginosa* biofilm was grown on bronchial epithelial cells was employed to determine the effectiveness of formulations on bacterial biofilm growing in a relevant physiological environment. Results obtained show that the exposure of any of the epithelial cell lines to the various formulations produced no reduction in cell viability as determined by CTB assay. The amounts of IL-8 produced varied across the different epithelial cells with BEAS-2B cells releasing the most amounts. Co-spray dried combinations of lactoferrin or apo lactoferrin with tobramycin or gentamicin reduced the established biofilm mass by 4- to 5-log units across all epithelial cell types. Neither the lactoferrin/apo lactoferrin nor tobramycin/gentamicin formulations

had such an effect. This data suggests that lactoferrin or the aminoglycosides are non toxic to epithelial cells and that the combined use should be effective in the treatment of patients with resistant *P. aeruginosa* lung infections.

7.2 Introduction

There is an ever increasing pressure on the pharmaceutical industry to ensure that pharmaceutical products meet prescribed claims, are devoid of side effects or adverse effects and are effective irrespective of a patient's genetic predispositions. It therefore means that, the more we know about the physicochemical properties of a drug candidate during the pre-screening stages, the more likely it is that the drug candidate will progress to be used as a medicine. Consequently, the last step of drug development usually involves *in vivo* studies in animals to assess the toxicity, absorption, metabolism and clearance of the drug compound. However, ethical arguments against the use of animals in research have been made for well over two decades (Rowan and Andrutis, 1990) and continue to be made at the present time by various humane societies all over the world.

7.2.1 The growing case for the use of non-animal alternatives for toxicity testing

In the UK, the growing public criticism against the use of animals in research has driven the evolution of relevant legislation and regulatory policies such as the Animals (scientific procedures) act of 1986 (a replacement for cruelty to animals act of 1876) which guides the use of animals in research. In fact, the regulatory policy (governing the use of animals in research) in the UK is considered to be one of the tightest regulations in the world. This is due to the strict requirements needed for personal, project and institutional licenses and also the requirement of explicit cost benefit assessment that has to be put forward for every application requesting the use animals in research (The Animal Procedures Committee, 1986).

Hence, the use of alternative methods based on the 3Rs approach (Replacement, Reduction and Refinement) during toxicity studies is now becoming a popular approach among the scientific community. Independent scientific agencies like the National Centre for the Replacement, Refinement and Reduction of animals in Research (NC3Rs) and the Fund for the Replacement of Animals in Medical Experiments (FRAME) are typical examples of institutions which currently exist to drive the development and validation of alternatives to animal toxicity testing (www.nc3rs.org.uk; www.frame.org.uk). It is arguably the case that *in vitro* assessments do not always correspond to *in vivo* responses, there is still a need for better non animal alternatives specifically for toxicity testing. This is because, the development of *in vitro* models that predict starting doses for *in vivo* drug study would save considerable animal numbers.

The scientific interest to develop non-animal experiments as alternative approaches to toxicity testing has gradually increased, international laboratories including the European Union Reference Laboratory for Alternatives to Animal Testing **(**EURL ECVAM) are fully dedicated to the work of developing and validating these non-animal alternatives. For example, the slug mucosal irritation assay (Adriaens, 2006), BCOP assay (Gautheron et al., 1992) and the Isolated Chicken Eye tests (Prinsen and Koëter, 1993) have both been developed and validated to replace the Draize rabbit eye test (Draize et al., 1944), which was the most widely used toxicity test for substances applied to the skin or mucosal surfaces (Oecd, 2012).

7.2.2 The use of *in vitro* cell culture techniques in cytotoxicity assessments

In vitro cell/tissue culture techniques are techniques currently being used by biomedical researchers for non-animal based toxicity testing as they provide physiologically-

relevant response and are cheaper alternatives for carrying out preliminary biological tests on lead compounds. Tests are simpler, robust, fast and provide good experimental control, saving time when compared to animal studies (Hertzfeld and Myers, 1987; Eisenbrand *et al.*, 2002).

Various *in vitro* and *ex vivo* cell/tissue models are currently being used to study lung related diseases (Choe *et al.*, 2003; Choe *et al.*, 2006b; Choe *et al.*, 2006a). *In vitro* and *ex vivo* cell culture models have also proved to be good predictors of *in vivo* toxicities of new drugs since they provide the ability to focus on the effects of the drug on one particular cell type and in so doing are more likely to reflect what happens in the human body to that particular cell type compared to animal models (Bérubé *et al.*, 2010). However, a disadvantage of *in vitro* cell culture models maybe that in animal models, many cells types exist in synergy. So, cells cultured in vitro are not truly representative of all the epithelial cell types that exist *in vivo*. In addition, the cell culture system lacks all the hormones, nervous system and immunity which are present in animal models. However, In vitro models are good predictors of toxicities of new drugs to be employed for managing respiratory diseases, as during development, the drugs can be examined for inflammatory cytokine production, mucus hyper secretion, goblet cell hyperplasia (GCH), and epithelial hyperplasia and metaplasia (Rogers, 2003). The need to carefully select the most suitable model that is appropriate for determining the possible effects of inhaled therapies in terms of how they cause injury or hinder repair in the respiratory tract is crucial for improving the rate of bench-to-bedside progression of potentially new therapies.

For the purpose of investigating the cytotoxicity and pro-inflammatory effects of the presented formulations and co-formulations, four immortalised human bronchial

epithelial cell lines (CALU-3, BEAS-2B, IB3-1 and C38) were selected as models for the response of human bronchial epithelial cells to these novel formulations.

7.2.2.1 CALU-3 cells

CALU-3 cells are derived from a bronchial adenocarcinoma, they form tight junctions and express a high level of functional CFTR, they therefore secrete Cl^- in response to cAMP-elevating agents via apical CFTR (Haws et al., 1994). CALU-3 cells are routinely used to model *in vitro* airway epithelial cell responses because they produce features consistent with differentiated and functional cells (Zhu et al., 2010). The CALU-3 cell line was chosen because they are an adequate model of the bronchial epithelium with respect to morphology and simple solute barrier functions. It is however important to mention that the cancerous origins of CALU-3 cells may interfer with cellular responses when employed as *in vitro* models hence raising concerns over how representative of the “normal airways” they are.

7.2.2.2 BEAS-2B cells

BEAS-2B cells were originally derived from normal human bronchial epithelial cells obtained during autopsy. To produce an immortalised cell line, the epithelial cells were infected with replication-defective simian virus 40 (SV40 virus) or an Adenovirus 12-simian virus 40 hybrid (Ad 12-SV40 hybrid virus), and then subsequently cloned (Reddel et al., 1988). BEAS-2B cells have been very useful in studies involving airway structure and function, including phenotyping and cytokine regulation (Atsuta *et al.*, 1997; Mullol *et al.*, 1996), investigating the direct cytotoxic effects of environmental tobacco smoke on airway epithelial cells (Sun et al., 1995), chemokine expression in CF epithelia (Schwiebert et al., 1999) and assessment of cytotoxicity of occupational particles (Veranth et al., 2007). Also BEAS-2B have been employed understand the mechanisms of pneumococcal infection (Adamou et al., 1998) and to study the expression and

activity of drug metabolizing enzymes (Eaton *et al.*, 1996; Proud *et al.*, 1994). BEAS-2B cells have also been shown to retain electron microscopic features of epithelial cells (as shown by the presence of keratin during indirect immunofluorescence for cytokeratin) as well as being nontumorigenic following s.c. injection of the SV40 transformed cells into nude mice thereby making them a useful cell line for studying multistage bronchial epithelial carcinogenesis (Reddel et al., 1988). BEAS-2B have been used for all the above mentioned studies because they are a fairly easy epithelial cell line to maintain in culture while studying the effects of chemicals on them.

7.2.2.3 IB3-1 cells

IB3-1 cells are an immortalized cell line created in 1992 from a primary culture of bronchial epithelial cells isolated from a patient with cystic fibrosis. The cells were transformed with a hybrid virus, adeno-12-SV40, making them immortalised and suitable substrates for studies involving mutant CFTR (Zeitlin et al., 1991). IB3-1 cells retain characteristics of epithelial cells and the fact that they are deficient in the cyclic AMP-mediated protein kinase A activation of chloride conductance which is a diagnostic feature of CF; (Flotte et al., 1993), makes them suitable for use as a CF model. Genotypically, the cell line is a compound heterozygote containing the delta F508 mutation and the nonsense mutation, W1282X, with a premature termination signal (Schneider et al., 1999).

7.2.2.4 C38 cells

The C38 cells are essentially derived from the IB3-1 cell line, but have the additional transfection of wild-type CFTR in order to correct the CF phenotype (Anderson et al., 2008). Inclusion of C38 cells aids to isolate airway epithelial cell responses that occur specifically due to ΔF508 and W1282X CFTR mutations, in other words, assessing our

formulations on both IB3-1 and C38 cells enables the identification of responses specific to defective CFTR in airway epithelium.

By employing these four immortalised epithelial cells for our investigation, a basis for comparison of the responses between non-CF (CALU-3, BEAS-2B and C38) and CF (IB3-1) airway epithelial cells is established thereby eliminating bias which may have resulted if only one cell line had been exclusively employed. The IB3-1 cell line expressing the ΔF508 and W1282X mutant forms allows for simulation of a disease specific response (in this case cystic fibrosis lung model) thereby making it well suited for determining the effects of various antimicrobial agents in their eventual, intended target- the CF airway. Furthermore, by employing the use of immortalised cells, there was an added advantage of unlimited cell passages, making the assessment/investigation more cost-effective compared to using animals or even primary cells.

7.2.3 *In vitro* methods to assess cytotoxicity of compounds in the airway

In vitro cytotoxicity tests which employ relevant airway cells have proved most useful for determining the toxicity of compounds in the early developmental stages. Several indices have been suggested as possible indicators for inhaled therapy cytotoxicity including; cellular metabolic activity, assessment of cell membrane breakdown (measurement of endothelial leakage), estimation of cell numbers, cilliary beat frequency (supplies information on epithelial viability and function) and assessment of electrical resistance (measures the integrity of the epithelial layer). The first and most readily observed effect following exposure of cells to toxic compounds are morphological alterations of the cell layer which may be visualized by light microscopy (for gross

modifications) or transmission or scanning electron microscopy (for fine ultrastructural modifications). Other indices which are specific indicators of respiratory system toxicity are; changes in epithelial cell viability and changes in epithelial cell attachment

In vitro cytotoxicity tests carried out in isolated cell models derived from human origin have shown to be useful in predicting human acute toxicity (Ekwall *et al.*, 2000; Bondesson *et al.*, 1989) and for predicting *in vivo*-equivalent and biologically relevant doses for a particular compound (Bérubé *et al.*, 2010) *In vitro* test have provided meaningful information on parameters such as genotoxicity, induction of mutations and programmed cell death. The popularity of *in vitro* cytotoxicity assays has been widely driven by the need to evaluate the potential toxicity of large numbers of compounds making sure to limit animal experimentation whenever possible while using small quantities of a test compound.

7.2.3.1 Techniques for assessing the cell viability of *in vitro* cell cultures

Three basic principles generally govern *in vitro* cell viability measurements and they include; measurement of cellular metabolic activity, assessment of cell membrane breakdown and estimation of cell numbers

1. ***Measurement of cellular metabolic activity***: this principle assumes that a reduction in cellular metabolic activity signifies cellular damage, for example, the CellTiter-Blue® assay uses an optimized reagent containing resazurin, which is reduced to fluorescent resorufin by metabolically active cells. Resorufin production can be determined by the use of a fluorometer and gives an indication of cell viability, where low relative fluorescence units (indicative of low levels of resorufin) are considered to signify low cellular metabolic activity.

2. ***Assessment of cell membrane breakdown***: the cell membrane is a functional barrier around the cell, it permits the exchange of materials into and out of the cells through specific transporters, receptors and secretory pathways, when damaged they become 'leaky' and this forms the basis of assessing cell viability. For example, in viable cells, the enzyme lactate dehydrogenase (LDH) is present in the cytosol and cannot be measured extracellularly. Once cell damage occurs, e.g on exposure of cells to a damaging test compound, the presence of extracellular LDH indicates cellular toxicity. Other endpoint indicators of cell membrane damage measure the uptake of various dyes which is usually not able to penetrate intact cells.

3. ***Estimation of cell numbers***: this method is most often suited for adherent cell types such as epithelial cells. On exposure of these cell types to a damaging test compound, dead cells become detached from the cultureware. It is therefore possible to directly quantify the number of live cells by discarding the non-adherent cells and counting the adherent cells or measuring the total protein (in adherent cells) or quantifying the DNA levels (which is proportional to the number of remaining live cells). This method is usually not very accurate as a number of factors are responsible for epithelial cell detachment from the cultureware.

Changes in cellular metabolism is the better predictor of early cell injury when compared to changes in cell membrane permeability or live cell number determinations. We chose to assess the cytotoxicity of the various antimicrobial agents on the epithelial cells by measuring their cellular metabolic activity.

