UNIVERSIDADE DO ALGARVE

FACULDADE DE CIÊNCIAS E TECNOLOGIA

Departamento de Química e Farmácia

Inhalable mannitol microparticles as antigen carriers

Joana Cruz Henriques Silva

Dissertação para obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob a orientação de:

Professora Doutora Ana Margarida Moutinho Grenha

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// Abstract

Even with scientific advances, lower respiratory tract infections pose a serious danger to human life worldwide. Lower respiratory tract infections remained the deadliest infectious disease in 2019 and were the fourth most prevalent cause of death globally, according to the World Health Organization. Different bacteria and viruses, such as *S. pneumoniae* or *SARS-CoV-2*, can cause respiratory infections. Thus, prevention is crucial in the management of lower respiratory tract infections. Immunisation strategies regarding the prevention of lower respiratory infections include parenteral immunisation or even oral antigen delivery. In any case, it seems that lung protection is insufficient because lung infection often develops. A pulmonary immunisation technique seems promising because the lung is the primary site of infection. The use of the pulmonary route ensures that a local immune response is produced along with systemic immunisation, while avoiding adverse effects, such as liver metabolism, or protein degradation associated with the oral route. Appropriate drug delivery systems, such as microparticles, might be the adequate means to comply with the aerodynamic requisites needed for successful lung delivery.

Spray-dried microparticles comprising mannitol and a commercial formulation of bacterial lysates, produced by spray drying, are proposed for lung immunisation. Varying the mannitol/bacterial lysates mass ratio, several formulations of bacterial lysates-loaded microparticles were successfully produced. The association efficacy of the bacterial lysates was confirmed, reaching values of 50% and 65%. Aerodynamic evaluation was performed by cascade impaction using a methodology of gravimetry or the direct drug determination by Bradford method. The microparticles showed mass mean aerodynamic diameters between 5.9 and 7.3 µm, with the potential to reach the lung, mainly its upper part.

Keywords: Respiratory tract infections, Pulmonary delivery, Mannitol, Bacterial lysates, Microparticles.

Resumo

Mesmo com os avanços científicos, as infeções das vias respiratórias inferiores representam um sério perigo para a vida humana em todo o mundo. As infeções das vias respiratórias inferiores continuaram a ser a doença infeciosa com a maior taxa de mortalidade em 2019 e foram a quarta causa de morte a nível mundial, de acordo com a Organização Mundial de Saúde. Diferentes bactérias e vírus, tais como *S. pneumoniae* ou *SARS-CoV-2*, podem causar infeções respiratórias. Assim, a prevenção é crucial numa abordagem terapêutica para o tratamento das infeções das vias respiratórias inferiores. As estratégias de imunização relativas à prevenção de infeções respiratórias incluem uma imunização parenteral ou mesmo a administração oral de antigénios. Em qualquer caso, a proteção pulmonar parece insuficiente porque a infeção pulmonar acaba por se desenvolver frequentemente. Uma técnica de imunização pulmonar parece promissora porque o pulmão é o local primário da infeção. A utilização da via pulmonar assegura que uma resposta imunitária local é produzida juntamente com a imunização sistémica, evitando ao mesmo tempo efeitos adversos, tais como o metabolismo hepático, ou a degradação proteica associada à via oral. Sistemas apropriados de administração de fármacos, tais como micropartículas, podem ser os meios adequados para cumprir os requisitos aerodinâmicos necessários para uma administração pulmonar bem-sucedida.

Micropartículas contendo manitol e uma formulação comercial de lisados bacterianos, produzidas por técnica de secagem por atomização, são propostas para uma imunização pulmonar. Variando a relação massa manitol/lisados bacterianos, foram produzidas com sucesso várias formulações de micropartículas carregadas de lisados bacterianos. A eficácia da associação dos lisados bacterianos foi confirmada, atingindo valores entre os 50% e 65%. A avaliação aerodinâmica foi realizada por impactação de cascata utilizando uma metodologia de gravimetria ou a determinação direta do fármaco pelo método de Bradford. As micropartículas mostraram diâmetros aerodinâmicos entre 5,9 e 7,3 µm, com potencial para atingir o pulmão, principalmente a sua parte superior.

Palavras-chave: Infeções do trato respiratório, Administração pulmonar, Manitol, Lisados bacterianos, Micropartículas.

Resumo Alargado

As infeções respiratórias apresentam uma elevada prevalência e incidência a nível mundial, sendo a principal causa de morte por doenças infeciosas. Mesmo com o desenvolvimento científico crescente, continua a não existir uma terapêutica realmente eficaz, o que se torna preocupante principalmente com o aumento das resistências aos antibióticos. As infeções respiratórias podem ser provocadas por diversos microrganismos, como a bactéria *S. pneumoniae* ou o vírus, *SARS-CoV-2*. Conhecer a patogénese das infeções provocadas por estes microrganismos é um passo essencial para desenvolver novos fármacos e terapêuticas.

Como a entrada destes microrganismos no organismo e o seu local primário de ação é o sistema respiratório, parece promissor considerar uma abordagem terapêutica que envolva a administração pulmonar. A utilização desta via para administração de fármacos está descrita e ocorre há muitos anos e oferece várias vantagens, tanto para um tratamento a nível local como sistémico. Por exemplo, no tratamento da asma é desejável a administração direta dos fármacos e a obtenção de um efeito local, uma vez que o fármaco é direcionado para o local de ação na dose mais pequena possível, melhorando potencialmente o perfil de segurança. Por outro lado, se o objetivo for a administração sistémica, o pulmão tem uma grande superfície disponível para absorção, a qual está dotada de elevado fluxo sanguíneo, características favoráveis para permitir a absorção do fármaco para a circulação sistémica, além de também evitar o metabolismo hepático característico da administração oral. A via pulmonar é já amplamente utilizada para a administração de anestésicos ou até mesmo de insulina.

A prevenção, através da imunização, das doenças infeciosas pode ser uma estratégia terapêutica que ajude a combater o aumento das resistências aos antibióticos. Contudo, a maioria das vacinas atualmente disponíveis são administradas através da via parenteral, trazendo diversas desvantagens como a necessidade de pessoal treinado para a administração, bem como a exigência de condições especializadas de armazenamento e transporte. Por outro lado, também é conhecido que as vacinas administradas por via parenteral conferem uma fraca imunidade mediada por células T e proteção das mucosas. Para além dos benefícios da via pulmonar já discutidos, a vacinação pulmonar parece ser uma alternativa à via parenteral, principalmente para doenças infeciosas que afetem diretamente o sistema respiratório. Também parece lógico administrar os antigénios no

local de ação do agente infecioso, desencadeando tanto uma resposta imunológica local como sistémica. Embora as vacinas inaladas sejam promissoras, ainda existem muitos desafios a ultrapassar. Por exemplo, o antigénio tem de ser veiculado num sistema aerodinamicamente adequado para alcançar o pulmão e deve ser apresentar suficiente imunogenicidade para assegurar uma ativação adequada das células do sistema imune.

As formulações em pó seco podem trazer soluções que os seus equivalentes líquidos não conseguem atingir, aplicando-se também às vacinas. A estabilidade à temperatura ambiente, eliminando a necessidade de uma cadeia de frio, o volume e peso diminuído que representam e a sua adequabilidade para ser usadas nos dispositivos de inalação já comercializados são alguns dos benefícios que as vacinas sob a forma de pó seco podem trazer. Os pós secos para inalação podem ser produzidos por várias técnicas, como a secagem por atomização. Esta técnica é umas das mais tipicamente descritas para produzir partículas com uma gama de tamanhos adequada para inalação, uma vez que é necessário um tamanho de partícula aerodinâmico entre 1 e 5 μm para atingir o pulmão. A utilização da secagem por atomização para produção de pós secos para inalação tem sido bastante explorada, devido à flexibilidade da técnica, que permite ajustar as características das partículas em função dos parâmetros do processo, assim permitindo que exibam as características aerodinâmicas adequadas para alcançar o pulmão. Contudo, a lista de materiais aprovados para a administração pulmonar é muito restrita. O manitol é uma das opções disponíveis e pode ser utilizado não só como portador, mas também como um material da matriz das micropartículas.

Neste contexto, este trabalhou propõe o desenvolvimento de micropartículas à base de manitol, produzidas pela técnica de secagem por atomização, para a veiculação de antigénios para o pulmão. A formulação de antigénios baseia-se numa formulação comercial constituída por lisados bacterianos e outros excipientes. As micropartículas produzidas foram caracterizadas quanto à sua morfologia, propriedades aerodinâmicas e eficácia de associação dos lisados bacterianos.

Os baixos rendimentos de produção impediram que a simples conversão da formulação comercial de lisados bacterianos numa formulação inalável tivesse sucesso. Como resultado, o manitol foi escolhido como excipiente, e micropartículas contendo várias quantidades de lisados bacterianos foram produzidas com sucesso utilizando a técnica de secagem por atomização, exibindo pouca variação entre si. Os diâmetros de Feret variaram entre 2,77 μm e 3,32 μm, e os lisados bacterianos foram associados com

sucesso às micropartículas com uma eficácia de associação variável entre 50% e 65%. As propriedades aerodinâmicas foram caraterizadas por impactação de cascata, testando-se dois métodos para a quantificação das micropartículas sedimentadas nas diversas partes do equipamento: por gravimetria ou por quantificação da proteína depositada em cada parte do equipamento. A formulação selecionada registou um diâmetro aerodinâmico de 5,9 µm e obteve-se uma fração de partículas finas de 19%, identificando-se a necessidade de aperfeiçoamento desta formulação numa fase futura com vista à melhoria destes parâmetros. Além disso, notou-se que, embora mais prático, o método gravimétrico é pouco adequado para determinar as propriedades aerodinâmicas das micropartículas, dado que se verificou uma sobrestimação do diâmetro aerodinâmico. A utilização de diferentes excipientes ou solventes para melhorar o procedimento de atomização é uma potencial variável para otimização na fase seguinte.