7.2.3.2 Interleukin-8 (IL-8) assessment

In vitro quantitative determinations of human IL-8 in an isolated cell culture model is a potentially useful tool for predicting *in vivo* inflammatory properties of new therapeutic agents. Interleukin-8, also known as neutrophil activating peptide 1 (NAP-1), granulocyte chemotactic protein 1(GCP-1), monocyte-derived neutrophil chemotactic factor (MONAP) and leukocyte adhesion inhibitor (LAI) (www.Genenames.Org) is a non glycosylated protein of 8 kDa. It is made up of 72 amino acids, belongs to the chemokine superfamily and specifically chemoattracts neutrophils (Baggiolini *et al.*, 1989; Zwahlen *et al.*, 1993). Upon appropriate stimulation, IL-8 is produced by various cells including; monocytes, alveolar macrophages, endothelial cells, fibroblasts keratinocytes, hepatocytes chondrocytes, glioblatoma cells, mesothelial cells and epithelial cells (www.copewithcytokines.org). While fibroblast, epithelial cells and hepatocytes secrete IL-8 in response to IL-1-alpha, IL-1-beta, and Tissue Necrosis Factor- alpha (TNF-alpha) stimulation, monocytes, endothelial cells, epithelial cells and alveolar macrophages additionally express IL-8 when induced by bacterial lipopolysaccharide (Yoshimura *et al.*, 1987; Strieter *et al.*, 1988; Strieter *et al.*, 1989; Kunkel *et al.*, 1991). In the cystic fibrosis lung, enhanced macrophage production of proinflammatory cytokines including IL-1, IL-6, IL-8 TNF-alpha is produced in response to *P. aeruginosa* infection (Bonfield et al., 1995). This sustained production of cytokines and chemokines such as IL-8 in the CF airway has been implicated in the sustained neutrophil influx into the airways (Berger, 2002; Sagel *et al.*, 2007) and consequently contributes to the vicious circle of mucus retention, infection and the inflammatory cascade observed in the airways of CF patients (Ratjen, 2009).

7.2.4 Submerged versus Air-liquid interface cell culture models

Submerged cell cultures generally refer to *in vitro* cell culture models where cells are grown under submerged conditions in cultureware. Submerged cell culture techniques are most frequently used for expanding immortalised cell lines in cultureware flasks, maintaining excised tissues and also for investigating the toxicity of test agents (Zhu et al., 2010). Although many cells in the body do grow under submerged conditions, the cells in the airway are not submerged hence this method of cell culture does not recapitulate the features of respiratory epithelial cells *in vivo*. The airway epithelium differs greatly in morphology and distribution of differentiation markers compared to cells under submerged conditions (Auger et al., 2006). The cells grown at air-liquid interface (ALI) more closely resemble *in vivo* cells (Auger et al., 2006). However, submerged cell cultures have been readily employed in *in vitro* screening assesments for the acute toxicity of chemical agents not just because they are relatively cheaper to setup and manage but also because they highly reproducible (Van De Sandt *et al.*, 1999). However, their use is limited to toxicity testing of water-soluble test agents since the agents will be required to dissolve in the cell culture media in order to obtain accurate responses. On the other hand, although airway epithelial cell cultures grown at the ALI more closely resemble *in vivo* apical surfaces (especially due to the presence of cilia and mucus) compared to cells growing under submerged cell culture conditions, there are limited techniques which can be employed to analyse cells growing at ALI because disruptions to differentiated cell layers will possibly result in cell damage (Bielemeier, 2012)

7.2.5 Co-cultures of bacterial biofilms on human epithelial cells

The design of *in vitro* cell models for screening compounds have generally relied on the application for which they are intended, consequently, a variety of cell models exist for

assessing test compounds. For compounds intended for antimicrobial applications it is pertinent that the cell model methods employed in their screening *in vitro* are robust and well suited for the application for which they are intended. Therefore, in order to better understand the *in vitro* activities of novel antimicrobials intended as anti-biofilm agents, *in vitro* testing methods should incorporate biofilms and human epithelial cells. To this end, Anderson et al., (2008) have reported the development of a static co-culture biofilm system in which *P. aeruginosa* biofilms were grown on human CF-derived lung epithelial cells *in vitro*. This model has so far been exploited to screen tobramycin's anti-biofilm activity (Anderson et al., 2008), combinations of tobramycin with deferoxamine or deferasirox (Moreau-Marquis et al., 2008) and aztreonam alone and in combination with tobramycin (Yu et al., 2012). An immediate benefit of employing this co-culture model, especially in trying to simulate the CF lung *in vitro* is that it recapitulates several aspects of CF lung disease such as the commonly observed airway infections and in doing so provides a more disease-relevant model akin to what is observed in the CF lung. In order to assess the efficacy of the various formulations produced in this study, the method developed by Anderson et al., (2008) was adopted.

7.3 Aims and Objectives

This final chapter of work addresses the effects of the spray-dried formulations on human airway epithelial cells *in vitro*. Two markers will be examined; cellular viability (which gives an indication of the cytotoxic nature of the formulations and co-formulations) and IL-8 secretion (which gives an indication of the pro-inflammatory effects of the compounds). In addition, static co-culture of bacterial biofilms of *P. aeruginosa* will be grown on epithelial cells and our formulations added to these cell culture models to allow us to measure the antibacterial effects of these compounds on *P. aeruginosa* biofilms *in situ* in a relevant physiological system.

All compounds were to be tested on four different cell types in order to determine generalised cell responses rather than cell-type specific response. Thus, cultures of, CALU-3, BEAS-2B, IB3-1 and C38 are to be employed. The different formulations were administered at 10mg/ml concentration to the four cell types under submerged cell culture conditions for 24 hours, and CellTiter-Blue® reagent used to assess metabolic activity. In addition, the supernatants collected from these experiments will be analysed for IL-8 in order to evaluate the pro-inflammatory potential of the compounds. In order to assess the effectiveness of formulations on bacterial biofilm growing in a relevant physiological environment, an overnight culture of *P. aeruginosa* was introduced onto the apical (uppermost) surface of the epithelial cultures and allowed to form a biofilm. After 24 hours of treating the static co-culture biofilm with the various formulations, the cells were lysed and the Internalised bacterial colonies (in the apical portion of the Transwell insert) enumerated. The overall objective of this study was to investigate possible cytotoxicity/pro-inflammatory potentail of the antimicrobial formulations, to assess their efficacy at reucing biofilm in situ and hence to evaluate the *in vivo* usefulness of employing our formulations as therapeutic agents for managing airway infections in people with cystic fibrosis.

7.4 Materials

CellTiter-Blue® reagent was from Promega (Hampshire, UK), human IL-8 enzyme linked immunosorbent assay (ELISA) development kit was from Peprotech EC Ltd (London, UK), Nunc Maxisorp 96 well plates, Tween 20, ITS supplement, BSA and Arginine were all from Sigma (Poole, UK), Dulbecco's PBS, and all cell culture consumables and reagents were from PAA Laboratories Ltd (Somerset UK), for culture at ALI, Transwell cell culture inserts (polycarbonate emembrane, 0.4 μm pore size) were from VWR

7.5 Methods

7.5.1 Cell culture

Four human bronchial epithelial cell lines; BEAS-2B, IB3-1, C38 and CALU-3 were used in this study. For routine, submerged cell culture, cells were maintained in 75cm^2 tissue culture flasks in full medium, this was a 1:1 mixture of Hams F12/DMEM with 10% (v/v) FCS, 2mM L-glutamine and 0.1 U/mL, penicillin and 0.1 mg/mL streptomycin. Cells were passaged as described in section 2.2.20.2 when 80-90% confluent. For co-culture experiments, cells were counted and seeded onto Transwell® inserts as described in section 2.2.20.5 .

7.5.2 Treating submerged cultures with formulations.

All cell types were seeded at a density of 1 x 10^5 cells/ml in 0.5 mL full medium per well in 24-well plates and allowed to adhere overnight. Cells were serum starved by overnight incubation with antibiotic-free ITS medium (Ham's F 12/DMEM with ITS supplement and L-glutamine) before the formulations were added. The various protein

formulations, with and without aminoglycosides, were diluted in antibiotic-free ITS medium to yield a final concentration of 10 mg/mL. 0.5 mL of the test solutions were added into the wells and the cells were then incubated for a further 24 hours.

7.5.3 CellTiter-Blue® (CTB) viability assay

Following exposure of the submerged cells to the various agents for 24 hours, 0.1 mL of CTB was added to each well and cells were incubated at 37 °C for 3 hours. Upon completion of incubation, a 200 µL aliquot from each well was transferred into a black 96 well plates and the fluorescence intensity measured using a standard multi-well fluorescence plate reader (Spectramax Gemini XS, Molecular Devices, Berkshire UK) with excitation and emission wavelengths of 560 and 590 nm, respectively.

7.5.4 Quantitative measurement of IL-8 production by ELISA

Culture supernatants from epithelial cells (CALU-3, BEAS-2B, IB3-1 and C38) exposed to the various protein-antibiotic formulations, were harvested and centrifuged to remove dead cells and cellular debris before the supernatant was taken for IL-8 measurement. IL-8 was measured using a commercially available ELISA kit (Human IL-8 ELISA Development kit, by PeproTech EC Ltd, (London, UK) according to the manufacturer's instructions with some minor modifications outlined in section 2.2.20.7.2

7.5.5 Static Co-culture Biofilm assay

A model previously described by Anderson et al., (2008) was employed for this investigation with some modifications. Briefly, the four epithelial cell lines (CALU-3, BEAS-2B, IB3-1 and C38) were seeded at a density of 1×10^5 cells/ml on the apical

portion of a Transwell inserts, with 900 µL of epithelial cell growth medium in the basal portion of the insert. The transwells were incubated at 37 °C, 5 % CO_2 for four days.
On the fourth day, the epithelial growth medium on the apical portions of the Transwell® was removed and the epithelial cells are now able to differentiate at the air-liquid interface to form cilia and mucus (Matsui *et al.*, 1998b). The epithelial cell growth medium in the basolateral compartment was replaced. Subsequently, all the liquid permeating to the apical portion of the inserts (apical liquid) were removed and the basolateral medium replaced every other 4 or 5 days until the cells had assumed the required tight junction resistance. This had been previously reported by Willetts (2012) and Bielemeier (2012) to be around 11 to 14 days post apical medium removal.

With the epithelial cells growing at the ALI and following the formation of tight junctions, 100 µL of *P. aeruginosa* was innoculated at a concentration of 3 x 10^7 CFU/mL onto the apical side of the Transwells and the plates incubated for 1 hr at 37 °C, 5 % CO_2. After 1 hr, unattached bacteria were gently removed by aspirating the supernatant on the apical side before replacing with 100 µL of antibiotic free and serum free DMEM/F12 airway epithelial medium containing 0.4 % arginine – to prolong the viability of airway cells incubated with *P. aeruginosa* under static conditions (Anderson et al., 2008), and the entire set-up incubated at 37 °C, 5 % CO_2 for 2 hours. After 2 h, the arginine containing epithelial medium in the apical portion of the transwell were replaced with 100 µL of the various antibiotic agents diluted to 1mg/mL in antibiotic free and serum free DMEM/F12 airway epithelial medium and incubated at 37 °C, 5 % CO_2 for 24 hours. A control assembly was also setup which constituted the antibiotic free, serum free DMEM/F12 airway epithelial medium on cells.

Following 24 h treatment of the epithelial cells, the cells were washed three times with phosphate-buffered saline (PBS) to remove planktonic bacteria and then treated with

100 µL of 0.1% Triton for 15 minutes to lyse the cell. The lysate was vortexed for 3 minutes, serially diluted and plated onto Mueller-Hinton agar plates. The plates were then stored at at 37 °C for 18 h, following which bacterial colonies were counted to determine the number of colony-forming units (CFU)/well which remained adherent to the cells

7.6 Results

All the results have been summarised in tables 7.1 (cytotoxicity, pg 333), 7.2 (IL-8 secretion, pg 334) and 7.3 (static co-culture biofilm assay, pg 336)

7.6.1 Effect of exposure of CALU-3 cells to various antimicrobial agents on

7.6.1.1 Cytotoxicity

The cytotoxicity of the various antimicrobial agents towards CALU-3 cells in submerged cultures was evaluated using CTB. The protocol is based on the ability of viable cells to metabolise a highly purified resazurin into the fluorescent end product, resorufin.

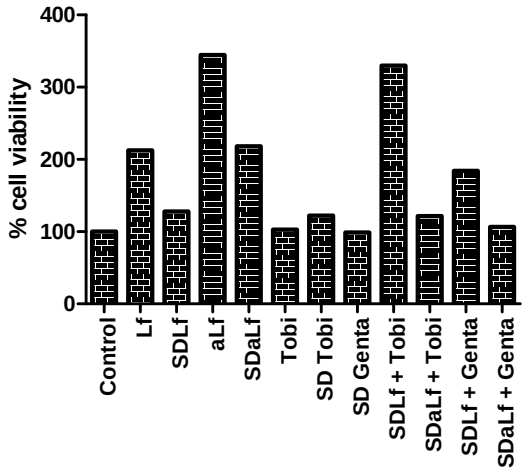


Figure 7 -1: Viability of CALU-3 cells in the presence of various formulations. Cells were seeded then grown in the presence of the indicated antimicrobial or in the case of the control, in medium alone. The presence of viable CALU-3 cell was determined using CTB assay and percent cell viability determined by converting the fluorescence read-out to percent assuming the fluorescence value of the control (i.e where no antimicrobial agent was included) to be 100% viable.