Abbreviations

ACE2 - Angiotensin converting enzyme 2

ACI - Andersen cascade impactor

AE - Association efficiency

ANOVA - One-way analysis of variance

APCs - Antigen-presenting cells

AT1 - Alveolar type 1

AT2 - Alveolar type 2

BgaA - β-galactosidase

BL - Bacterial lysates

BSA - Bovine serum albumin

CbpA - Choline-binding protein A

CBPE - Phosphorylcholine esterase

CFC - Chlorofluorocarbon

COVID-19 - Coronavirus disease

CPS - Polysaccharide capsule

CTL - Cytotoxic T lymphocyte

DAMPs - Damage-associated molecular patterns

DCs - Dendritic cells

DNA - Deoxyribonucleic acid

DPIs - Dry powder inhalers

DPV - Dry powder vaccines

dsRNA - double-stranded ribonucleic acid

ED - Emitted dose

EDX - Energy dispersive X-ray

Eno - Enolase

ER - Endoplasmic reticulum

FDA - Food and Drug Administration

FESEM - Field emission scanning electron microscopy

FPD - Fine particle dose

FPF - Fine particle fraction

HFA - Hydrofluoroalkane

Hib - Haemophilus influenzae type B

HPMC - Hydroxypropyl methylcellulose

iBALT - inducible bronchus-associated lymphoid tissue

IgA - Immunoglobulin A

IgG - Immunoglobulin G

IgM - Immunoglobulin M

IP - Induction Port

LC - Loading capacity

LRTI - Lower respiratory tract infection

Man/BL - Mannitol/bacterial lysates

MD - Metered dose

MHC - Major histocompatibility complex

MMAD - Mass median aerodynamic diameter

mRNA – messenger ribonucleic acid

NanA - neuraminidase

PAF - platelet-activating factor

PAMPs - Pathogen-associated molecular patterns

PepO - Endopeptidase

Pht - Pneumococcal histidine triad protein

PLY - Pneumolysin

pMDIs - pressurized metered-dose inhalers

PRRs - Pattern recognition receptors

PspA - Pneumococcal surface protein A

RNA - Ribonucleic acid

SIgA - Secretory immunoglobulin A

SMIs - Soft mist inhalers

StrH - β-N-acetylglucosaminidase

Tfh - Follicular helper T cells

URTI - Upper respiratory tract infection

ZmpA - Pneumococcal zinc metalloprotease

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1. Introduction

1.1. Respiratory tract infections

1.1.1. Epidemiology

The respiratory tract is vulnerable to pathogens because it is in direct contact with the environment through gas exchange, which can lead to the development of infections. These infections can be caused by different agents such as viruses, bacteria, fungi, or parasites (1). When the infection affects the nasopharyngeal area, it is an upper respiratory tract infection (URTI), while if it involves the trachea, bronchi, and lung parenchyma, it is a lower respiratory tract infection (LRTI) (2). The latter are the most worrying as in 2019, according to the World Health Organization, LRTIs remained the world's deadliest infectious disease, being the 4th most common cause of death in the world (3).

Even with advances in science, LRTIs play a major role in increasing mortality from communicable diseases, regardless of the country's income group. In all countries between 2000 and 2019, there was a decrease in mortality from LRTIs, with these representing 6% of total deaths in 2000 and 4.7% in 2019, but this decrease was not as strong as expected, mainly due to antibiotic resistance and the lack of new therapies (4). In addition to the above, there are still differences between the impact these diseases have on mortality in each country. For example, in low-income countries such as Sub-Saharan Africa, LRTIs remains the second leading cause of death behind neonatal conditions, unlike in high-income countries where they are only ranked sixth behind diseases like ischaemic heart disease or stroke (3,4).

Another major concern with LRTIs is that they are the leading cause of death in children under the age of 5, with an estimated 740,000 deaths in 2019, however, there has been a significant improvement since 2010 (1,120,000 deaths) (4). The highest LRTI mortality rate in 2017 occurred in Central and Western sub-Saharan Africa with 239.7 and 338.7 deaths per 100000 children, respectively. Central Asia also had a high rate with 145.4 per 100000 children. Western Europe, North America and Australasia had the lowest rates (Figure 1.1.) (5).

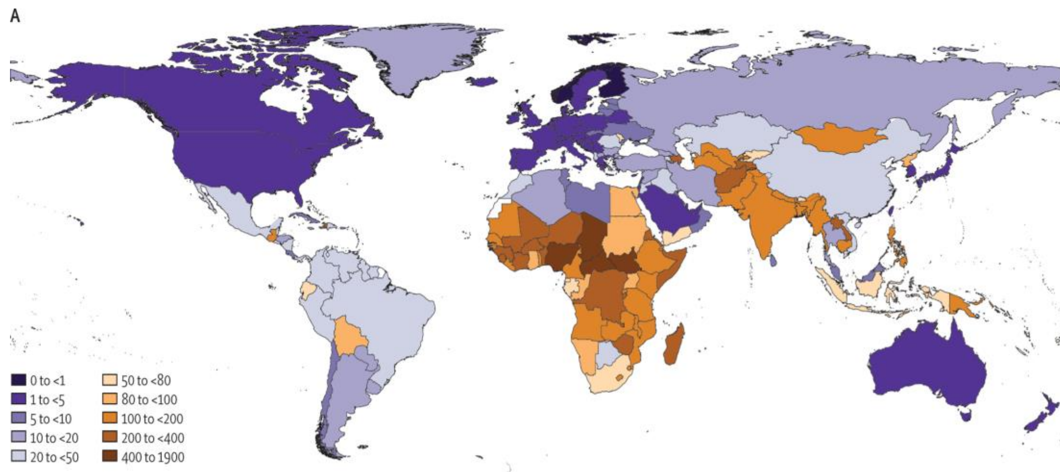

Figure 1.1. Mortality rate from LTRI in children under 5 years of age, 2017 (5).

Lack of access to vaccines, antibiotics, and health care in general at a preventive and early stage of the disease may contribute to the high mortality of infections in Africa. Other contributing factors are air pollution and childhood undernutrition (5–7).

Similarly to what happens in most high-income countries, the estimated deaths by LTRI in Portugal have been increasing over the years (Figure 1.2.). In 2019 about 685 per 100000 people died from these infections (3). One explanation may be the increase in antibiotic resistance (8,9). The older population is the most affected, as they are also the most vulnerable due to underlying diseases and aging itself (10,11). Children under 5 years of age, contrary to what happens in Africa, do not have a high mortality rate.

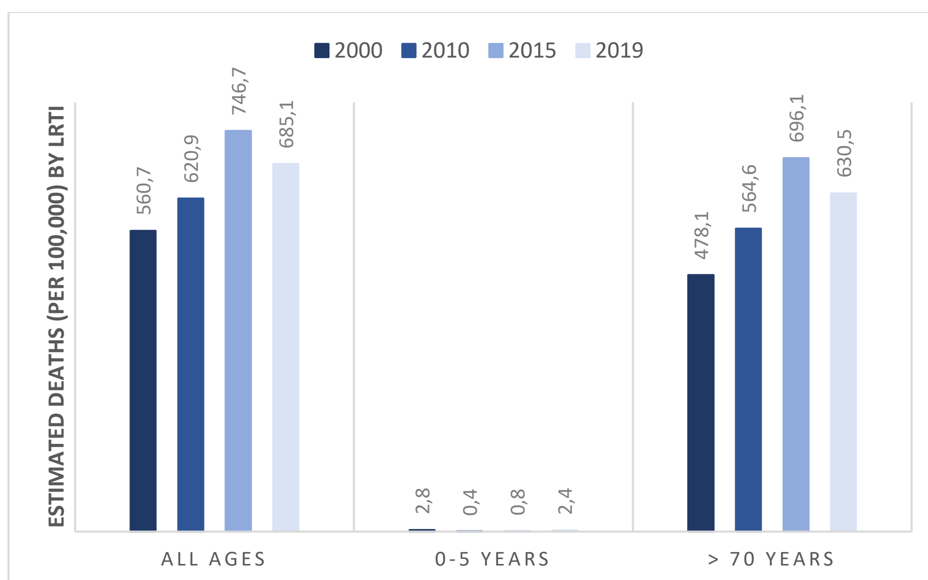

Figure 1.2. Graphic of estimated deaths by LRTI in Portugal, adapted from (4).

1.1.2. The respiratory microbiome and respiratory infections

The microbiome consists of all the microorganisms and their products that colonise surfaces in the human body. Each major compartment of the human body appears to have a unique microbiome with species specific to that environment (12). Fungi, bacteria, viruses, and archaea are all part of the human microbiome. This microbial ecosystem seems to play an important role in human health/disease as it allows the stimulation of the immune system, enabling the development of mucosal and/or epithelial barriers that contain the proliferation of pathogens (13).

The advance of new technologies for genome sequencing, allowed the development of the Human Microbiome Project which aimed to identify the community of microorganisms in different parts of the organism: vagina, skin, oropharynx, gut, and nasal cavity. Prior to this project, culture methods were those used to identify bacteria which led to the lower respiratory tract being thought to be a sterile site (14). The respiratory microbiome includes all microbes associated with the airways and lung tissue and is most accurately described as pertaining to the lower airways below the larynx. In addition, the microbiome of the oropharynx and nasal cavity are separate from the lower respiratory tract microbiome and have previously received more attention because they are easily accessible for sampling (12).

During the first year of life, the development of the microbiome will depend on many factors, such as mode of birth, genetic predisposition, breastfeeding, antibiotic exposure, vaccination, and geographic and seasonal variations (13). The onset of this development begins at birth, in babies born vaginally there is a predominance of *Lactobacillus*, *Prevotella*, or *Sneathia* spp. in the lower respiratory tract, very similar to the microbiome in the maternal vaginal environment. In contrast, babies born via caesarean section have a microbiome dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp., which are bacteria found predominantly on the skin (15,16). Generally, the changes in microbial composition are most dramatic in the first two months of life, with a single dominant profile in the first weeks, i.e. first a *Streptococcus* followed by a profile dominated by *S. aureus*. Gradually, these profiles are replaced by others with the dominance of other species such as *Moraxella*, *Corynebacterium*, *Dolosigranulum,* and/or *Haemophilus* (16). The timing of microbiological changes can influence susceptibility to various diseases (17). This was proposed by previous studies showing

that early colonisation with *Streptococcus pneumoniae*, *H. influenzae* or *M. catarrhalis* in the respiratory tract at four weeks of life led to an increased risk of bronchiolitis and pneumonia in infants (18).

The microbiome of the lower respiratory tract, in terms of quantity and diversity, is relatively smaller than is found in other body compartments, with about 10-100 bacterial cells per 1000 human cells (19). The composition of the lung microbiome can be affected by several factors, which will also increase or decrease the risk of lung disease. The three key factors are: microbial immigration, microbial elimination capacity and, regional growth conditions. The main routes of microbial immigration into the lungs are microaspiration, airborne inhalation and direct diffusion through mucosal surfaces (20). Of these, microaspiration is probably the most important, since the microbiota of the oral cavity and the lungs overlap in community composition and several previous studies have shown that subclinical microaspiration is ubiquitous among healthy individuals (21–23). The elimination of microbes from the lower respiratory tract is an active and constant process that requires several mechanisms such as coughing, mucociliary clearance and innate and adaptive immunity (24). By mucociliary clearance and coughing, organisms can be mechanically removed from the lungs. The microbes are trapped in the mucus secreted by the goblet cells and, with the help of the ciliated epithelial cells and the cough mechanism, they move up the respiratory system until they reach the laryngopharyngeal area where they can be expelled or swallowed (12). Another major defence of our lungs is the immune system which has a great capacity to recognise, signal and eliminate foreign organisms. Finally, the conditions that favour the growth of microorganisms within the lungs are determinant for the risk of developing disease in susceptible individuals (25). The environmental factors that have an impact on bacterial growth are: pH, nutritional availability, temperature, O2 tension, local microbial competition, concentration, and activity of inflammatory cells. However, these factors are less determinant of the risk of developing disease in healthy individuals than the other two factors discussed above (21,26,27).

Changes in these mechanisms can affect the clearance of microorganisms and cause an imbalance in the microbiome that increases susceptibility to infection. But not only that, environmental factors such as allergens and smoking can also trigger an imbalance in the host microbiome, along with tissue damage, resulting in a dysbiosis state (28,29). As consequence, there are changes at the epithelial level, increased colonisation,

activation of the innate immune system and, consequently, recruitment of immune cells, and disruption of the barrier, allowing pathogens to invade the tissues (Figure 1.3.). This leads to inflammatory processes to fight the infection until the danger signals disappear (30).

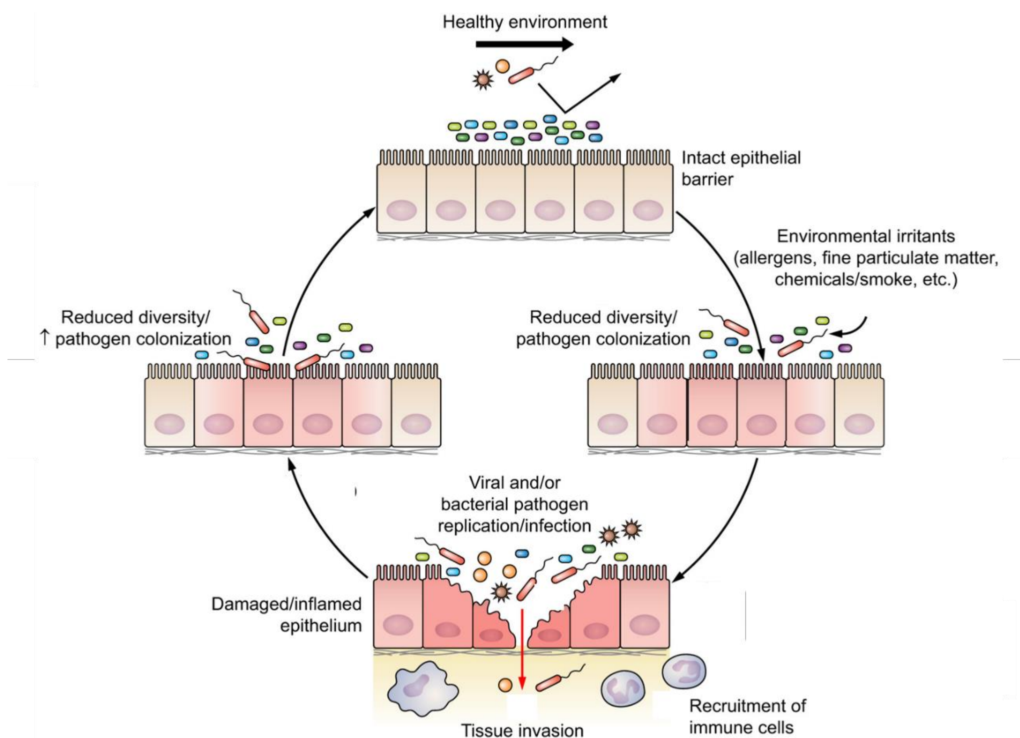

Figure 1.3. Equilibrium between host mucosal surface and microbial environment, adapted from (30).

1.1.3. Pathogenesis of lower respiratory tract infections: *Streptococcus pneumoniae* and *SARS-CoV-2* examples

1.1.3.1 *Streptococcus Pneumoniae*

There are many factors that can cause a pathogen to trigger an LRTI, such as pneumonia. As discussed above, the resident microbiota and a strong immune system are essential to prevent progression and assist in eliminating the invading agent. The bacterium *S. pneumoniae* is the main etiologic agent causing pneumonia so is important to know its process of pathogenesis (31).

S. pneumoniae is a gram-positive, extracellular, opportunistic pathogen and is also an ubiquitous human commensal of the upper respiratory tract, specifically the nasopharynx. In the first years of life, there is colonisation of the nasopharynx which is

repeated over time by different pneumococcal strains, although the prevalence of colonisation decreases with increasing age (32–34). When the pathogen reaches the nasopharynx, it encounters several defence mechanisms of the host and competition with the local microbiota (35,36). The main processes for an efficient colonisation are: setting the first contact with the epithelium and epithelial receptors, interaction with complement system, metal binding, reduction of neutrophil activity and mucus degradation (37). For this purpose, *S. pneumoniae* expresses several virulence factors that are summarised in Table 1.1. Each strain of *S. pneumoniae* has different virulence factors and this is a key factor impacting the evasion of the host immune system and the progression of the disease (38). Furthermore, it can help finding new strategies of prevention and treatment (38,39). After the nasopharynx colonisation, the first stage of *S. pneumoniae* life cycle and the only known natural human reservoir, transmission can occur through aerosolised droplets or contaminated objects (37).

The polysaccharide capsule (CPS), outside the cell wall, is unquestionably the main virulence factor of *S. pneumoniae* (39). Its role in virulence involves inhibition of phagocytosis mediated by innate immune cells and prevention of recognition by host receptors and complement factors. CPS is negatively charged, as are mucus and host macrophages, which may explain the ability of bacteria not to be trapped in mucus for removal as there is electrostatic repulsion (40,41). In addition to the CPS, the cytolytic toxin pneumolysin (PLY) has been shown to be crucial for prolonged colonisation and invasive disease (42,43). PLY is a bacterial toxin that is responsible for pore formation, which consequently leads to cell lysis and allows bacterial access to the cytosol of host cells, triggering the production of proinflammatory chemokines and cytokines (44–46). Other properties that PLY exhibits are the ability to inhibit mucociliary clearance in human lungs, separate the tight junctions between cells, which allows tissue penetration and facilitates invasion, and expose new sites of pneumococcal attachment. The human epithelium has several glycoconjugate proteins. These can be degraded by exoglycosidases such as neuraminidase (NanA), β-galactosidase (BgaA), and β-N-acetylglucosaminidase (StrH), which cleave sialic acid, galactose and N-acetylglucosamine, respectively. When glycoproteins are cleaved, they lose their function, allowing the release of sugars as a nutrient source, degradation of mucus and exposure of hidden receptors, facilitating bacterial adhesion (47–49). Pneumococcal surface proteins, such as pneumococcal surface protein A (PspA), choline-binding protein

A (CbpA), enolase (Eno), and pneumococcal histidine triad protein (Pht), directly and indirectly block complement deposition by binding to various factors of the classical and alternative complement pathway (50–52).

Table 1.1. Virulence factors of Streptococcus pneumoniae and their function in pathogenesis, adapted from (37).

Virulence factor	Function in pathogenesis
PLY	· Cytotoxic and pro-apoptotic for a large variety of host cells · Activates classical complement pathway and decreases serum opsonic activity · Highly pro-inflammatory at sub-lytic levels · Activates TLR4, NLRP3 inflammasome and p38–MAPK pathways
NanA	· Cleaves terminal sialic acid from host mucin and cell surface glycoconjugates · Unmasks receptors for adhesins · Triggers TGF-β signalling to facilitate endothelial invasion
BgaA	· Sequentially cleaves sugars from host glycoconjugates
StrH	· Sequentially cleaves sugars from host glycoconjugates
CPS	· Prevents entrapment by mucus during colonisation · Inhibits opsonophagocytosis by preventing the interaction of iC3b and the Fc fragment of IgG bound to deeper bacterial surface structures with receptors on phagocytic cells
PspA	· Limits C3 deposition on pneumococcal surface · Protects against bactericidal effects of free lactoferrin
CbpA	· Binds C3 and factor H and limits C3b deposition on pneumococcal surface · Binds PIGR and laminin receptor through separate domains · Facilitates adherence and invasion of respiratory epithelium and blood–brain barrier
Eno	· Binds and activates plasminogen · Facilitates tissue invasion
Pht	· May reduce C3 deposition on pneumococcal surface by binding factor H · Putative adhesins · Facilitate Zn acquisition together with AdcAII
PepO	· Binds fibronectin and plasminogen · Facilitates adherence and invasion · Binds C1q to inhibit classical complement pathway
CbpE	· Decreases neutrophil activity by inactivation of host PAF · Binds plasminogen
ZmpA	· Cleaves human IgA1
ChoP	· Binds PAFR on surface of epithelial and endothelial cells, facilitating adherence and invasion

Endopeptidase (PepO), released from the pneumococcal surface, binds to C1q and thus also interferes with the immune response (53). Pneumococcal phosphorylcholine esterase (CBPE) attaches and inhibits platelet-activating factor (PAF), a host-derived

inflammatory phospholipid, which results in the inactivation of neutrophils. Another effective virulence factor is pneumococcal zinc metalloprotease (ZmpA), also known as immunoglobin A (IgA) 1 protease, which cleaves the region of the crystallisable fragment of human IgA1, the most abundant immunoglobin in the respiratory tract, eliminating the binding capacity it has, causing the bacteria to evade this defence mechanism (54).