The data presented in figure 7 -1 indicate that, overall, exposure of CALU-3 cells to the various formulations did not cause the death of the epithelial cells rather incubation of CALU-3 cells with Lf resulted in the doubling of the fluorescent intensity from 5,387 FU for the control (where no antimicrobial was present) to 11,444 FU (i.e. a 112 % increase in cell viability when compared with the control). Incubation of CALU-3 cells with SDLf resulted in a fluorescent intensity of 6,887 FU (i.e. approximately 28 % increase in cell viability above the viability of cells in the control). Incubation of CALU-3 cells with aLf resulted in a fluorescent intensity of 18,555 FU representing approximately 244% increase in cell viability above the % cell viability of the cells in the control (where no antimicrobial was added). The fluorescence of the CALU-3 cells upon incubation with SDaLf, was 11,746 FU was obtained indicating that there was approximately 118 % increase in cell viability above the % cell viability of cells in the control. However, the observed increase % of viable cells was less than observed cell viability values obtained with aLf.

Upon incubating CALU-3 cells with Tobi and SD Tobi, the fluorescence intensities were; 5,536 and 6,586 FU representing 2 and 22 % increase above the % cell viability of the control while the fluorescence intensity was 5,325 FU when CALU-3 cells were incubated with SD Genta, representing a 1 % decrease in cell viability compared with the % cell viability of cells in the control.

Incubation of submerged CALU-3 cells with SDLf + Tobi resulted in a fluorescence intensity of 17,774 FU which was approximately 230 % above the viability of the control, however, upon incubating CALU-3 cells with SDaLf + Tobi, a fluorescence intensity of 6,544 FU was obtained this represented approximately 22 % increase in cell viability when compared with the control.

Upon incubating the submerged CALU-3 cells with SDLf + Genta, the fluorescence value obtained was approximately 9,916 FU representing an 84 % increase above the control. Finally, incubation of submerged CALU-3 cells with SDaLf + Genta resulted in a fluorescent intensity of approximately 5,728 FU following CTB assessment indicating a 6 % increase compared with the the control.

7.6.1.2 IL-8 secretion

The secretion of IL-8 by CALU-3 cell on exposure to the different compounds for 24 h was investigated in order to determine if the various formulations had any pro-inflammatory properties.

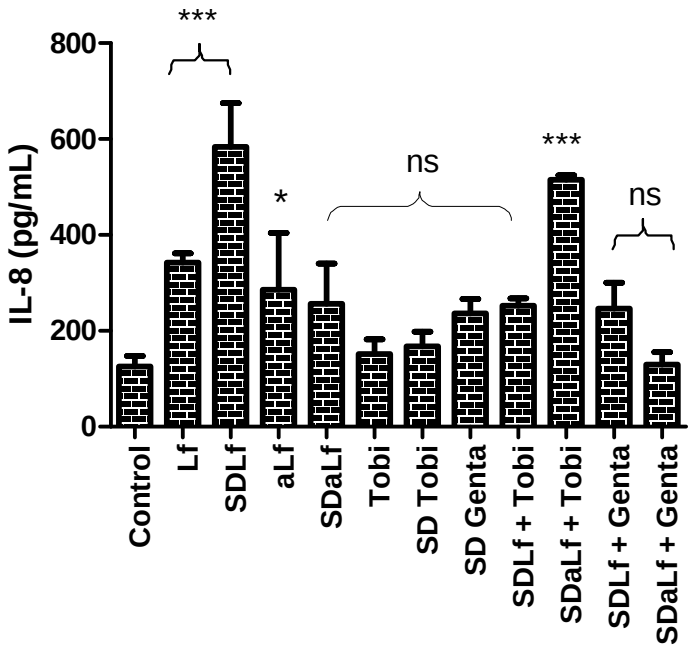


Figure 7 -2: Secretion of IL-8 from CALU-3 cells following 24 hours exposure to various antimicrobial formulations or in the case of the control, in the absence of antimicrobial agents. At the end of the incubation period, the IL-8 concentration in the supernatant was measured using ELISA. Data represent the mean of three individual measurements from the same experiment and are expressed as mean ±SD. Comparisons of the concentrations of IL-8 secreted by CALU-3 cells upon exposures to the various antimicrobials were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; ns indicates no significance compared to the control (no antimicrobial agent added), *** indicates $p < 0.001$ compared to the control, * indicates $p < 0.05$ compared to the control.

The concentrations of IL-8 secreted by CALU-3 cells upon exposure to the various antimicrobial agents. Where no antimicrobial agent was present, it was found that CALU-3 cells secreted 125.5 ±22 pg/mL of IL-8. Upon incubating the cells with the various lactoferrin only formulations, a significant increase ($p < 0.05$) in IL-8 secretion compared to the control (figure 7 -2). In particular, when the CALU-3 cells were incubated with Lf, the cells secreted 342.5 ±19 pg/mL of IL-8 and upon incubation of

CALU-3 cells with SDLf, 583.8 ±91 pg/mL of IL-8 was secreted by the cells. On the other hand, incubation of CALU-3 cells with aLf resulted in the secretion of 285.9 ±118 pg/mL of IL-8 while incubation of CALU-3 cells with SDaLf resulted in the secretion of 256.5 ±84 pg/mL of IL-8.

There was however, no effect of exposing the CALU-3 cells to the various aminoglycoside only formulations as 151.6 ±31, 167.4 ±31 and 236 ±30 pg/mL of IL-8 was secreted by the cells in response to tobi, SD Tobi and SD Genta respectively. Upon exposing the CALU-3 cells to SDLf + Tobi, there was no significant difference in the amounts of IL-8 secreted compared with the control. Incubation of CALU-3 cells with SDaLf + Tobi led to the secretion of significant ($p < 0.001$) amounts (514.8 ±10 pg/mL) of IL-8 compared to the concentrations of IL-8 secreted constitutively in the control or upon exposure of CALU-3 cells to the SDLf + Tobi. Exposure of CALU-3 cells to SDLf + Genta elicited the secretion of 246.1 ±54 pg/mL of IL-8 while exposure of CALU-3 cell to SDaLf +Genta elicited the secretion of 129.8 ±26 pg/mL of IL-8, the amounts of IL-8 produced by CALU-3 cells upon incubation with SDLf + Genta and SDaLf + Genta was not significantly different to the control (that is when no antimicrobial was present)

7.6.1.3 *P. aeruginosa* biomass on CALU-3 cells cultured at ALI.

Static co-cultures of *P. aeruginosa* biofilms on CALU-3 cells were developed and exposed to antimicrobial agents in order to determine the effectiveness of formulations on bacterial biofilm. The number of bacterial colonies which survived treatment was quantified using the spread plate method.

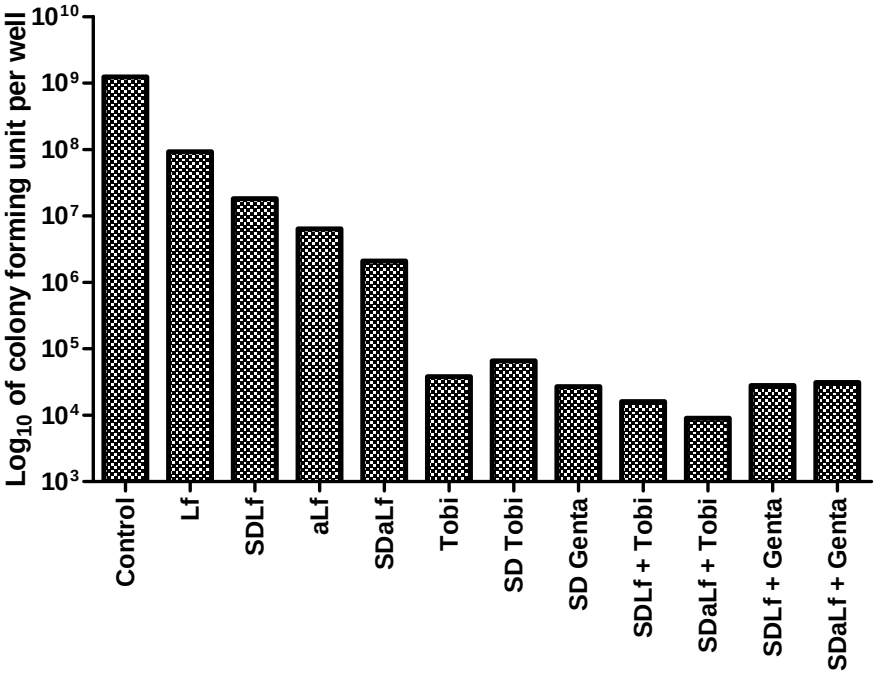


Figure 7 -3: *P. aeruginosa* biomass on CALU-3 epithelial cells following treatment with various antibiotics or combinations. The control shows bacterial biomass on the cells in the absence of protein or antibiotics. Data are expressed as log of colony forming units per well, for single well analysis of each treatment. The concentration of the various protein-antibiotic used in these experiment was 10 mg/mL. The treatment was maintained for 24 hours before the remaining surface attached biofilms were dislodged by sonication and the number of cfu/well determined. Although the Lf preparations were not effective in reducing colony counts when applied in isolation, they appeared to enhance the activity of the antibiotics when used in combination.

The control (i.e. when no antimicrobial agent was present) had the highest number of bacterial colonies at 1.24 x 10^9 cfu/well. Upon treating the static co-culture with Lf or SDLf, there was a 1 log reduction in the number of bacterial colonies to 9.2 x 10^8 and 1.82 x 10^8 cfu/well for Lf and SDLf, respectively. When the static co-culture of biofilms and CALU-3 cells were treated with aLf and SDaLf, the number of residual bacterial

colonies was 6.4 x 10^6 and 2.1 x 10^6 cfu/well indicating that there was a 2 log reduction in bacterial colonies.

Upon treating the static co-culture with aminoglycoside only antimicrobial agents, the number of cfu/well was decreased by approximately 5-log units compared with the control where there was no antimicrobial treatment; the residual bacterial colony was 3.8 x 10^4, 6.6 x 10^4 and 2.7 x 10^4 cfu/well following treatment with Tobi, SD Tobi and SD Genta respectively.

Treatment of the static co-culture of biofilms and CALU-3 cells with co-spray dried lactoferrin and aminoglycosides (tobramycin and gentamicin) resulted in approximately 5-log reduction in the number of bacterial colonies compared with the control (where there was no treatment with antimicrobial agents). The residual number of bacterial colonies was 1.6 x 10^4, 9 x 10^3, 2.8 x 10^4 and 3.1 x 10^4 cfu/ well following treatment with SDLf + Tobi, SDaLf + Tobi, SDLf + Genta, SDaLf + Genta respectively.

7.6.2 Effects of exposure of various antimicrobial agents to BEAS-2B cells

7.6.2.1 Cytotoxicity

The cytotoxicity of the various antimicrobial agents on BEAS-2B cells in submerged cultures were evaluated. The fluorescence unit obtained by carrying out a CTB assay was converted to percent cell viability by assuming the fluorescence of the control (i.e where no antimicrobial agent was present) to be 100 %.

BEAS-2B cells did not show any significant reduction in metabolic activity following 24 hours incubation with the various agents. However, there were slight increases in the

number of viable BEAS-2B cells when they were incubated with the various antimicrobial agent compared with when no antimicrobial agent was included (control).

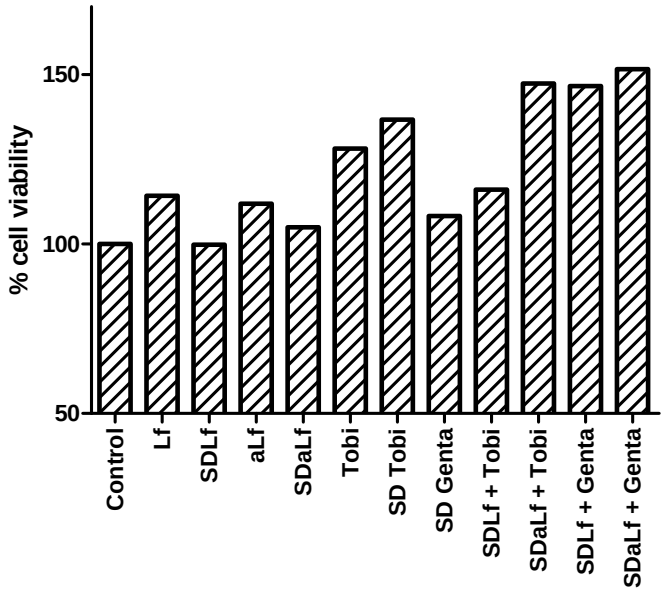


Figure 7 -4: Percent cell viability of BEAS-2B cells in the presence of various antimicrobial formulations. Cells were seeded into 24 well plates then grown in submerged culture in the presence of the indicated formulation or in the case of the control, in cell culture medium alone. The cell viability was determined using CellTiter-Blue® assay and the percent cell viability determined by converting the fluorescence read-out to percent assuming the fluorescence value of the control (i.e where no antimicrobial agent was included) to represent 100% viability. The same plates were measured three times to ascertain a mean measurement of the readout.