With all these factors, we can state that this microorganism has various tools at its disposal that can easily promote colonisation and invasion of the lower respiratory tract until pneumonia develops. The first barrier to be faced in the lungs is the mucociliary escalator, which traps the particles and microorganisms in the mucus and through an upward movement, expels them from the lung. To counteract this host defence mechanism, neuraminidases, the negative charge of CPS and ZmpA come into play, degrading and neutralising mucus and IgA1, as discussed above. The degradation of the mucus uncovers the respiratory epithelium which produces various antimicrobial peptides. By shedding the capsule, pneumococci counteract this defence and allow a closer and stronger interaction with the surface of the epithelial cells, starting the actual infection process (55,56).

Once the infection reaches the alveoli, the consolidation phase begins, which is characterised by severe inflammation and swelling of the affected lobules. Immune cells then accumulate in the alveoli, and the swelling is suspended by strands of fibrous tissue and erythrocytes leak out (red hepatization) (56). Subsequently, the lungs darken (grey hepatization) as leucocytes enter the lesion and bacteria are smothered by macrophages. Resolution of infection continues for a several days as the capsule-specific antibodies ensure efficient opsonisation and inflammatory mediators fade away (56,57).

1.1.3.2 SARS-CoV-2

A coronavirus referred to as SARS-CoV-2 became known in late 2019 when it emerged in the city of Wuhan, China, and caused an outbreak of viral pneumonia (58). It quickly spread around the world due to its high transmissibility, becoming a global pandemic (59,60). The coronavirus disease (COVID-19), characterised by flu-like symptoms (cough, fever, fatigue) in milder cases and in more severe cases dyspnoea and

pneumonia, has caused millions of deaths worldwide, especially in people over 60 years of age and with associated comorbidities (61–63).

SARSCoV-2 belongs to the genus Betacoronavirus of the family Coronaviridae of the order Nidovirales (64). It is an enveloped virus with a positive-sense ribonucleic acid (RNA) chain. Furthermore, it transcribes structural proteins like membrane protein, envelope protein, spike protein and nucleocapsid protein; non-structural proteins which are involved in the replication process, and accessory proteins. The structural proteins, together with the lipid membrane supplied by the host, form the enveloped virion. The non-structural proteins have diverse functions in the pathogenesis of the virus. They regulate transcription, helicase activity, immunomodulation, and prevent antiviral activity. The accessory proteins do not play a predominant role in replication but are associated to immunoevasive activities (65–67).

Transmission of the virus can occur from an infected person through droplets from speech or through aerosols, smaller particles that have the ability to remain in the air or in surfaces for a long period of time and can come into direct contact with the eyes, nose, or mouth or be inhaled by another person (68–70). When SARS-Cov-2 enters the body, the first cells that it targets are probably the multicellular cells in the nasopharynx or trachea, or the sustentacular cells in the nasal olfactory mucosa (71–73). Angiotensin converting enzyme 2 (ACE2) surface receptor has been shown to be an important target of the virus, as it is to this receptor that the spike protein will bind to start the process of entry into the host cell. The spike protein has two functional regions, known as S1 and S2. The different subunits of the S1 region are responsible for the association and stabilisation of the virus to the receptor and the S2 domain provides structural rearrangements that are essential for the fusion of the viral and host cell membranes (74,75).

Upon entry, the release and uncoating of viral RNA is immediate to initiate transcription and the production of viral proteins, including replicase proteins that form replication factories from endoplasmic reticulum (ER) membranes (76,77). These replication factories contain double membrane vesicles in which transcription occurs, protecting the double-stranded RNA (dsRNA) transcription intermediates from detection by cytoplasmic pattern recognition receptors (PRRs), thereby delaying the onset of the host antiviral response. After the production of new proteins, these translocate to the membrane of the endoplasmic reticulum and transit through the ER-to-Golgi intermediate

compartment, where interaction with newly produced genomic RNA results in budding into the lumen of secretory vesicular compartments. Finally, virions are released from the infected cell by exocytosis (78). With the formation of new viral particles, the infection progresses rapidly to the lower respiratory tract.

When it reaches the alveoli, SARS-CoV-2 primarily infects alveolar type 2 (AT2) cells rather than alveolar type 1 (AT1) cells, which was observed both in vivo and in vitro. AT2 cells are the precursor cells of AT1 cells, which are responsible for gas exchange and exist in greater numbers on the lung surface. The function of the AT2 is to secrete the surfactant needed to lubricate the alveoli and prevent them from collapsing (79–84). Therefore, infection in AT2 is responsible for damage to the alveoli, but this effect can also be caused by excessive local inflammation. Upon infection, AT2 cells adopt a phenotype associated with lung damage, in which there is no complete differentiation of AT2 cells (85–87). The damaged epithelium and endothelium of the alveoli allow fluid to enter the alveoli, but there is also an attraction and activation of platelets that initiate the coagulation cascade and thus the deposition of fibrin (88–92). At the same time, immune cells, such as monocytes and neutrophils, can be attracted and activated, and these have dysfunctional phenotypes that may further promote inflammation and coagulation, and neutrophils promote the formation of microthrombosis (93,94). All these factors eventually lead to a highly inflamed and flooded lung tissue, which impedes oxygen exchange and leads to hypoxia.

1.2. Pulmonary drug delivery

Pulmonary administration of drugs has been used for many years and offers several advantages, mainly if the focus is on the treatment of respiratory diseases such as asthma where a local effect is desirable, as the drug can be targeted to its site of action in the smallest possible dose, potentially improving the safety profile. On the other hand, if the aim is systemic administration, the lung has a large surface available for absorption with high blood flow, favourable characteristics to allow the absorption of the drug into the systemic circulation and is already widely used for the administration of anaesthetics and more recently for the administration of insulin, a formulation already marketed in the United States (95). In fact, it has been demonstrated that there is greater absorption of

drugs in the lung than in any other mucosal surface (96). However, the lungs have great capacity to eliminate foreign substances, including drugs, affecting their therapeutic efficacy. Therefore, the efficacy of a drug after lung delivery depends on three main factors: the aerodynamic properties that define how far it can reach, the physical, chemical and biological properties that determine the rate at which clearance from the lung is achieved, and finally, if the formulation is not only the drug itself, but includes a drug carrier, this must avoid its own pathways of clearance within the lungs (97). Due to the limitations of the oral and parenteral routes and the above-mentioned advantages of the pulmonary route, the latter has been investigated as a potential alternative. Table 1.2. compares the advantages and disadvantages of the different routes of administration (98–100).

Table 1.2. Advantages and limitations of pulmonary, oral, and parenteral routes of administration.

Route	Advantages	Limitations
Pulmonary	· Avoidance of first-pass metabolism · Lower side effects · Low concentration of drug required · Elevated vascularisation	· Complexity of respiratory tract · Lung defence mechanisms · Specific aerodynamic requisites to reach different zones of respiratory tract
Oral	· Convenient · Great compliance · Painless	· Slow onset · Interaction/degradation with food or acid from stomach · First pass metabolism
Parenteral	· Rapid absorption and fast drug onset of action · No first pass effect. · Suitable for unconscious patients · 100% bioavailability · Low concentration of drug required	· It is generally riskier · Invasive route · Painful

1.2.1. Lung deposition

The effectiveness of inhalable therapies against respiratory diseases strongly depends on the aerodynamic properties of the drug or drug carriers, on the patient's breathing pattern, and the morphology of the lungs (101,102). The aerodynamic diameter of a particle is defined theoretically as the equivalent to the diameter of a unit density ($\rho 0$) sphere which has the same terminal velocity in still air as the particle(103). One of the

most common theoretical approaches to calculate this diameter takes into account not only the size of the particle and its density, but also its shape:

$$d_{aer} = d\sqrt{\frac{\rho}{\rho_0 \chi}}$$

where d is the geometric diameter of the particle, ρ is the particle density and χ is the particle dynamic shape factor denoting deviation of shape from sphericity. There are experimental techniques allowing the determination of aerodynamic parameters, which are based on cascade impaction. Cascade impactors are thus the official instruments for aerodynamic assessment according to the European Pharmacopoeia and the United States Pharmacopoeia, and several apparatuses are described for the effect. The Andersen Cascade Impactor (ACI, Figure 1.4.) was the one used in the present work. The aerosol particles are transported by the air flow into the impactor, pass through several stages and are deposited on the collecting surfaces, named plates. Each stage of the impactor has a "cut-off diameter", that is, the particles with a diameter greater than that are deposited in this stage, while those with a smaller diameter follow the air flow. The result of this progressive separation is that the particles are divided into size intervals based on their aerodynamic diameter (104–106).

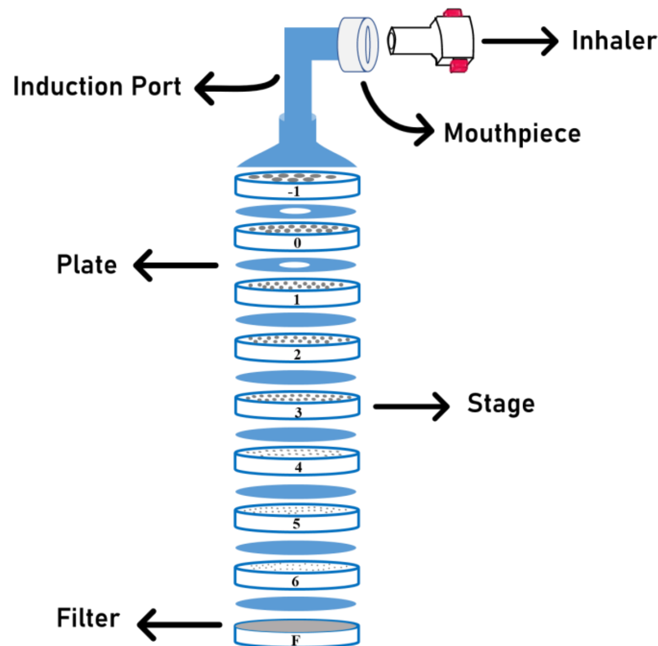

Figure 1.4. Schematic diagram of Andersen Cascade Impactor.

After entering the lung, particulate matter, including drugs or drug carriers, will observe deposition. This will occur according to different mechanisms, depending on the interplay of parameters mentioned above. Gravitational sedimentation, impaction, and diffusion are the three mechanisms that support particle deposition in the lung (Figure 1.5.). In the throat or at a bifurcation in the respiratory tract, the airstream changes course. If a particle's momentum is high enough, it will strike the walls of the airways rather than moving with the airstream as it changes. The inertial impaction, which is the main mechanism for deposition in the nose, mouth, pharynx, larynx, and the large conducting airways, is particularly significant for large particles having a diameter above 5 µm, and even more so for particles with a diameter greater than 10 µm (102). The airstream's velocity drops as the conducting airways continue to branch, making impaction a less significant deposition mechanism.

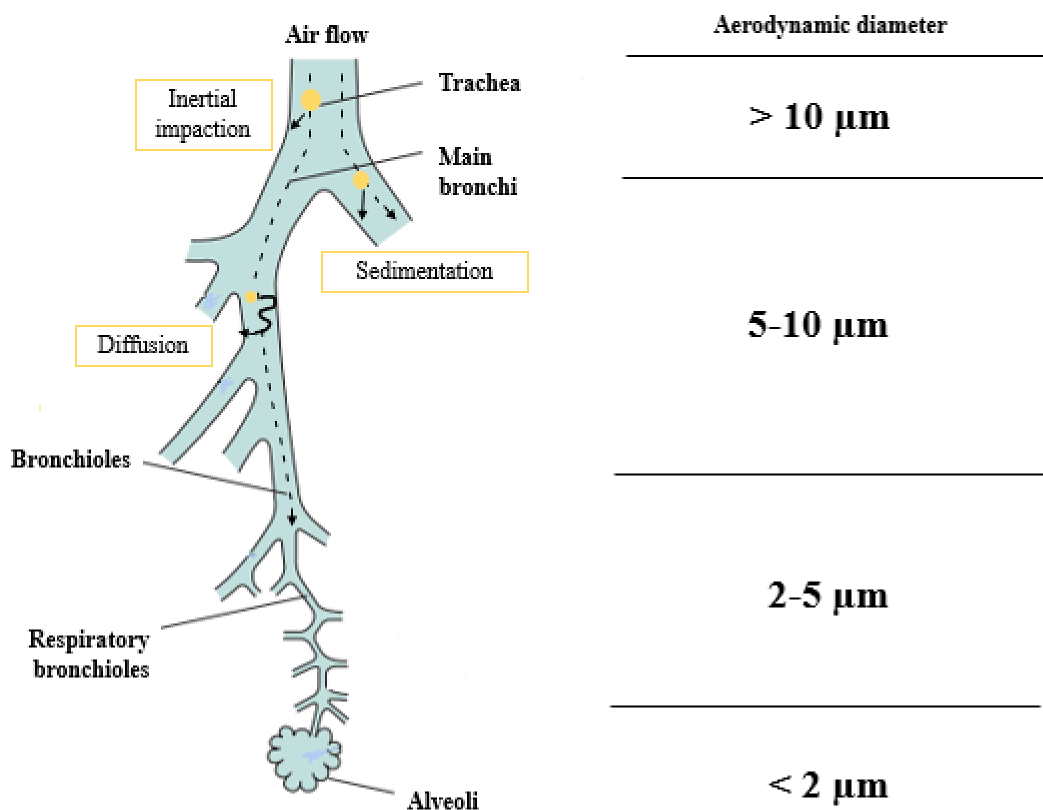

Figure 1.5. Schematic diagram of the three mechanisms of lung deposition and the aerodynamic diameters of each lung zone.

For particles that have escaped impaction-based deposition and are in the 2 µm to 5 µm size range, sedimentation is a significant deposition mechanism in the small airways and alveoli. However, this mechanism depends not only on the particle size and density, but also on the residence time in the airways. Smaller particles with less than 2 µm do not have the strength to leave the air currents and experience random and intense motions (Brownian diffusion) that can lead to the collision with the walls. Particles smaller than <0.5 µm are easily exhaled because they are too tiny for effective impaction or sedimentation (101,102). Hydrostatic suction pressure is another deposition mechanism, but it does not have as significant an impact as those mentioned previously. This occurs when a particle passes close to a liquid-filled wall. The electrostatic suction pressure will suck the particle in, as it does not have enough strength to overcome this pressure.

The patient's breathing pattern is also important in lung particle deposition. For example, increasing inhalation flow enhances deposition in the larger airways by inertial impact, while increasing inhalation volume increases peripheral dispersion of particles in the lung. Holding the breath after inhalation causes more particles to be deposited through sedimentation and diffusion(107). Slow and deep inhalation until the entire lung capacity is exhausted, followed by breath-hold before exhalation, results in the best aerosol deposition (102). If the patient has respiratory problems and consequent airway changes, it will constrain particle deposition.

1.2.2. Pulmonary administration

One of the crucial points for successful pulmonary administration is the inhalation device. The ideal device should produce an aerosol of the right size for the intended purpose of administration and allow reproducibility of the dose. In addition, it should be easy to handle, transportable and relatively affordable (108).

Currently, nebulizers, pressurized metered-dose inhalers (pMDIs), and dry powder inhalers (DPIs) are the three primary types of aerosol-generating devices described in Pharmacopoeias. More recently soft mist inhalers (SMIs) have emerged and can also be used in inhaled drug therapy. pMDIs, nebulisers, and SMIs work with liquid formulations where the drug is dissolved or in suspension, while in DPI the formulation is in the form of a dry powder (107).

pMDIs are the most common devices; in these, the drug is delivered in a pressurised container with a metering valve while suspended in liquid propellants or dissolved in them along with additional excipients, such as surfactants. When the metering valve is activated, a specific dose is delivered as an aerosol. The presence of the propellant eliminates the need for a sprayer, because as the propellant encounters ambient temperature and pressure turns into a gas, leaving a fine dispersion of the remaining components of the formulation (in the form of particles or droplets) which have the ideal characteristics to be inhaled effectively (107,109). The portability, affordability, and disposability of pMDIs are their main benefits. Also, the hermetically sealed container protects the drugs from oxidative deterioration and microbial contamination. pMDIs do, however, have drawbacks. When the device is activated, the first propellant droplets leave the device quickly and impact in the oropharyngeal regions, causing a significant loss of drug. The average size of the droplets that are released is usually greater than 40 µm, and propellants may not evaporate quickly enough for the droplets to become small enough to settle in the deep lung. Another issue with pMDIs is their improper usage by patients, as these devices require coordination between dose activation and inspiration (107).

Nebulisers were the first inhalation devices to hit the market (108). They work with liquids and transform them into aerosols. These devices are generally used for drugs that cannot be used in other devices. A major advantage is their ability to withstand high doses of drug over a long period of time. Nebulisers also have the advantage over pMDI and DPI systems that the drug can be inhaled during normal tidal breathing through a mouthpiece or face mask. As a result, they are beneficial for patients who have difficulty using pMDIs, such as young children and the elderly. However, there are some disadvantages related with portability, prolonged administration time and non-reproducibility of doses (107).With regard to the application of propellant-driven pMDI technology, environmental concerns have arisen. Although the original chlorofluorocarbon (CFC) propellants have been phased out due to their effects on the ozone layer, the hydrofluoroalkane (HFA) replacement propellants are greenhouse gases that are 2,000 times more potent than carbon dioxide (110). For this reason, the pharmaceutical industry had to adapt and look for alternatives, and DPIs emerged. Currently, DPIs account for more than half of the global inhaler market share and are forecast to represent 54% by 2030 (111). These devices have the significant benefit of delivering drugs in a solid state in the form of dry powder. The drug is placed in hard

gelatine or hydroxypropyl methylcellulose (HPMC) capsules that are placed in a device before use or is pre-loaded into an inhalation device (107,109). As portable as MDIs, DPIs combine powder technology with device design to disseminate dry particles as an aerosol in the patient's inspiratory airflow rather than using propellants. In most cases, DPIs only contain, in addition to the drug, a carrier, which aids on improving drug flow properties. These are breath-activated devices, thus not requiring on inhalation-actuation coordination as pMDIs (112,113).

DPIs, however, have several drawbacks. The patient's inhalation capacity, which may be compromised in cases of respiratory disease, is determinant for the removal of the dose from the device and for disaggregation objectives, either if it is disaggregation of drug particles from the carrier (e.g. lactose) or drug carrier disaggregation. A balance must be reached between inertial impact on the upper airway and disaggregation of the particles. In fact, if there is an increase in airflow, the particles will leave the device faster and more likely impact in the upper airways. Nevertheless, they will also disaggregate more easily, allowing the carrier particles to impact the throat and the smaller drug particles to reach the lung. DPIs contain the drug in powder form, which is much more stable than liquid formulations and advantageous for countries where temperatures are high because of the simplicity of storage conditions. However, they are more susceptible to external weather conditions, which could weaken the stability of the formulation. For example, increased humidity can lead to aggregation of powders. (107–109,112,113).

SMIs have emerged more recently and can be alternatives to DPIs and pMDIs. There is currently only one inhaler on the market, the Respimat® Soft Mist™ inhaler, which is suitable for asthma and chronic obstructive pulmonary disease. The energy of a compressed spring inside the inhaler powers the device, which does not require propellants. Individual doses are delivered through a specially designed mouthpiece system like a slow-moving aerosol cloud. It is more moisture resistant because the aerosol is produced from a solution rather than a powder, making it suitable for humid regions, an advantage over DPIs. Although the breathing technique required is similar to that of a pMDI, the aerosol is delivered from the device much more slowly than it would be from a pMDI, eliminating the need to use a spacer (114).

1.3. Immunisation

1.3.1. General concepts in immunisation

Since the first vaccination trials conducted by Edward Jenner and Louis Pasteur, several vaccines have been developed for a variety of infectious diseases that were previously among the most serious diseases affecting humanity. For example, as vaccination became more widespread, the incidence of diseases such as diphtheria, measles, mumps, pertussis, rubella, polio, and tetanus, which had once victimized millions of people, especially children, decreased significantly. Although we can intuitively understand that vaccination is a life-saving weapon, there is still a need to improve certain aspects: efficacy and safety, reduction in cost, as well as ensuring that they are distributed to those who need them most, especially in developing countries (115).