The fluorescence for the control was 16,368 FU, upon incubating BEAS-2B cells with Lf, a fluorescence of 18,702 FU was obtained indicating a 14 % increase when compared with the control. Incubation of BEAS-2B cells with SDLf resulted in a fluorescence of 16,336 FU, indicating there was no change compared to the fluorescence of the control. Upon incubating the iron depleted aLf and SDaLf with BEAS2B, a fluorescence of 18,318 and 17,175 FU was obtained indicating 11 and 4 % increase in viability compared to control.

Incubation of BEAS-2B cells with the aminoglycosides also showed increased fluorescence compared with the control. When BEAS-2B were incubated with Tobi, SD Tobi or SD Genta the fluorescence was 20,978, 22,376 and 17,721 FU, respectively. This represents an increase of 28, 36 and 8 % compared to the control.

Incubating BEAS-2B cells with SDLf + Tobi and subsequently quantifying viable cells by the CTB assay showed that there was a 16 % increase in percent cell viability compared with the control. However, when BEAS-2B cells were incubated with SDaLf + Tobi, a fluorescence of 24,121 FU was obtained indicating approximately 47 % increase in percent cell viability compared with the control. Similarly, assessment of cell viability of BEAS-2B cells incubated with co-spray dried lactoferrin/ apo lactoferrin and gentamicin indicated a 47 and 52 % increase in percent viable cells when compared with the control.

7.6.2.2 IL-8 secretion

Upon exposing BEAS-2B cells to the various formulations, there was no significant difference in IL-8 production between the control and 10 of the different antimicrobial formulations (Lf, aLf, SDaLf, Tobi, SD Tobi, SD Genta, SDLf + tobi, SDaLf + tobi, SDLf + Genta and SDaLf + Genta). In contrast, exposure of BEAS-2B cells to SDLf resulted in a significant ($p < 0.05$) increase in IL-8 secretion compared with the constitutive concentration of IL-8 secreted by the control cells where no antimicrobial agent was added.

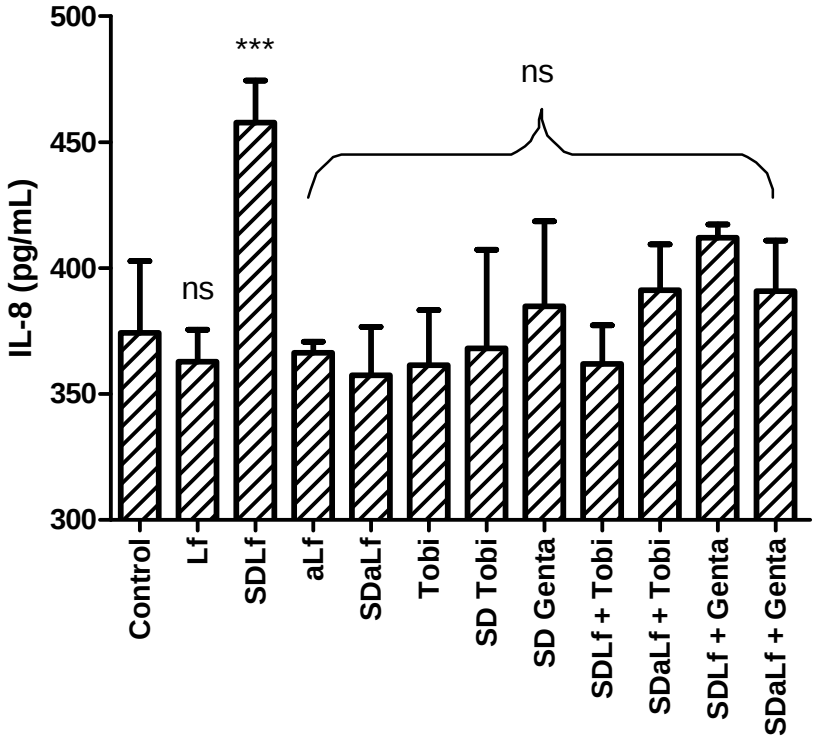


Figure 7 -5: IL-8 production by BEAS-2B cells following treatment with various formulations. The cells were seeded and grown in submerged culture for 24 h in the presence of the various protein-aminoglycoside combination at 10mg/ml or in the case of the control, with no antimicrobial additions. At the end of the incubation period, the IL-8 concentration in the supernatant was measured using ELISA. Data represents the mean of three individual measurements from the same experiment and are expressed as mean ±SD. Comparisons of IL-8 secretion by the BEAS-2B were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; ns indicates no significance compared to the control (no antimicrobial agent added) and *** indicates $p < 0.001$ compared to the control.

7.6.2.3 *P. aeruginosa* biomass on BEAS-2B cells cultured at ALI

The surviving *P. aeruginosa* colonies on BEAS-2B cells following treatment with various antimicrobial agents were evaluated. The control (i.e. no antimicrobial agent added) had the highest number of bacterial cells (8.8×10^8 cfu/well).

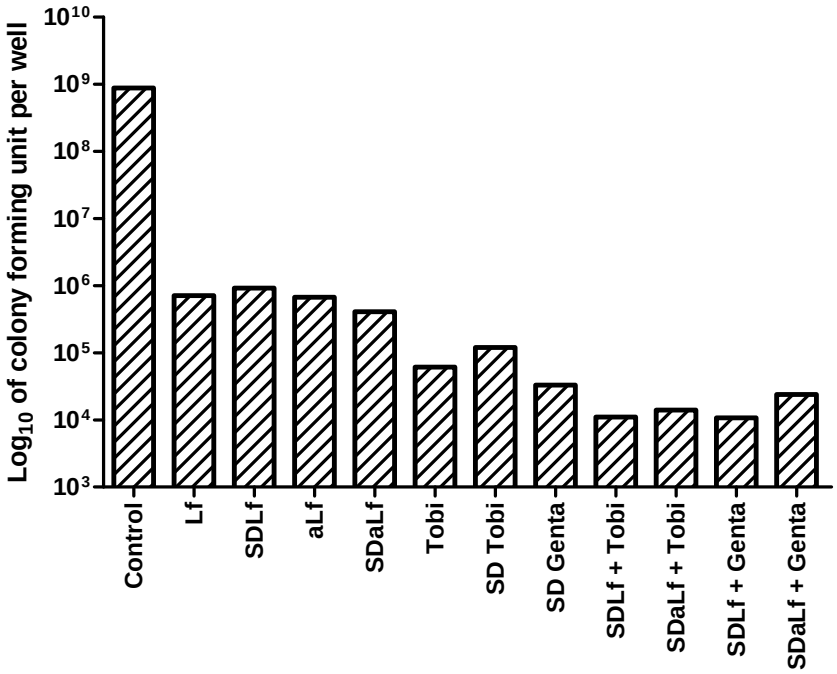


Figure 7 -6: Effect of various antimicrobial formulations on *P. aeruginosa* biomass on BEAS-2B cells. Static co-cultures of biofilms of *P. aeruginosa* on BEAS-2B epithelial cells were treated for 24 h with and without (control) the addition of the various antimicrobial preparations at a concentration of 10 mg/mL. After 24 hours the surface attached biofilms were dislodged by sonication, and the number of CFU/well determined. The data shown are the mean values of triplicate colony counts from the same well expressed as log of colony forming units per well.

Upon treatment of static co-cultures of *P. aeruginosa* biofilms on BEAS-2B cells with the lactoferrin only formulations, there was a 3-log reduction in colony forming units per well – i.e. 7.1×10^5, 9.9×10^5, 6.7×10^5 and 4.1×10^5 cfu/well for Lf, SDLf, aLf and SDaLf respectively – compared with 8.8×10^8 (the number of colonies in the control). Similarly, when the static co-culture of *P. aeruginosa* biofilms on BEAS-2B cells was treated with the aminoglycoside only antibiotics – Tobi, SD Genta and SD Genta – there was approximately 4-log reduction in colony forming units per well – i.e. 6.1×10^4, 1.2×10^5 and 3.3×10^4 cfu/well respectively – compared with the colony forming units in the control. On treating the static co-culture of biofilms on BEAS-2B cells with combinations

of protein-aminoglycoside antimicrobials, there was an even further reduction in residual *P. aeruginosa* biomass on the BEAS-2B cells by -log reduction when compared with the number of colony forming unit in the control.

7.6.3 Effects of incubating IB3-1 and C38 cells with various antimicrobial agents

7.6.3.1 Cytotoxicity

IB3-1 and C38 cell types are similar cell types except for the cystic fibrosis phenotype present in the IB3-1 cells which has been corrected by the stable transfection of wild type, functioning CFTR in C38. While IB3-1 cells are best suited for our investigational purposes, taken together, cellular responses obtained from both cell types can help determine *in vitro* cellular responses specific to a cystic fibrotic cell.

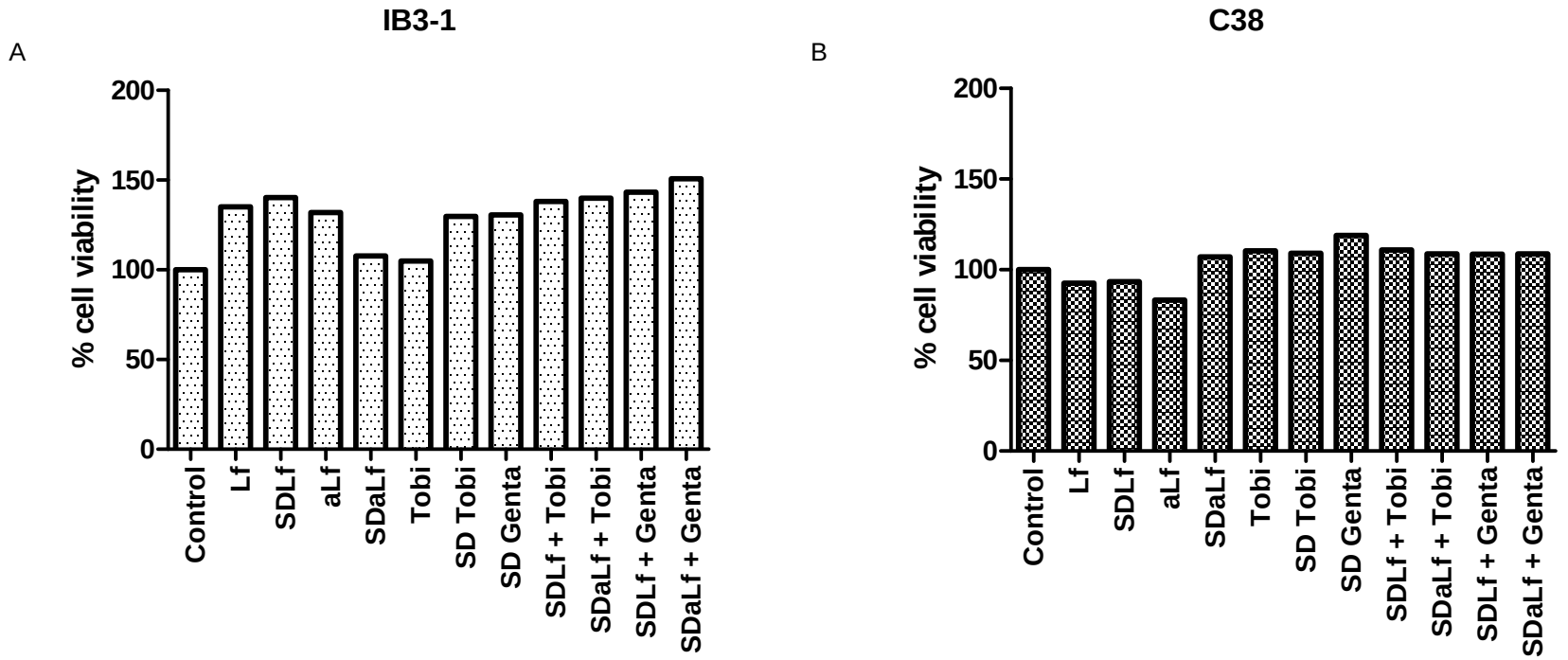


Figure 7 -7: Percent cell viability of IB3-1 and C38 cells in the presence of various antimicrobial formulations. Cells were seeded then grown in the presence of the indicated compound or in the case of the control,no additions. (A) % cell viability of IB3-1 cells. (B) % cell viability of C38 cells. The cellular metabolic activity of IB3-1 and C38 epithelial cells were determined using cell titre blue (CTB) assay and the percent cell viability determined by converting the fluorescence read-out to percent assuming the fluorescence value of the control (i.e where no antimicrobial agent was included) to represent 100. The same plates were measured three times to ascertain a mean measuremen of the readout.