Immunisation is the process of inducing a protective immunity against a pathogen that causes disease. This process can be passive or active and can come from artificial or natural sources. Natural sources are the result of contact with the environment, people, and animals. Artificial sources, on the other hand, result from medical treatment (116). The transfer of already formed antibodies to an unimmunised person is known as passive immunisation. The unimmunised person receives antibodies that provides temporary protection; once antibodies are eliminated, the protection against the pathogen disappears because no organism-specific memory B or T cells were developed (115,116). As mentioned earlier, passive immunisation can occur from a natural source, the most frequent example being the transmission of maternal antibodies to the foetus and infants via the placenta or milk, respectively. Human gamma globulin and antivenin injection are two examples of artificial passive immunisation (115,117). At present, several conditions still justify the use of passive immunisation, including immune deficiency, especially congenital or acquired B-cell defects; exposure to life-threatening toxins or venom and, exposure to pathogens that can cause death more rapidly than an effective immune response can develop (115,117).

In order to produce protective immunity and long-lasting immunological memory, active immunisation aims to initiate the adaptive immune response by stimulating the production of antigen-reactive T and B cells, which leads to the development of protective

memory cells. This is the goal of vaccination. Active immunisation can be achieved either naturally through exposure to the infectious agent, such as the SARS-CoV-2 virus, or artificially through administration of a vaccine. Subsequent exposure to the pathogen, if active immunisation is effective, is not serious because the organism can eradicate the pathogen or prevent disease caused by its components or products (115,116). The market offers a variety of vaccine types with different levels of immunogenicity, stability, and safety (Figure1.6.).

The original pathogen forms, weakened by laboratories, are present in live and attenuated vaccines, such as those used to prevent measles, mumps, and chickenpox. These vaccines offer prolonged exposure of the immune system to the immunogens (epitopes) in the attenuated organism and more accurately resemble the growth patterns of the "real" pathogen because of their ability to grow, even briefly. This often leads to increased immunogenicity and more effective generation of powerful memory cells. However, because these vaccines contain live pathogens, they present higher risk and refrigeration is required to maintain efficacy, with the risk that the pathogen may revert to its original virulent form. Therefore, vaccines based on live organisms should not be administered to immunocompromised individuals (115).

Heat or chemical treatment is another method often used to make a pathogen suitable for inclusion in a vaccine. This eliminates the pathogen and prevents it from replicating, but still allows triggering an immune reaction to at least some of the immunogens (antigens) present in the body. The key epitopes of the surface antigens must be preserved in their current structure after inactivation. Due to the considerable results of protein denaturation during heat inactivation, it is often unsatisfactory and is likely to dramatically modify any epitopes that depend on higher levels of protein structure. The development of a strong immune response is inferior compared to attenuated vaccines, requiring several boosters. An example of this type of inactivated vaccine is the influenza or hepatitis A virus vaccine (115,116).

Subunit vaccines are purified macromolecules derived from pathogens. Inactivated pathogenic exotoxins, also known as toxoids, isolated capsular polysaccharides or surface glycoproteins and purified recombinant major protein antigens are the three applications of this method that are most frequently used. The recombinant hepatitis B vaccine is an example of a subunit vaccine, since it contains only the epitopes, which are the regions of an antigen that T cells and antibodies can most easily recognise

and bind to. Fewer specific antigens are used in these vaccines, which reduces the risk of negative reactions; however, this specificity makes it more difficult to choose which antigens should be in the vaccine (115).

Tetanus and diphtheria vaccines are examples of toxoid vaccines as they are made by inactivating bacterial toxins with formalin. The immune system reacts to the bacterial toxins in reaction to these toxoids (115).

Conjugate vaccines are a unique type of vaccination subunit, with the Haemophilus influenzae type B (Hib) vaccine serving as an example. In a conjugate vaccine, antigens or toxins from a microbe are bound with polysaccharides from the outer coating of the pathogen, which increases the ability of macrophages and neutrophils to recognise and phagocytise them (115,118).

It is important to remember that while live attenuated vaccines enhance cell-mediated responses and prolong the delivery of immunogenicity, a disadvantage is that they occasionally revert to pathogenic forms. Recombinant vectors prevent this significant drawback of reversion while maintaining the benefits of the live attenuated vaccine method. In safe attenuated viruses or bacteria that are used as live carriers, specific genes encoding essential antigens of particularly lethal pathogens can be added. The individual gene product(s) of the pathogen are expressed by the attenuated organism, which acts as a vector by reproducing within the host that received the vaccination. The potential for reversion is essentially non-existent because the pathogen's genome is missing most of its sequence (115).

Deoxyribonucleic acid (DNA) vaccines are made from plasmid DNA, which is injected into the recipient's muscle and contains antigenic proteins. In order to route the antigen through endogenous major histocompatibility complex (MHC) class I presentation pathways, which should help trigger greater cytotoxic T lymphocyte (CTL) responses, this technique relies on host cells to take up the DNA and create the immunogenic protein in vivo. The DNA appears to fuse with the host chromosomal DNA or be maintained in an episomal state for brief periods of time.

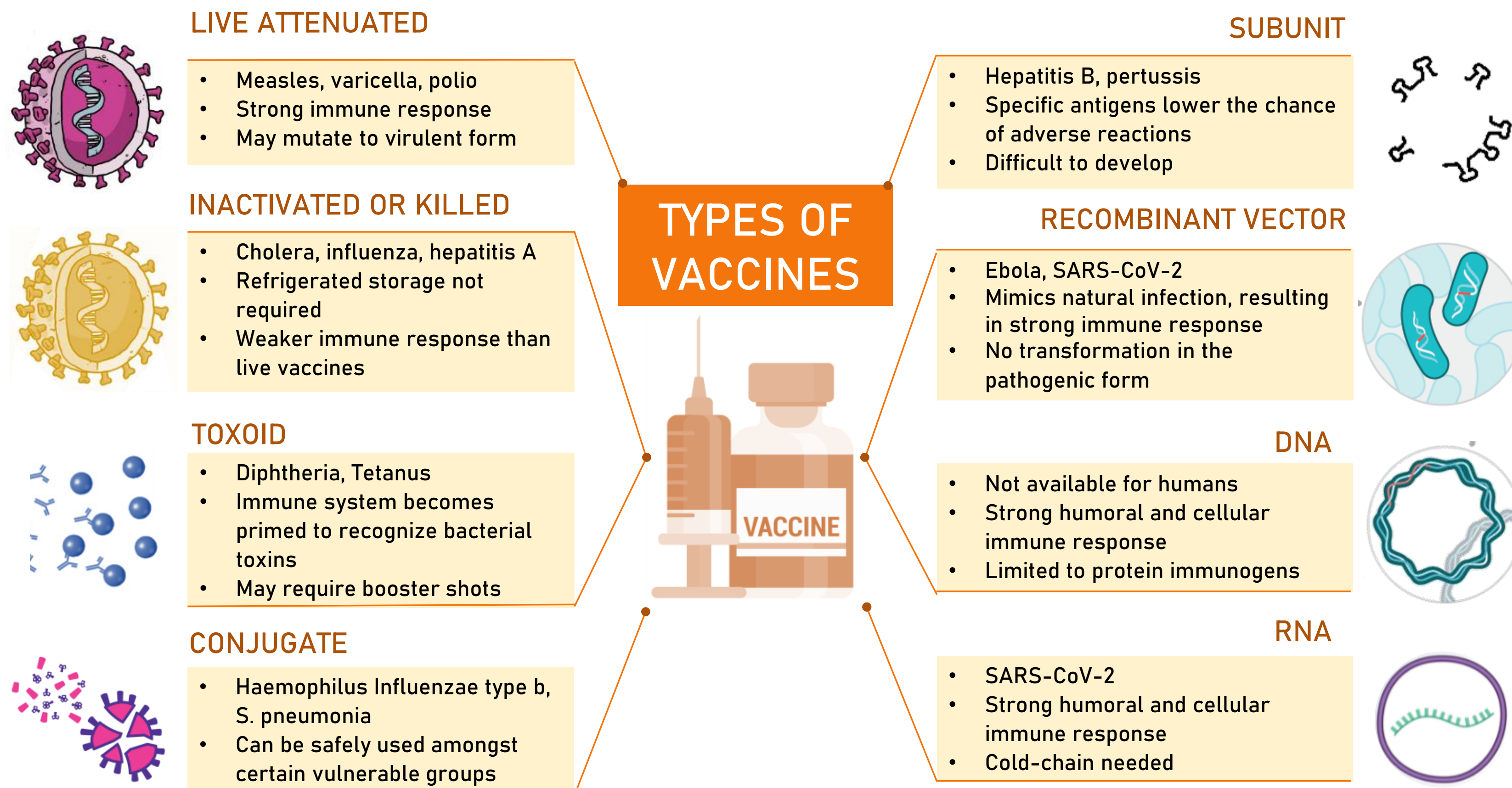

Figure 1.6. Different types of vaccines and their advantages and limitations, adapted from (115).

The aim is to reach dendritic cells (DCs) and other antigen-presenting cells (APCs) at or near the injection site. Direct or indirect administration to local APCs is essential for the establishment of antigenic responses to these vaccines, as muscle cells express little MHC class I and no co-stimulatory molecules (115,119,120).

Another type of vaccines are RNA vaccines, which are now well known due to COVID-19 pandemic. These vaccines are composed by messenger RNA (mRNA) fragments that encode a protein of the pathogen with antigenic potential. DCs phagocytose and pick up these mRNA fragments. The DCs read the mRNA and manufacture the viral antigens that the mRNA encodes using their internal machinery (ribosomes). The proteins will be recognised as foreign by the body and trigger the immunity process. Within a few days of administration, the body breaks down the mRNA fragments. The great advantages of these vaccines are their efficacy, safety, and ease of production. Since they are not created using pathogenic particles or inactivated pathogens, they are less infectious and there is also no risk of the RNA integrating with host DNA. The only mRNA vaccines currently approved are those against the SARS-CoV-2 coronavirus. These vaccines employ mRNA, which instructs cells to make copies of the 'spike protein' (121–123).