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On exposure of IB3-1 cells to the various formulations there was no reduction in metabolic activity (as determined by fluorescence) of the cells below the level observed in the control. Incubation of IB3-1 cell with the various lactoferrin only formulations – Lf, SDLf, aLf and SDaLf – produced fluorescence of 17,377, 18,048, 16,972 and 13,866 FU which represents a 35, 40, 32 and 8 % increase in the cell viability of the IB3-1 cells respectively, when compared to the control (12,879 FU).

Upon incubation of IB3-1 cells with the various aminoglycoside only formulations, a fluorescence of 13,501, 16,699 and 16,809 FU was obtained for Tobi, SD Tobi and SD Genta respectively following CTB assay and this represented a 4, 29 and 30 % increase in cell viability respectively above the % cell viability levels of the control.

Incubation of submerged IB3-1 cells with various protein-antimicrobial combinations resulted in increased fluorescence following CTB assay, hence indicating increased % cell viability. The fluorescence values were 17,776, 18,016, 18,445 and 19,395 FU, representing 38, 40, 43 and 50 % increase in cell viability above the % cell viability levels of the control.

Incubation of C38 cells with the various lactoferrin only formulations – Lf, SDLf, aLf and SDaLf – produced a fluorescence of 22,483, 22,713 and 20,231 FU which was about 8, 7 and 17 % lower than the % cell viability of the control (24,299 FU) where no antimicrobial agent was added, however, incubation of the C38 cells with SDaLf produced a fluorescence of 26,013 FU (7 % increase in cell viability when compared with the control).

Upon incubating the C38 cells with the various aminoglycoside only formulations, a fluorescence of 26,832, 26,525 and 28907 FU was obtained for Tobi, SD Tobi and SD

Genta respectively following CTB assay and this represented a 10, 9 and 18 % increase in cell viability respectively above the % cell viability levels of the control.

Incubation of submerged C38 cells with various protein-antimicrobial combinations produced increased fluorescence an indication of increased % cell viability. The fluorescence values were 26938, 26417, 26365 and 26424 FU, representing 10, 8, 8 and 8 % increase in cell viability above the % cell viability levels of the control.

7.6.3.2 IL-8 secretion

The secretion of IL-8 by both IB3-1 and C38 cells on exposure to the different compounds for 24 h was investigated to determine whether the exposure of the epithelial cells to the different antimicrobial agents may contribute to CF airway inflammation through increased expression of the neutrophil chemoattractant IL-8. This was determined by comparing the expression of IL-8 in the CF airway epithelial cell (IB3-1) with the expression of IL-8 in the non-CF airway epithelial cell (C38).

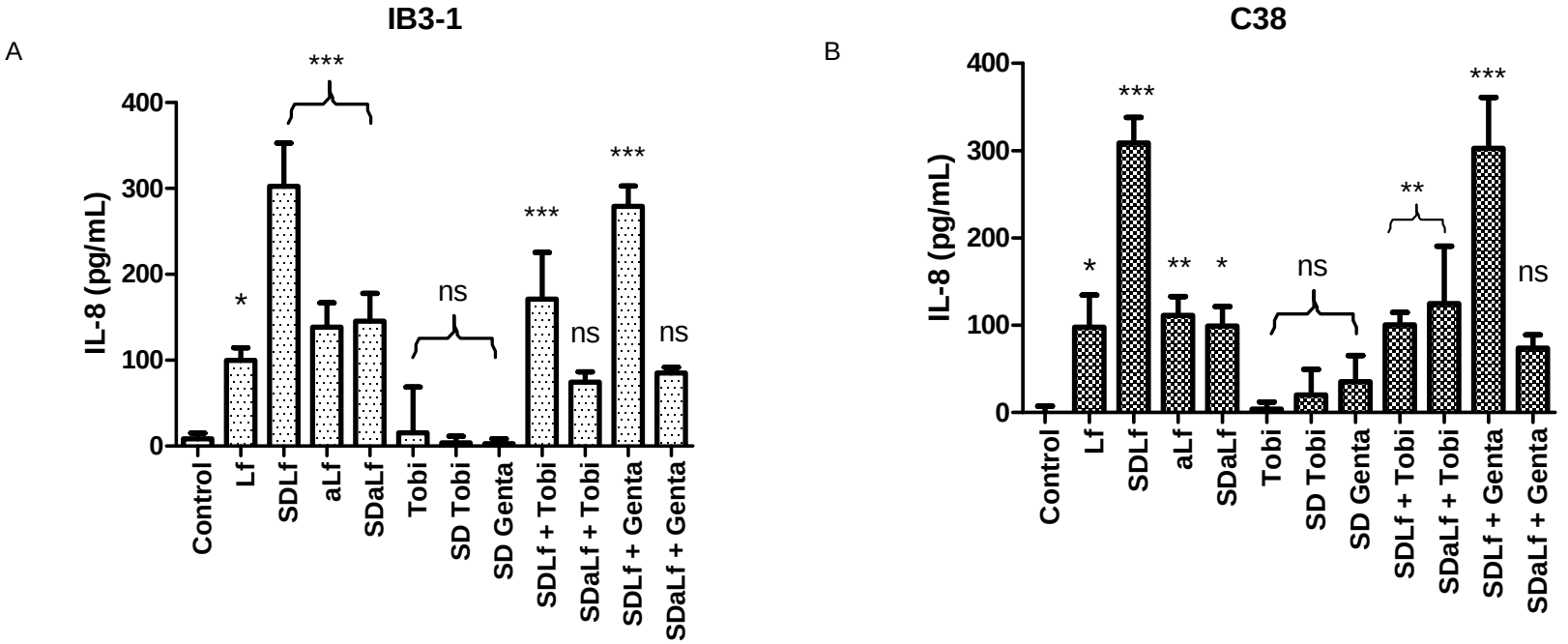

Figure 7 -8: IL-8 production by IB3-1 and C38 cells following treatment with formulations. Cells were seeded and grown in submerged culture for 24 h in the presence of the antimicrobial agents at a concentration of 10mg/ml or in the case of the control, with no antimicrobial additions. At the end of the incubation period, the IL-8 concentration in the supernatant was measured using ELISA. Data represents the mean of three individual IL-8 quantifications from the same experiment and are expressed as mean ±SD. (A) IL-8 production by IB3-1 cells upon exposure of the IB3-1 cells to the antimicrobial formulations (B) IL-8 production by C38 cells upon exposure to antimicrobial agents. Comparisons of IL-8 secretion by the airway epithelial cells were made by one-way analysis of variance (ANOVA) using graphpad prism 5 (GraphPad Software Inc., Version 5.0) followed by Dunnett post-test; ns indicates no significance compared to the control (no antimicrobial agent added), ** indicates $p < 0.01$ compared to the control, and * indicates $p < 0.05$ compared to the control, and *** indicates $p < 0.001$ compared to the control.

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The mean concentration of IL-8 which was produced by IB3-1 and C38 cells constitutively were neglible; 8 and 0 pg/mL repectively. However, upon exposure of submerged IB3-1 and C38 cells to the various lactoferrin formulations, there was increased production of IL-8 compared to the control (where no agent was added). The concentrations of IL-8 produced by incubating IB3-1 cells with Lf, SDLf, aLf and SDaLf were 99, 302, 139 and 146 pg/mL respectively. While the concentrations of IL-8 produced by incubating C38 cells with Lf, SDLf, aLf and SDaLf were 97, 308, 111 and 98 pg/mL respectively.

Incubation of IB3-1 cells with the various aminoglycoside only formulations – Tobi, SD Tobi and SD Genta – elicited the production of 15.5 ±53, 3.8 ±8 and 2.8 ±6 pg/mL of IL-8 respectively, while incubation of C38 cells with the various aminoglycoside only formulations – Tobi, SD Tobi and SD Genta – elicited the production of 3.98 ±8, 20.0 ±29 and 35.3 ±30 pg/mL of IL-8 respectively.

Upon exposure of IB3-1 cells to the various protein-aminoglycoside combinations – SDLf + Tobi, SDaLf + Tobi, SDLf + Genta, SDaLf + Genta – resulted in the secretion of 171.1 ±54, 74.5 ±12, 279.2 ±24 and 85.3 ±6 pg/mL of IL-8 respectively (Figure 7 - 7A). Incubation of C38 cells with the the various protein-aminoglycoside combinations – SDLf + Tobi, SDaLf + Tobi, SDLf + Genta, SDaLf + Genta – resulted in the secretion of 100.3 ±14, 124.9 ±66, 302.6 ±59 and 73.8 ±15 pg/mL of IL-8 respectively.

7.6.3.3 *P. aeruginosa* biomass on IB3-1 and C38 cells cultured at ALI

Following treatment of co-cultures of biofilms on IB3-1 and C38 cells with the various antimicrobial agents, the surviving *P. aeruginosa* colonies on both cell types were enumerated. The controls (i.e. no antimicrobial agent added) had a colony forming unit per well of 3.9 x 10^9 and 5.8 10^9 cfu/mL for the IB3-1 and C38 cells respectively. Upon

treating the co-culture of biofilms on both IB3-1 and C38 cells with the different lactoferrin only formulations – Lf, SDLf, aLf and SDaLf – the residual bacterial biomass was reduced by between 2- and 3-log units compared with the number of colonies per well in the respective controls. However, upon treating co-culture of biofilms on both IB3-1 and C38 cells with aminoglycoside only formulations, there was approximately 5-log reduction in number of colonies on the IB3-1 cells (4.2×10^4, 1.2×10^4, 2.2×10^4 cfu/well for Tobi, SD Tobi, SD Genta) and between 3- and 5-log reduction in number of colonies on the C38 (6.7×10^5, 1.1×10^6 and 7.1×10^4 cfu/well for Tobi, SD Tobi, SD Genta). Treatment of co-culture of biofilms on IB3-1 and C38 cells with protein-aminoglycoside combinations (SDLf + tobi, SDaLf + tobi, SDLf + Genta and SDaLf + Genta) resulted in the reduction of the cfu/well by between 5 and 6 log units compared with the cfu/well in the control.

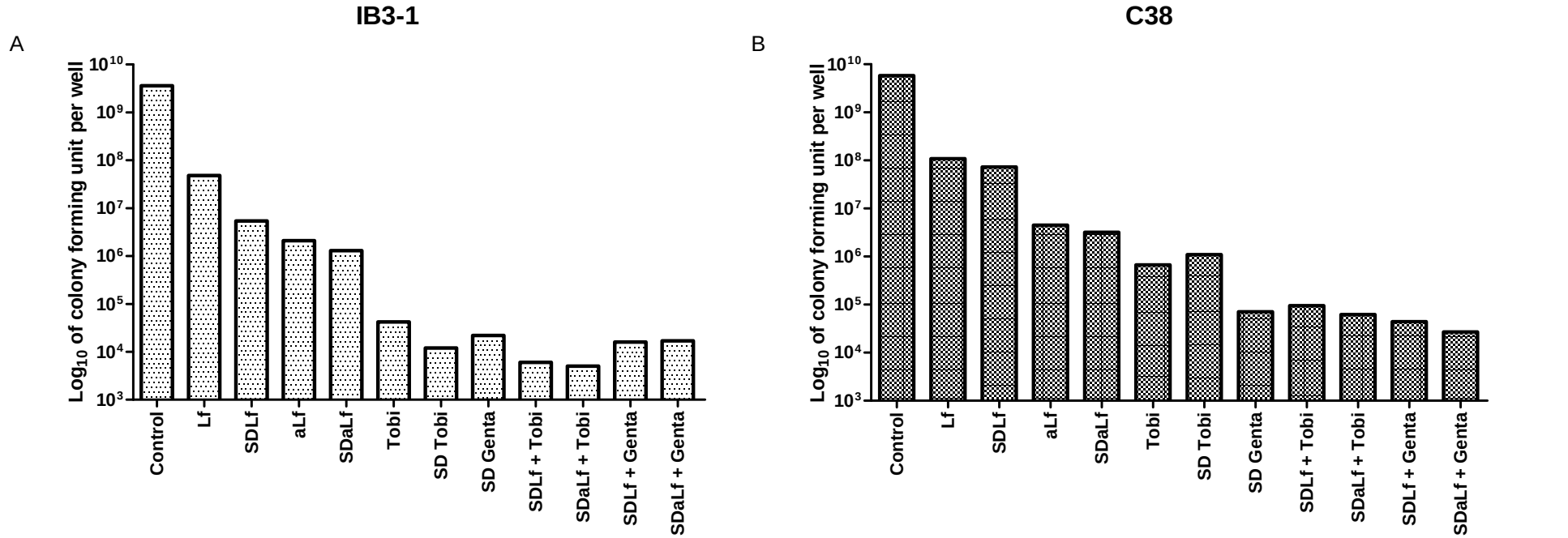

Figure 7 -9: Effect of various antimicrobial formulations on *P. aeruginosa* biomass on IB3-1 (A) and C38 (B) epithelial cells. Static co-culture of *P. aeruginosa* biofilm and the epithelial cells were incubated for 24 h with and without addition of the various compounds at a concentration of 10 mg/mL. The treatment was maintained for 24 hours before the remaining surface attached biofilms were dislodge by sonication and the number of CFU/well determined. (A) bacterial colony forming unit on IB3-1 epithelial cell. (B) bacterial colony forming unit on C38 epithelial cell. The data shown are the mean values of triplicate colony counts from the same well expressed as log of colony forming units per well.