Infectious disease outbreaks can be prevented and controlled using vaccines. They support global health security and will be a crucial weapon in the fight against antibiotic resistance. However, despite significant progress, immunisation rates have plateaued in recent years and declined after 2020 and many infectious diseases remain uncovered by vaccinal strategies.

1.3.2. Pulmonary immunisation

Most vaccines currently available are administered by injection. This administration modality brings several disadvantages, such as the need for trained personnel for administration, requirement for specialised storage and transport conditions, increased risk of cross contamination due to the possibility of needle-stick injuries and needle re-use, all resulting in the increase in the cost of vaccination (124). Another drawback is pain and discomfort that may decrease people's compliance. In

addition, injectable vaccines are capable of eliciting robust systemic humoral responses but confer weaker T-cell mediated immunity and mucosal protection (125,126).

In addition to the benefits of the pulmonary route already discussed, pulmonary vaccination appears to be an alternative to the injectable route, particularly when respiratory conditions are focused. It also seems logical to administer the antigens at the site of action of the infectious agent, triggering both a local and a systemic immunological response. Although inhaled vaccines are a step forward, there are still many problems to overcome. For example, the vaccine must be aerodynamically suitable to reach the lungs and must be immunogenically active enough to ensure adequate activation of the immune cells (127). It is therefore important to understand how the immune system reacts to (or is compromised by) distinct respiratory pathogens in order to design more potent or widely applicable vaccines.

When immune cells contact with pathogens, the identification of damage-associated molecular patterns (DAMPs), such as those of infected cells, or pathogen-associated molecular patterns (PAMPs), through toll-like receptors causes the APCs to take up the antigen. Following this, the DCs often move along the afferent lymphatic arteries to nearby draining lymph nodes, where the antigen is then delivered to immature T and B cells (127,128). In addition to following the traditional route of antigen presentation, when the human respiratory system is infected, specialised tissues known as inducible bronchus-associated lymphoid tissue (iBALT) may develop. These tissues are composed of B-cell follicles and plasma cells and are occasionally surrounded by tightly packed zones of T cells and APCs. Here, immature and effector B and T cells receive the antigens directly; there is no need to migrate in the lymphatic system (128–130). Microfold cells with particular functions transmit antigens and particles through the mucosal epithelium of the iBALT. Directly below the epithelium, DC are situated so that they can collect incoming antigens. The DC layer is followed by B-cell follicles and surrounded by T-cells, where activation occurs. At the margin of the T-cell region, efferent lymphatic vessels collect fluids and tissue cells and transport them to upstream lymph nodes or into the circulation. These ectopic lymphoid tissue structures are stimulated by inflammatory factors after infection and maintained by follicular DCs (131,132). Activated B and T cells can have various functions, for example, some B cells will differentiate into long-lived plasma cells that reside in the bone marrow and secrete antibodies for decades; other B cells may differentiate into short-lived plasma cells that

reside in the nasal and pulmonary mucosa and secrete antibodies (133). Some CD4+ T lymphocytes will differentiate into cytokine-producing effector cells that stay in the lung and stimulate macrophage activation and recruitment of inflammatory cells (134), while other CD4+ T cells will likely differentiate into follicular helper T cells (Tfh) that stay in lymphoid tissues and support B cells (135). CD8+ T-lymphocytes will mainly take charge of killing the infected cells (136). After microorganism clearance, most of the reactive B and T cells will undergo apoptosis, leaving some memory cells dormant in the lymphoid organs and respiratory system, where they will remain until they encounter the antigen again (137).

The generation of immunoglobulins, both inside the parenchyma and for export to the mucosal surface of the lung, is one of the key tasks of B cells in the airways once activated. IgA is the immunoglobulin found in the highest concentration in a healthy respiratory tract and is considered to be the most crucial for improving lung defence (138). Dimeric IgA antibodies, which are formed by plasma cells in the subepithelial lamina propria under the influence of Tfh and epithelial cytokines (e.g. IL-2, IL-5, IL-6, IL-10), are an essential effector component of the mucosal immune system, in addition to systemic immunoglobin G (IgG) (139,140). IgA on mucosal surfaces are present in their secretory form, and are called secretory IgA (SIgA), which has the particularity of preventing proteolytic cleavage in the lumen (141). The principle of immune exclusion, which includes all defences against antigens passing through the epithelium of the respiratory tract, is initiated at this time in the lumen by effector activities of SIgA and immunoglobin M (IgM) antibodies. They have three main defence mechanisms: firstly, pathogens in the lumen may become cross-linked by SIgA, which slows or prevents their ability to adhere to the epithelium, causing a barrier to infectivity. Secondly, they are able to bind to antigens that have already passed through the epithelial lining and expel them by the mechanism of receptor-mediated endocytosis. In addition, they have the ability to bind antigens within infected cells and export them by vesicular transport (141–143). Macrophages, monocytes and neutrophils can also be activated by SIgA, as they have the IgA FcαR receptor (CD89) (144).

In summary, vaccine administration through the respiratory tract mucosa not only causes systemic IgG- and cell-mediated responses, but also potentiates the induction of a potent local immune response, supported in particular by SIgA antibodies, which are crucial for respiratory tract immune defence.

1.4. Inhalation as a strategy in respiratory tract infections therapy

Dry powder formulations have been one of the solutions for inhalable medicines, as they bring advantages comparing with liquid counterparts. The benefits also apply to vaccines. Dry powder vaccines (DPV) are more stable, as they can be at room temperature, eliminating the need for a cold chain. This will especially help developing countries and tropical climates, as was already referred before. Bulk transport will also be facilitated because DPVs weigh and occupy less volume than liquid formulations. DPVs are also suitable for use in DPIs, which prevents re-use, cross-contamination and allows delivery of reproducible doses in one or just a few inhalations compared to liquid formulations (145,146).

With encouraging results, DPV administration by inhalation has been investigated in vivo for tuberculosis, hepatitis, influenza, and measles (147–152). A clinical trial also confirmed the safety and tolerability of a dry powder measles vaccine administered by inhalation (153).

There are several techniques described to produce dry powders for inhalation, including spray-drying and spray-freeze-drying. Spray-drying is already applied in the industrial pharmacy for this end and is one of the most typically described to produce particles with a size range suitable for inhalation, as aerodynamic particle size between 1 and 5 µm is required to reach the lung. Particles above this size tend to impact the surface of the upper airways. For vaccine delivery, the latter may not be completely disadvantageous because these particles can still reach the lymphoid tissue in the oropharyngeal region (145). Another important point for the formation of DPV is its stability both at the time of drying and storage. To provide stability we can use various excipients to protect the antigen by encapsulating it in a matrix of glass forming excipients such as sugars, a method that has been used for various biopharmaceuticals (154).

1.4.1. Spray-drying to produce inhalable dry powders

Spray-drying is a widely used technique in the pharmaceutical, chemical, materials, cosmetics and food industries to produce dry powders (155–157). It is a process

that converts a liquid into a dry powder by atomising it into a hot drying gas stream, typically air (158). This process can be divided into 4 fundamental phases: atomization of the liquid feed, drying of spray into drying gas, formation of dry particles and separation and collection of the dry product from the drying gas (159–161) (Figure 1.5.). The feed is introduced into the drying chamber and then atomised, forming a spray that corresponds to small droplets (micrometric scale) (162,163). As the hot drying gas simultaneously enters the drying chamber and encounters the droplets, the liquid evaporates rapidly and the solids form a dry particle. A cyclone collector is then used to separate the dried microparticles from the drying gas, and the microparticles will accumulate in the collection vessel (164).

This method allows the optimisation of the geometric size, density, and shape of the microparticles, by combining different operational parameters such as drying (inlet) temperature and drying gas flow rate, feed characteristics, feed rate, pressure and amount of atomizing air entering the drying chamber.

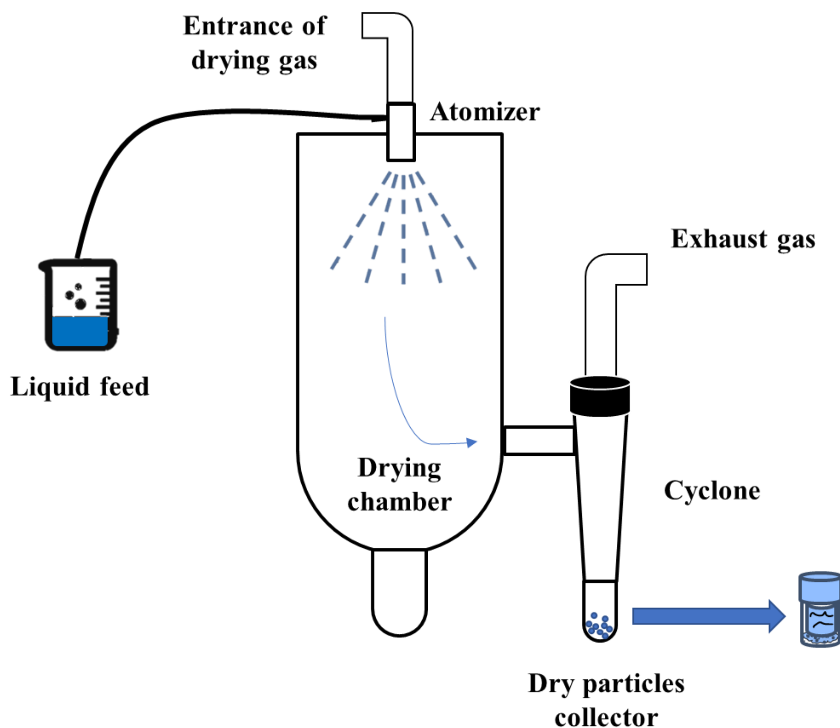

Figure 1.7. Diagram of the equipment and process of spray-drying.

The combination of these parameters depends very much on the final objective of the microparticles, but there is much space for optimisation (165,166). The parameters

referred above along with the humidity of the air and the properties of the feed, will directly affect droplet size, outlet air temperature, drying efficiency, and physical attributes of the final product, such as particle size, moisture content, and hygroscopicity. For example, a higher inlet temperature favours the production of drier products; however, lower temperatures prevent degradation or loss of active compounds or an increase in solid concentration in the feed results in larger particles (166).