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Table 7.1: Summary of the cell viability results from the four epithelial cell lines (CALU-3, BEAS-2B, IB3-1 and C38) upon exposure to the various antimicrobial agents. The effects of the individual agents on the cell viability of epithelial cells have been compared to the cell viability of the control (i.e. where no antimicrobial agent was present).

Antimicrobial agents	**CALU-3 cells**	**BEAS-2B cells**	**IB3-1 cells**	**C38 cells**
Lf	**Increase**	**Increase**	**Increase**	Decrease
SDLf	**Increase**	No change	**Increase**	Decrease
aLf	**Increase**	**Increase**	**Increase**	Decrease
SDaLf	**Increase**	No change	No Change	**Increase**
Tobi	No change	**Increase**	No Change	**Increase**
SD Tobi	No change	**Increase**	**Increase**	**Increase**
SD Genta	No change	No Change	**Increase**	**Increase**
SDLf + Tobi	**Increase**	**Increase**	**Increase**	**Increase**
SDaLf + Tobi	No Change	**Increase**	**Increase**	**Increase**
SDLf + Genta	**Increase**	**Increase**	**Increase**	**Increase**
SDaLf + Genta	No Change	**Increase**	**Increase**	**Increase**
Reference	Figure 7 -1 (*Pg 314*)	Figure 7 -4 (*Pg 319*)	Figure 7 -7A (*Pg 326*)	Figure 7 -7B (*Pg 326*)

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Table 7.2: Summary of IL-8 release from the four epithelial cell lines (CALU-3, BEAS-2B, IB3-1 and C38) upon exposure to the various antimicrobial agents. The amounts of IL-8 secreted by the four epithelial cells upon exposure to the various antimicrobial agent have been compared to the concentrations of basal IL-8 in the control cells (i.e. where no antimicrobial agent was present).

Antimicrobial agents	**CALU-3 cells**	**BEAS-2B cells**	**IB3-1 cells**	**C38 cells**
Lf	**+++**	-	**++**	**++**
SDLf	**+++**	**+++**	**+++**	**+++**
aLf	**++**	-	**+++**	**+**
SDaLf	-	-	**+++**	**++**
Tobi	-	-	-	-
SD Tobi	-	-	-	-
SD Genta	-	-	-	-
SDLf + Tobi	-	-	**+++**	**+**
SDaLf + Tobi	**+++**	-	-	**+**
SDLf + Genta	-	-	**+++**	**+++**
SDaLf + Genta	-	-	**++**	-
Reference	Figure 7 -2 (*Pg 317*)	Figure 7 -5 (*Pg 323*)	Figure 7 -8A (*Pg 329*)	Figure 7 -8B (*Pg 329*)

Key:

(+++) Significantly ($p < 0.001$) more IL-8 was secreted compared with the control

(++) Significantly ($p < 0.01$) more IL-8 was secreted compared with the control

(+) Significantly ($p < 0.05$) more IL-8 was secreted compared with the control

(-) No significant difference in the amount of IL-8 secreted compared to the control

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Table 7.3: Summary of residual *P. aeruginosa* colonies on the *in vitro* co-culture models following treatment of the co-culture models with the various antimicrobial agents. At the end of each drug treatment, the cells were washed three times with phosphate-buffered saline (PBS) to remove planktonic planktonic bacteria and then 0.1% Triton was added to lyse the epithelial cells and dissociate the biofilms. The lysate was vortexed for 3 mins and the number of colonies determined by the spread plate method. The residual colonies following treatment with the various antimicrobial agents have being compared to the colony forming units in the control cells at the end of the experiment.

Antimicrobial agents	CALU-3 cells	BEAS-2B cells	IB3-1 cells	C38 cells
Lf	1-Log reduction	3-Log reduction	1-Log reduction	2-Log reduction
SDLf	1-Log reduction	3-Log reduction	2-Log reduction	3-Log reduction
aLf	2-Log reduction	3-Log reduction	3-Log reduction	3-Log reduction
SDaLf	2-Log reduction	3-Log reduction	3-Log reduction	3-Log reduction
Tobi	5-Log reduction	4-Log reduction	4-Log reduction	5-Log reduction
SD Tobi	5-Log reduction	3-Log reduction	3-Log reduction	5-Log reduction
SD Genta	5-Log reduction	5-Log reduction	5-Log reduction	5-Log reduction
SDLf + Tobi	5-Log reduction	5-Log reduction	5-Log reduction	6-Log reduction
SDaLf + Tobi	5-Log reduction	5-Log reduction	5-Log reduction	6-Log reduction
SDLf + Genta	5-Log reduction	5-Log reduction	5-Log reduction	5-Log reduction
SDaLf + Genta	5-Log reduction	5-Log reduction	5-Log reduction	5-Log reduction

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7.7 Discussion

In this study, four airway epithelial cell lines (CALU-3, BEAS-2B, C38 and IB3-1) were used to evaluate the acute cytotoxicity properties of eleven formulations in terms of their cell viability and inflammatory response (in particular IL-8 secretion). For the assessment of cellular metabolic activities, the one-step CellTiter-Blue assay was employed to provide a general indication of cell viability of the the epithelial cells following exposures to the various antimicrobial formulations. The assay (which was an end point assay) involved incubating epithelial cells which had been previously exposed to the various formulations with highly purified resazurin (a fluorogenic redox indicator). Actively growing cells are able to convert resazurin (dark blue with little intrinsic fluorescence) to resorufin (a pink, highly fluorescent compound) (Promega Corporation, 2009) thereby providing information on the metabolic (viability) apparatus of the cell.

Even though CTB as a metabolic marker has been shown to correlate with number of viable cells under normal assay conditions, it does have limitations as it is unable to distinguish between cell proliferation and increased cellular ATP levels. However in this experiment, increased viability of the epithelial cells gave an indication of the metabolic status of mitochondrial enzymes in the various epithelial cells. Another interesting point to note at this juncture is that while this method was suitable for determining the cell viability of submerged cell culture it was unsuitable for determining the viability of the epithelial cells following co-culture with biofilms. This is because the assay is not specific for mammalian cells only and the results obtained reflect the metabolic activity of both actively growing bacterial and mammalian cells (Promega Corporation, 2009).

Overall, the viability of the various epithelial cells were not affected by the different formulations indicating that they were not cytotoxic under the test conditions for period of time assessed (24 hrs). Generally, C38 cells were found to be the most metabolically active cells as in the absence of any added antimicrobial agent (i.e the control). The

ranking order of resazurin metabolism by the four epithelial cells were: C38 > BEAS-2B > IB3-1 > CALU-3.

In order to investigate the possible secretion of inflammatory markers by the four airway epithelial cell lines upon exposure to the eleven antimicrobial agents, IL-8 secretion was chosen as the indicator to monitor since it had been previously shown to be secreted by the four epithelial cell lines in detectable quantities when stimulated (Bielemeier, 2012; Babu et al, 2004; Witschi and Mrsny, 1999). IL-8 is a well known acute inflammatory chemo-attracter for immune cells such as neutrophils and mast cells (Richman-Eisenstat et al., 1993), therefore induction of massive amounts of IL-8 secretion by the various antimicrobial formulations is an indication that *in vivo* application of the formulations would most likely result in acute inflammatory responses (Davies, 2002). In order to achieve a successful antimicrobial formulation that is best suited for *P. aeruginosa* eradication, there is a need to ensure that the antimicrobial therapy of choice does not antagonise the activities of other anti-inflammatory agents, like ibuprofen (Konstan *et al.*, 1991; Konstan *et al.*, 1995) which may be co-administered during multiple drug therapy.

Under basal, unstimulated conditions, the IL-8 concentrations secreted by the various cells were found to vary markedly amongst the four cell types. While BEAS-2B cells secreted the highest amount of IL-8 (374.2 ±28 pg/mL), corrected CF cells (C38) secreted the least amount of IL-8 (0 ±7 pg/mL). Our findings are consistent with a previously published report by Schwiebert and co-workers (1999) which showed that under basal conditions, there is increased IL-8 secretion in BEAS-2B cells compared to IB3-1 cells. Specifically, this study employed 6 epithelial cell lines (3 non-CF airway epithelial cell lines: BEAS-2B, 16HBE14o-, and A549; and 3 CF airway epithelial cell lines: IB3-1, CFBE41o-, and ΣCFTE29o-) and found that while BEAS-2B cells expressed > 20 ng/10^6 cells of IL-8 protein under basal conditions, the actual amount of IL-8 secreted by the IB3-1 cells were not detectable by the IL-8 specific ELISA method

which they employed in their investigation. However, our data also suggests that more IL-8 was secreted by the IB3-1 cells compared to the C38 cells and this may be related to previous reports by DiMango et al., (1998) and Tabary et al., (2006) who have also demonstrated that the very low levels of IL-8 secreted by C38 cells maybe as a result of minimal nuclear factor kappa B (NF-Kb) present in the C38 cells under unstimulated conditions. This suggestion has also been echoed in other published reports by Tabary et al., (1999), Tabary et al., (2001), Venkatakrishnan et al., (2000) and Weber et al., (2001).

Upon exposure of CALU-3 cells to the 11 antimicrobial agents, there were inconsistencies in the in the levels of IL-8 expressed. While Lf, SDLf and SDLf + Tobi, elicited the secretion of significant ($p < 0.001$) amounts of IL-8 compared with the basal levels of IL-8 secreted in the control, exposure of CALU-3 cells to all the other eight antimicrobial agents produced no significant differences when compared to the levels of IL-8 expressed under basal conditions in the control (Figure 7 -2, Table 7.2)

Upon exposing BEAS-2B cells to the eleven antimicrobial agents, only SDLf induced a significant increase ($p < 0.001$) in IL-8 protein expression, while all the other antimicrobial agents produced no significant change in IL-8 expression when compared to the basal IL-8 levels secreted by the control (Figure 7 -5, Table 7.2). IL-8 expression by both IB3-1 and C38 cells upon exposure to the different antimicrobial agents showed that while exposures of both cell types to the lactoferrin containing antimicrobials elicited significant (between $p < 0.001$ to $p < 0.05$) increases in IL-8 expression by the airway epithelial cells compared to the basal IL-8 levels secreted by the respective controls, there was no significant change in IL-8 levels expressed by both cell types (IB3-1 and C38 cells) upon exposure to the aminoglycoside only antimicrobials compared with basal IL-8 levels expressed by the respective controls (Figure 7 -7, Table 7.2). Taken together, the data tells us more about the bovine lactoferrin which was used for these studies. Although the choice to use bovine lactoferrin for this pilot study was made

based on using the most economical purified lactoferrin we could obtain to prove our concept, the lactoferrin we obtained which was ~ 95% pure, may have contained other proinflammatory cytokines like interleukin 1 and 6, tumor necrosis factor and interferon gamma (Bocci *et al.*, 1991; Stelwagen *et al.*, 2009). Therefore, these cytokines may be responsible for the exaggerated IL-8 expression observed by the various airway epithelial cells which may also account for the protein bands seen on the silver stained SDS-PAGE gels (chapter 6, figure 5 -11b).

To better understand the potential usefulness of the various formulations in preventing or disrupting established biofilms on the CF-derived airway cells, a static co-culture of *P. aeruginosa* biofilm on airway epithelial cells was employed. We found that the lactoferrin only formulations generally reduced the number *P. aerugionsa* colonies per well by between 1 and 3 log units when compared to the control (where no antimicrobial agent was applied to the co-culture of *P. aeruginosa* and the various epithelial cells). Also, the ability of the protein (Lf) to reduce bacterial growth was also found to be dependent its ferric ion (Fe^{3+}) content as iron depletion seemed to be crucial in promoting a greater reduction in residual bacterial colonies. SD Tobi and SD Genta reduced the colony forming units by between 4- and 5-log units compared with the control, representing a further 1-log reduction when compared to the lactoferrin only formulations. This finding possibly contributes to previously published reports by Moreau-Marquis et al., (2009) which found that 1mg/mL tobramycin significantly ($p < 0.05$) reduced the biomass of estabalished biofilms.

Upon treating the static co-culture models of *P. aeruginosa* and airway epithelial cells with co-spray dried lactoferrin-tobramycin formulations, the pattern of bacterial colony reduction was the same with the number of *P. aeruginosa* colonies reduced by between 5 and 6 log units/well compared to the control. Our data suggests that the various combination therapies were no worse than the aminoglycoside only formulations in treating *P. aeruginosa* airway infections in both CF and non-CF cells with the SDLf +

Tobi and SDaLf + Tobi formulations performing slightly better on the C38 cells. This is significant because half the concentration of SD Tobi or SD Genta killed approximately the same number of bacterial colonies when combined with lactoferrin of apo lactoferrin. To the best of our knowledge, this is the first study reporting combinations of lactoferrin and aminoglycoside are effective in killing established *P. aeruginosa* biofilms growing on non-CF and CF airway epithelial cells.

In summary, we studied some of the cellular effects of eleven antimicrobial formulations on four different airway epithelial cells (three non-CF and one CF airway epithelial cells) as well as on static co-culture systems of *P. aeruginosa* biofilms on the four airway epithelial cells and found that the various formulations were generally not toxic on the four airway epithelial cells used for the investigation, also, when 11 different antimicrobial agents were administered on static co-culture models of *P. aeruginosa* and epithelial cells, overall, it was found that combinations of lactoferrin and tobramycin/gentamicin were no worse than tobramycin alone at reducing biofilm mass growing on both non-CF and CF airway epithelial cells even though the formulations only contained 50% tobramycin/gentamicin. However, as this was the first study of its kind, further investigations are still needed to determine if there are other beneficial effects a combination therapy of lactoferrin and tobramycicn will contribute to, especially with regards to the management of airway infections.

Chapter Eight

General discussion

8.1 Summary of the thesis

Pulmonary delivery of peptides and protein drugs has come a long way since attempts were first made to deliver insulin via inhalation in the 1920s. Although important advances have been achieved so far, the production of efficacious peptides and protein formulations suitable for pulmonary administration has not been without challenge. Peptide and protein powders are finding increasing applications in dry powder drug delivery systems, however the methods available for preparing these peptide and protein powders are still limited due to the sensitive nature of peptides and proteins to the various processing environments. This is particularly evident when the dry powders are intended for lower airway delivery, since this requires that they possess a suitable aerodynamic particle size (usually < 5 µm), density, morphology and surface properties. These requirements present a challenge as the protein would be expected to undergo some amount of physical stress which may adversely impact the three dimensional structure of the proteins thereby resulting in negative outcomes in their functions following processing. However, to overcome some of these challenges, researchers carefully select suitable carriers (for example, liposomes, polymeric microspheres) and devices that are able to facilitate the delivery of the peptides and proteins to specific areas of the lungs. Consequently, pulmonary delivery of peptides and proteins is gradually emerging as a serious, non-invasive delivery option for protein administration.

With the emergence of antibiotic resistant microorganisms, development of antimicrobial peptides and proteins appears an attractive alternative to antibiotics, however, pulmonary delivery of these antimicrobial therapies is yet to be achieved. This is a classic example of an area where the science of pulmonary delivery of peptides and proteins may find ready application since inhaled or nebulised antimicrobial peptides and proteins could potentially be employed to manage multi-drug resistant bacterial infections in the airway. A disease condition which would benefit immensely from

inhaled therapy is cystic fibrosis (CF). This is a congenital, recessively inherited disorder affecting 1 in 2500 newborns of the Caucasian population, where the eradication of chronic respiratory infection resulting from abnormalities of fluid and electrolyte transport is rarely possible with the currently available antibiotics (Ratjen *et al.*, 2010).

It has been known for a while that the innate immune system, of which the antimicrobial peptides and proteins are part, is responsible for keeping the airways free from infections (Ganz, 2003; Travis *et al.*, 2001; Rogan *et al.*, 2006). Currently, there is no established link between the defective chloride channel, the pathological changes in the airways and the persistent airways infections typical of CF and many theories have been proposed. Two major hypotheses have been advanced to explain the pathogenesis of airway infections in CF (i.e. the 'low volume' (Matsui *et al.*, 1998b) and 'high salt' (Smith *et al.*, 1996) hypotheses). Both of these theories hinge on inactivation of innate antimicrobial peptides. However, it appears that one of the most plausible explanations for the *persistence* of *P. aeruginosa* infections in the airway of people with CF, is the increased concentrations of Fe^{3+} in the airway surface liquid (Stites *et al.*, 1998; Reid *et al.*, 2007b). Therefore, sequestering all the free iron may help decrease the incidence of *P. aeruginosa* in the CF airway. To this effect, various researchers have previously advanced the inclusion of an antimicrobial protein like lactoferrin which has iron chelation abilities as well as contributes to the enhancement of microbial susceptibility to various antibiotics

The overall objective of the present body of work has been to show that it is possible to formulate antimicrobial proteins with particularly good inhalable properties either alone or in combination with aminoglycosides in order to maintain or improve upon the antimicrobial activity against planktonic and biofilm cultures of *P. aeruginosa*. By co-formulating lactoferrin with antibiotics (chapter 6) it was found that it is possible to

produce such an antimicrobial combination (fig 6 -4, pg 268, fig 6 -5, Pg 269). This is important because it suggests that; 1) it is possible to incorporate an antimicrobial protein like protein into currently relevant inhaled antimicrobial therapies, 2) combinations of antimicrobial proteins and antibiotics could help slow the rising instances of antimicrobial resistance, 3) such combinations may also offer the opportunity to decrease adverse effects arising from excessive dosing of currently prescribed antimicrobials. For the above reasons, co-spray dried lactoferrin-tobramycin/gentamicin combination is novel and possesses several interesting properties that make it suitable as a drug candidate for treating *P. aeruginosa* infections in people with CF.

Considering that aggressive antimicrobial therapy is the major reason for the dramatic increase in the life expectancy of people with CF over the last few decades, it is not surprising that the almost continuous, intensive antibiotic use, has resulted in the rising prevalence of multi-drug resistant bacterial. In addition, the lack of new anti-infective drugs coming on to clinical use means that all the previously perceived gains (mostly seen in the area of improvements to quality of life) are gradually being rolled back. For people with CF, for whom treatment of symptoms is the only strategy which could effectively manage their condition (since there is currently no cure), it has been suggested that to curtail the rising incidence of multi-drug resistant bacterial infection, it is pertinent that the currently available antimicrobial agents are effectively utilised and this includes employing combination therapies of antimicrobial proteins and antibiotics.

Our results show that a combination therapy of an antimicrobial protein and an antibiotic can be formulated for delivery directly to the respiratory tract as dry powders. This, thereby provides an effective strategy to reduce the prevalence of antibiotic-resistant *P. aeruginosa* strains in the airways. This is particularly an advantage since such combination therapies will utilize the sum of the individual components to inhibit the

development of resistant bacteria. For example, in the case in study, aminoglycosides, though highly potent against planktonic *P.aeruginosa* (Davis, 1987; Hancock, 1981), are poorly effective against slow growing bacterial cells seen when microbes reside within biofilms (Chernish and Aaron, 2003). Lactoferrin, though able to prevent biofilm formation by enhancing 'twitching motility' (Singh *et al.*, 2002; Rogan *et al.*, 2004), is not a very potent antimicrobial agent- requiring very high doses to effectively eliminate large populations of planktonic cells. For example, in the current study, 100 mg/L of lactoferrin was required to kill 1000 colonies of *P. aeruginosa* per hour compared to 2 mg/L of lactoferrin-tobramycin combination: fig 5 -2, pg 223 and 6 -3, pg 265). Therefore, making a novel formulation approach, exemplified by our formulations, is an attractive strategy to employ in developing inhaled antimicrobial agents which are effective against biofilm forming bacteria.

8.2 Current treatment strategy for respiratory infections in people with CF

Currently, the treatment strategy for respiratory infections in people with CF recognises that one antimicrobial agent may not be effective to completely eradicate the range of chronic infections. Hence, a combination of two or even three antimicrobial agent maybe used either together or successively during clinical management of airway infections (Ryan *et al.*, 2011). In addition the treatment strategies also involves the use of both inhaled, nebulised and systemic antibiotics (also either alone or combination) (Ryan *et al.*, 2011) but commonly treatments are nebulised and sytemic. For the control of *P. aeruginosa* specifically, the approved antibiotics are tobramycin and colistin with others (aztreonam; ciprofloxacin; amikacin; and combined fosfomycin-tobramycin) still under clinical evaluation (Ryan *et al.*, 2011). While all these antibiotics have been shown to be

effective against planktonic bacteria, there is currently no antimicrobial agent indicated for the specific management of *P. aeruginosa* biofilm infections in the airways. We now know that *P. aeruginosa* has the ability to resist antibiotics either intrinsically, since they constitutively express β-lactamases and efflux pumps as well as by switching to a biofilm mode of growth where they are able to tolerate inflammatory response mechanisms and antimicrobial therapy, or following the acquisition of resistance genes (e.g. possession of genes for β-lactamases or enzymes that generally inactivate aminoglycosides) (Hoiby *et al.*, 2005; Høiby and Pressler, 2006), so there is need to develop formulations which are able to overcome the bacterial resistance strategies. Investigators like MacLeod *et al*., (2009) have tried to solve this problem by developing novel 4:1 antimicrobial combinations of fosfomycin and tobramycin, and found that the combinations had a high activity both *in vitro* and *in vivo* against clinical isolates of *E. coli*, *H. influenzae*, *S. aureus* and *Klebsiella* spp. Similarly, Cai *et al*., (2009) investigated the effect of combinations of the aminoglycoside amikacin with fosfomycin or the aminoglycoside isepamicin with fosfomycin on a *P. aeruginosa* biofilm-infected rat model. This study found that the combination not only showed a positive effect *in vitro* but also offered an improved therapeutic effect against the biofilm-infected rat model. Another group of investigators, Trapnell et al., (2012) have taken these findings a step further to clinically evaluate the safety and efficacy of a 4:1 (160/40 mg or 80/20 mg) fosfomycin-tobramycin nebulised combination in 79 adults with CF. The subjects all had chronic *P. aeruginosa* infections and FEV_1 ≥25% but ≤75% and the trial found that the fosfomycin-tobramycin combinations were well tolerated and substantially improved FEV_1 %, although there was a lack of conclusive reduction of biofilm in the subjects. However if real advances are to be made there is a need to broaden the scope of research outwith combinations of antibiotics. There is a need to include antimicrobial agents to which microbes cannot develop resistance in these therapies. This is believed to be a strategy that may constitute significant advancement in combination antimicrobial research as

well as provide a more effective use of currently available antibiotics. To the best of our understanding, antimicrobial peptides and proteins make particularly attractive candidates for pharmaceutical development especially in combination with potent aminoglycosides.

8.2.1 Use of antimicrobial peptides as therapeutic agents

There are several characteristics of antimicrobial proteins and peptides that make them attractive candidates for therapeutic agents; one of the main ones of these has to be their ability to halt the rise in the incidence of multi-drug resistant “superbugs”. In addition; 1) they are relatively fast acting, 2) they are microbicidal (rather than microbiostatic) and 3) despite their ancient lineage, they have remained effective defensive weapons with little observed resistance developed by different strains of bacteria. This is a very essential property in the age of multi-resistant bacteria (Hancock and Patrzykat, 2002; Scott and Hancock, 2000). For these and several other reasons, the exciting idea of employing antimicrobial peptides as drugs has resonated a lot especially among biotechnology companies (Hancock, 2000).

However, these widely held opinions on the use of antimicrobial peptides as potent therapeutic agents are not without controversy. Indeed, one study by Andres *et al.,* (2005) seems to suggest otherwise. This study used time kill assays to show that human recombinant lactoferrin actually decreased the efficacy of tobramycin towards clinical isolates of *P. aeruginosa*. The authors deduced that lactoferrin-induced cell depolarization maybe responsible for a slow-uptake of antibiotics such as tobramycin- thereby decreasing effectiveness. Their investigation employed the use of iron saturated lactoferrin (personal communication with with Randall Alfano Ph.D of InVitria, a division of Ventria bioscience) which is likely to reduce the the antimicrobial activity of lactoferrin

(Figure 5 -2, 5 -4, 5 -5, 6 -5 and 6 -6), an observation that has also been previously reported by Arnold *et al.,* (1980). Also, the assessment method which was employed in their investigation involved the use of a high salt growth media (10 mM Tris-HCl buffer containing 100 mM NaCl) which would compromise the activity of Lactoferrin, since it has been shown that high salt inhibits the bactericidal activity of lactoferrin (Smith *et al.,* 1996; Viejo-Diaz *et al.,* 2003; Viejo-Diaz *et al.,* 2004). Therefore, it is possible that these observations, contradicting the current study, suggesting that Lactoferrin reduces tobramycin activity are actually a consequence of reduced lactoferrin activity because of the elevated sodium chloride levels in the culture medium and the saturation of the antimicrobial protein with ferric ion.

Therefore, antimicrobial proteins and peptides remain an attractive propostion for inhalable therapies and still resonate among biotechnology companies (Hancock, 2000). This is even more evident as increasingly more protein biomolecules (with structural resemblance to naturally occurring antimicrobial peptides) are being discovered or employed as new and innovative alternatives to chemical antibiotics in the treatment of infections.

8.3 Challenges of spray drying proteins

For protein formulations that are insufficiently stable in aqueous solution, drying provides a suitable method for improving stability. There are numerous techniques that can be used to dry proteins (for example, freeze drying, spray drying, spray-freeze-drying and supercritical fluid drying). However, only spray drying offers an effective and efficient one-step method to produce dry powders with defined size, shape, surface property and density, all aerodynamic characteristics that cannot be readily achieved with other particle engineering processes (Shoyele and Cawthorne, 2006; Depreter *et al.*, 2013).

Although this particle production technique is well suited for producing micronized solids in a controlled manner, it presents with other challenges when employed to formulate therapeutically active peptide and protein drugs since it also exerts a significant amount of stress that can destabilize these labile molecules. The thermal heat applied, pressure and mechanical stress the protein has to undergo, proteins adsorbing to the air-liquid interfaces during atomisation and dehydration stresses occurring during the drying process have all been shown to have the capacity to perturb and ultimately denature proteins (Maa and Prestrelski, 2000; Ameri and Maa, 2006). However, it was observed during process optimisation of this study, that a careful selection of operating parameters ensured the achievement of dry powders of suitable size distribution and substantial activity even without the inclusion of stabilizing excipients (table 3.4, pg152). It was also observed that spray drying proteins alone produced poor yields typically between 40 and 60 % even when the high performance cyclone was used (table 4.2, pg188). However, it was found that the yield was considerably improved by addition of either NaCMC (table 3.3, pg 150 and table 4.2, pg 188) or the aminoglycoside antibiotics (table 6.1, pg 262).

In these studies, the spray drying parameters were initially optimised during the preliminary studies described in chapter 3. Data obtained from that study indicated that it was possible to spray dry a protein and produce respirable, spherically shaped particles while maintaining the structural integrity of the protein. These parameters were then applied to a clinically relevant antimicrobial protein- lactoferrin. The data presented in chapter 4 indicate that the optimised protocol produced inhalable particles ($FPF_{6.4\mu m}$ > 50 %) that retained antimicrobial activity towards *P. aeruginosa*. The mass median aerodynamic diameter of the spray-dried lactoferrin particles was found to be lower than the starting material at between 4 and 6 µm (Fig 4 -10, pg 201), with this observation further validated by scanning electron micrographs which revealed that the spray drying

process produced spherically shaped particles closely resembling dehydrated blueberries (Fig. 4 -5, pg 194).

As with most spray-dried proteins, X-ray powder diffraction analysis of the various dry powders suggest that all the spray-dried antimicrobial agents were amorphous in nature due to the presence of a broad halo and lack of resolved reflections. Although there might have been varying degrees of crystallinity, this line of investigation was not pursued further, because an absolute degree of crystallinity can only be made when reference materials of amorphous and crystalline materials are available and even then the ratio of the amorphous to crystalline protein would need to be established in a calibration plot which cannot effectively inform on the role of mixing the proteins with aminoglycoside.

Interestingly, SDS-PAGE and western blot analysis of the various spray-dried lactoferrin preparations did not show any irregularities in terms of the molecular weight bands observed and also indicated that the spray-dried protein was still recognised by both the IG12 and 4C6 anti-bovine lactoferrin antibodies (Fig 5 -9, pg 237), a clear indication that the binding sites for these antibodies were not destroyed during spray drying.

8.4 Penetration of antimicrobial agents through *P. aeruginosa* biofilms)

Since microorganisms do not live as dispersed single cells, but rather as flocks or sludge or ‘biofilms’, many studies have tried to link the lack of antimicrobial penetration to the inherent antibiotic resistance of biofilms (Lewis, 2001; Mah and O'toole, 2001; Stewart, 1996; Xu *et al.*, 2000). Based on this, it was essential to have a better understanding on how the various antimicrobial formulations prepared over the course

of this study may penetrate the biofilm matrix formed by *P. aeruginosa*. In chapter six, we found that combinations of lactoferrin and aminoglycoside significantly enhanced the penetration of aminoglycosides (between 40 and 70 %, table 6.3, pg 266) through *P. aeruginosa* biofilms compared to penetration of aminoglycoside alone. Our experimental observations were also further verified by confocal laser scanning microscopy, a non-destructive visualization technique which was employed to gain an in-depth insight into the effect of the formulations on *P. aeruginosa* biofilms grown on coverslip glass. It was noticed that there was a mixture of live and dead cells in all the biofilms exposed to the various formulations, however, more dead cells were observed upon treating the biofilms with combinations of apo lactoferrin and tobramycin/gentamicin for 24 hours. Interestingly, we also observed a lower number of dead cells compared with live cells upon treating the biofilms with the aminoglycosides only antibiotics. This observation may help explain previous observations by Anwar *et al.,* (1989) and Chuchalin *et al.*, (2009) who suggested that complete elimination of *P. aeruginosa* from the airways is not achievable with inhaled tobramycin alone.

8.5 *In vitro* administration of protein-aminoglycoside antimicrobial combinations

Most importantly, we tested the various protein-aminoglycoside combinations on *P.aeruginosa* biofilms grown on both CF (IB3-1) and non CF (CALU-3, BEAS-2B and C38) airway epithelial cells to find out if formulations maintained their antimicrobial activities *in vivo*. To achieve this, we utilized a relevant co-culture model in which biofilms were grown on CF airway epithelial cells, a method which was previously used by Anderson et al., (2008) and Moreau-Marquis et al., (2009) to effectively model biofilms that form within CF airway. We found that on all four airway epithelial cells

employed for the study, the protein-aminoglycoside combinations caused a 5- log reduction in the number of colonies per well compared to lactoferrin only antimicrobials which still reduced the colony forming unit per well, but now by between 2- and 3- log units. The significance of this is that, if relatively low numbers of bacteria are present then using a spray-dried recombinant human lactoferrin or spray-dried recombinant apo lactoferrin alone is likely to be adequate if administered alone. Also as patients are routinely treated with other antibiotics, intermittent use of a recombinant dry powder inhaler could also significantly improve outcome of airway infections especially those caused by *P. aeruginosa*. Therefore, even though lactoferrin and apo lactoferrin failed to achieve the industry standard 3- log reductions at the end of the 24 hour treatment required for it to be designated as a bactericidal agent, it may still have many therapeutic uses.

We estimate that 30 mg of the apo lactoferrin/aminoglycoside combination will be required to reduce the bacterial colony population in the conducting zone of the airways by greater than 99 %. This calculation is based on the fact that the effective growth area of the transwell insert is 0.3 cm^2; the total cross sectional area of the conducting zone of the respiratory tract is 180 cm^2; and our results (table 7.3, pg 336) indicated that 0.1 mg of the combination effectively reduced the cfu/well by 5-log units. However, this may not be exact as it also depends upon the deposition profile of the formulation, the ability of formulation to synergise with resident antimicrobial peptides in the airways (Singh *et al.*, 2000), therefore, doses may need to be adjusted to reflect these significant variations. But overall, the significance of this is that the mass of tobramycin required will effectively be 73% less than the currently prescribed dose of tobramycin (4 x 28 mg capsules, twice daily), but will provide a comparable level of *P. aeruginosa* bacterial killing as tobramycin, this decrease in mass of tobramycin to be delivered should definitely translate to a decrease in incidences of adverse effects, improved patient compliance,

reduction in hospital visits (improvement in quality of life) and reduction in the use of intravenous antibiotic infusions.

Cytotoxicity assessments of the various formulations on four relevant airway epithelial cells (CALU-3, BEAS-2B, IB3-1 and C38) which are representative of the bronchial epithelium where the formulations would impact upon inhalation, were promising and showed that the various compounds did not decrease the viability of the submerged cultured epithelial cells following 24 hours of exposure. Evaluation of cytokine secretion showed that exposure of IB3-1 and C38 airway epithelial cells to various lactoferrin containing combinations resulted in the secretion of significant amounts of the pro-inflammatory cytokine, IL-8. This is a less than ideal situation to occur in the CF airways, however the data indicate that this response is not specific to CF as we see it in the corrected counterpart cells as well. Therefore, we believe that the increased IL-8 production might have resulted as a consequence of the non-human, non-purified source of lactoferrin used. We would hypothesise that using human recombinant lactoferrin would prevent this pro-inflammatory effect.

Chapter Nine

Conclusions and future work

9.1 Concluding remarks

The ubiquitous role of proteins in a plethora of biological processes makes them molecules with a high therapeutic potential. However, delivering them to active sites in the respiratory tract has remained a difficult challenge. This has principally been as a result of problems associated with their fabrication as micro-particulates that are suitable for lower airway deposition while simultaneously maintaining their stability and biological activity. This study, has sought to advance the specialist science of inhalation by investigating ways by which protein inactivation during spray drying can be substantially mitigated. Results from this study show that the extent of inactivation of the two proteins used in this study varied extensively depending on the spray drying parameters employed. In addition, it was also found that while the incorporation of suitable stabilization excipients like sodium carboxymethylcellulose (which helps to preserve the integrity of proteins) can help improve the activity of the model protein (alkaline phosphatase), it was however found to be detrimental to the antimicrobial activities of lactoferrin and apo lactoferrin.

In vitro aerosolisation assessment results also showed that the aerodynamic deposition of the various powder formulations produced in this thesis, were appropriately suited for the delivery of substantial doses of the therapeutic proteins to the lower airways. The results imply that a dry powder inhalable formulation consisting of antimicrobial proteins could be used as an enhanced therapeutic alternative to the currently available antipseudomonal therapies.

In the light of recent public health threats occasioned by the spread of antibiotic resistant microorganisms and the lack of new drugs in the drug delivery pipeline, some of the

strategies discussed in this thesis may readily find industrial or even commercial applications. However, even though much of the primary research is already being carried out, there is room for improvement as the strive to develop novel, effective and safe pulmonary delivered antimicrobial peptides and proteins that enable clinicians to remain ahead, especially in the fight against antibiotic resistant microorganisms residing in the respiratory tract. It is our believe that formulations prepared using the concepts presented in this thesis offer a paradigm shift in the way airway infections should be managed in the future, which until now have only involved antibiotic only preparations (either as a single therapy or a combination therapy).

Reiterating two of the most important findings of this study;

Although *P. aeruginosa* biofilms are highly resistant to antibiotics generally, lactoferrin or in particular apo lactoferrin in combination with tobramycin are able to significantly prevent *P. aeruginosa* biofilm formation in the first instance and to a certain degree their persistence once they have gained a foothold

Spray dried lactoferrin, spray dried apo lactoferrin and co-spray dried combinations of lactoferrin/apo lactoferrin with aminoglycosides (tobramycin and gentamicin) remain active following processing. This is important because the spray drying technique can be used to produce antimicrobial particles containing proteins which can be effectively employed to target localized infections of the respiratory tract.

9.2 Recommendations and future work

Although the results presented in this study have demonstrated the effectiveness of combinations of an antimicrobial proteins and aminoglycosides in preventing the formation of *P. aeruginosa* biofilms however, these studies could be further developed by a number of strategies:

9.2.1 Elucidation of mechanism of action of the various antimicrobial agents

Although during the course of the present study, efforts were made to better understand the mechanism by which the co-formulations exerted their superior antimicrobial activities on both planktonic and biofilms of P. aeruginosa, it would be insightful to analyse the chemistry of the various formulations in order gain a better information of how interactions at the functional group or molecular levels produce the observed enhancement of the antimicrobial activities of the various formulations.

9.2.2 Evaluation of the various formulations on clinical isolates of *P. aeruginosa*

Given that the various antimicrobial formulations produced in this research could be potentially useful in the treatment of airway infections especially those resulting from *P. aeruginosa*, a direct extension of this work should definitely involve investigations into the effectiveness of the various antimicrobial formulations on clinical isolates of *P. aeruginosa,* as well as, other relevant strains of microorganisms which are also deleterious to the airway mucosa or are more problematic to manage.

9.2.3 Combinatons of recombinant Lf, recombinant SLPI and recombinant lysozyme and relevant antibiotics

In the normal airway, the relevant antimicrobial peptides involved in innate defences include; Lf, SLPI, Lysozyme. Various studies have shown that these antimicrobial peptides act in synergy to prevent bacterial infection, but in the CF airway, these antimicrobial peptides though produced are inactivated by high salt concentrations, perharps, delivering recombinant forms of these antimicrobial peptides (recombinant human lactoferrin, recombinant secretory leukocyte protease inhibitor, recombinant lysozyme) as dry powders in combinations with relevant antibiotics could potentially offer greater therapeutic benefits for the management of bronchitis, chronic obstructive pulmonary disorders and cystic fibrosis. It would also be interesting to determine if the penetration of the various combinations of recombinant antimicrobial proteins and relevant antibiotics through viscous mucus could be enhanced through co-administeration with dornase alpha.

9.2.4 Assessment of long-term stability of the various spray dried and co-spray dried formulations

There is also much work to be done in the area of assessing the stability of the various formulations that were developed during the course of this research. This is necessary to enable the determination of the best storage conditions and suitable time periods within which the formulations should be used. Perhaps, the most direct extension of this work should entail carrying out this investigation

Chapter Ten

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10.1 References

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11 Conferences attended

April, 2010	UKICRS 2010 Symposium, University of Hertfordshire, UK
December, 2010	DDL21 Conference, Edinburgh, UK
July, 2011	APS Inhalation Conference, University of Bath, UK
December, 2011	DDL21 Conference, Edinburgh, UK
May, 2012	UKICRS 2012 Symposium, Aston University, UK
May, 2012	RDD 2012 Conference, Phoenix, Arizona, USA

12 List of publications

12.1 Abstracts

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