Microparticles made by spray-drying have a natural propensity to agglomerate due to the powerful cohesive forces that reduce flow characteristics. This has an impact on the storage of the microparticles, as well as their ability for lung deposition (167). Generally, spray-drying solutions include solvents, mainly water or hydrophilic solvents such as ethanol, a matrix excipient, and the active pharmaceutical ingredient. Matrix excipients help improve the mechanical qualities, pharmacological properties, or physical or chemical stability of the active pharmaceutical ingredient (168). The literature frequently describes the use of sugars and polysaccharides. These are most often used as bulking agents, dissolution enhancers and stabilisers, while other polymers and lipids are most often employed in controlled release formulations (169–171).

α-lactose monohydrate carrier particles constitute the majority of DPI formulations in the market. Therefore, there is much research on this excipient, its low toxicity profile and wide availability at reasonable cost being its main advantages. On the other hand, it shows incompatibility of the sugar reducing aldehyde group with various biopharmaceuticals such as peptides and proteins. Another drawback is that the quality of the product may change as result of manufacturing and storage procedures (172–177). The list of materials accepted for pulmonary administration is very restricted, so others are urgently required. Mannitol, benefiting from the status of approved material, appears as a potential alternative not only as a carrier, but also as a microparticle matrix material. This excipient was selected for the production of microparticles reported in this thesis and, therefore, will be discussed in more detail in the next section.

1.4.2. Mannitol

Mannitol is a natural six-carbon sugar alcohol belonging to the class of polyols that are characterised by the reduction of an aldehyde or ketone group to the

corresponding primary or secondary hydroxyl group. Many commonly eaten vegetables and fruits naturally contain mannitol (178), which is particularly common in squash, celery, carrots, pineapple, and seaweed (179).

Since its discovery, many of the significant physiological activities of this substance have come to light, and the food and pharmaceutical industries now make great use of it. It is, for example, used in sugar-free chewing gum as a sweetener or anti-stick agent (180,181). In pharmaceutical formulations is mainly used as an excipient in tablet formulations, where it is of great importance because it is non-hygroscopic and therefore can be used with active substances that are sensitive to moisture (182). It is also used in freeze-dried preparations, where it produces a rigid and homogeneous cake that improves the appearance of the freeze-dried product (183,184). It has been suggested as a plasticiser in soft gelatine capsules, as a component of sustained-release tablet formulations (185), and, as noted above, it is accepted for pulmonary administration, being an excipient in inhalable formulations (177,186). In medicine, it is used as a powerful osmotic diuretic and dehydrating agent and can reduce the risk of acute kidney failure in patients who have undergone kidney transplantation and prevents irreversible renal failure. It reduces intracranial pressure and is used in support systemic therapy for acute glaucoma. The bronchial challenge, a diagnostic test for asthma, can also be performed using mannitol, which at high doses induces an inflammatory response in the airways. More recently (October 2020), Bronchitol® (capsules of 40 mg mannitol for inhalation) was approved for the maintenance therapy of pulmonary symptoms associated with cystic fibrosis in adult patients (181,187,188)

2. Objectives

This thesis is aimed at producing spray-dried mannitol-based microparticles for lung delivery of antigens, considering the need to propose immunisation alternatives to address respiratory diseases. To reach the general objective, the following partial objectives were established:

1) To verify the ability of a commercial formulation of bacterial lysates (BL) to be converted into an inhalable formulation by a process of spray-drying;
2) To use spray-drying to produce mannitol microparticles encapsulating BL;
3) To characterise the produced microparticles regarding their morphology and aerodynamic properties;
4) To determine the efficiency of the association of BL.

3. Materials and Methods

3.1 Reagents

Mannitol, bovine serum albumin (BSA) and glycerol were purchased from Sigma-Aldrich® (Germany). Bradford reagent was purchased from Bio-Rad® (Germany). BL from Bronchovaxom® and ultrapure water (Millipore®, Portugal) were used throughout. All other chemicals were reagent grade.

3.2 Preparation of mannitol/bacterial lysates (Man/BL) and bacterial lysates (BL) microparticles by spray-drying

3.2.1. Preparation of Man/BL and BL dispersions

Man/BL-based microparticles were prepared from mannitol solutions prepared at the concentration of 2% (w/v). The preparation of the solutions involved the mechanical stirring of the previously weighed mannitol with ultrapure water. After dissolution, the BL were added, allowing stirring for at least 4 h before spray-drying. To prepare the dispersion with only the BL, ultrapure water was added gradually to a pre-determined amount of BL under stirring. Stirring was also allowed for at least 4 h before spray-drying. BL was used directly from the capsules of Bronchovaxom®, as separation of the other excipients was not possible.

Six different formulations were prepared: one without mannitol, produced only from the whole content of Bronchovaxom®, and the others containing mannitol. The concentration of mannitol was maintained constant at 2% (w/v), while BL concentration varied to reach Man/BL mass ratios between 10/0.2 and 10/0.9. Consequently, the solid content of the spraying dispersions was also variable, ranging from 1% to 7% (w/v).

3.2.2. Spray-drying of BL/Man and BL dispersions

The obtained dispersions were spray-dried in a Buchi B-290 laboratory mini spray-dryer (Buchi Labortechnik AG, Switzerland) equipped with a standard cyclone.

The spray flow rate was set at 473 L/h. As disclosed in Table 3.1., inlet and outlet temperatures ranged between 103 ºC and 72-83 ºC, respectively, the aspirator was set at 100% and the feed flow varied between 2.9 and 5.2 mL/min. The yield (%) of the spray-drying process was calculated using the following eq:

$$Production\ yield(\%) = \left(\frac{Weight\ of\ collected\ microparticles}{Initial\ weight\ of\ solids\ in\ dispersion}\right) \times 100$$

After spray-drying, the microparticles were collected to a flask and stored inside a desiccator until further use.

Table 3.1. Optimised parameters of spray-drying for the different microparticles. (mean ± SD; n>3)

Microparticles Man/BL (w/w)	Dispersion's solid content (w/v)	Inlet Temperature (ºC)	Outlet Temperature (ºC)	Aspirator (%)	Feed rate (mL/min)
BL 100%	1%	103 ± 1	72 ± 4	100	3.8 ± 0.1
10/0.2	3%		70 ± 2		3.6 ± 0.4
10/0.4	4%		67*		2.9*
10/0.5	5%		74 ± 9		3.9 ± 0.2
10/0.7	6%		83*		5.2*
10/0.9	7%		72 ± 3		3.7 ± 0.2

Man: mannitol, BL: bacterial lysates, *n=1

3.3. Microparticle characterisation

Microparticles morphology was visualised by field emission scanning electron microscopy (FESEM Ultra Plus, Zeiss, Jena, Germany). Dry powders were placed onto metal plates and 5-nm thick iridium film was sputter-coated (model Q150T S/E/ES, Quorum Technologies, Lewes, UK) on the samples before visualisation.

Energy dispersive X-ray (EDX) analysis was performed along with FESEM for the microparticles Man/BL = 10/0.2 and Man/BL = 10/0.9. This technique provided the

elemental details of near-surface elements of a sample and their global positional mapping.

The microparticle size was manually estimated as the Feret's diameter (distance between two parallel tangent lines on opposite sides of the particle) and measured as the mean of 300 microparticles for microparticles BL 100%, Man/BL = 10/0.2, Man/BL = 10/0.4, Man/BL = 10/0.9 (w/w).

Parameters of particle size distribution (D10, D50 and D90) were further determined from the cumulative particle size distribution, representing the size below which 10%, 50% or 90%, respectively, of all particles are found. Span was calculated using the following equation:

$$Span = \frac{(D90 - D10)}{D50}$$

3.4. Determination of BL association efficiency and microparticle loading capacity

The BioRad protein assay, based on the Bradford method, was used to determine the BL association efficiency (AE) and the loading capacity (LC) of microparticles. A calibration curve was made with BSA at concentrations between 10 and 60 µg/mL, starting from a 500 µg/mL stock solution. This determination was performed for microparticle formulations corresponding to BL 100%, Man/BL = 10/0.2, Man/BL = 10/0.5, Man/BL = 10/0.9 (w/w). For the formulations featuring mannitol, the BSA for the calibration curve was dissolved in a solution obtained from the solubilisation of mannitol microparticles in ultrapure water, while for microparticles containing BL only, ultrapure water was required. The sample preparation consisted in dispersing 20 mg (BL 100% or Man/BL = 10/0.2) or 10 mg (Man/BL = 10/0.5, Man/BL = 10/0.9) of microparticles in 1 mL of water. For the determination, 160 µL of each sample dilution were mixed with 40 µl of the Bradford reagent. Five min incubation at room temperature under light protection was allowed, after which the absorbance was read by spectrophotometry at 595 nm (Tecan-Infinite M200, Switzerland). Each assay was performed three times in duplicate.

The amount of protein quantified in a determined amount of BL was assumed as the basis to determine BL AE in the microparticles. Bronchovaxom® supplier indicates 7

mg of BL per capsule, which allowed the calculation of the correspondence between the amount of BL and that of protein. These assumptions were the basis to calculate the loading capacity of microparticles.

The AE (%) of BL and the LC (%) of microparticles were calculated using the following equations:

$$AE(\%) = \left(\frac{Real\ BL\ protein\ mass}{Theoretical\ BL\ protein\ mass}\right) \times 100$$

$$LC_{theoretical}\ (\%) = \left(\frac{Total\ theoretical\ amount\ of\ protein}{Initial\ weight\ of\ solids\ before\ spray\ drying}\right) \times 100$$

$$LC_{real}\ (\%) = \left(\frac{Real\ BL\ protein\ mass}{Mass\ of\ MP}\right) \times 100$$

3.5. Aerodynamic characterisation of microparticles using an Andersen cascade impactor (ACI)

For the aerodynamic testing of microparticles, 30 mg of each dry powder formulation to be tested were filled inside size 3 HPMC capsules (Quali-V-I, Qualicaps, Spain). The capsules were aerosolised using a high resistance RS01® inhaler (Plastiape Spa, Italy) and, in each aerodynamic test, the content of ten capsules was discharged, corresponding to 10 shots. The inhaler was connected to the ACI (Copley Scientific, UK) (Figure 1.4.) which operates at 60 L/min, ensuring a pressure drop of 4 kPa across the device. This was actuated for 4 seconds to allow 4L of air to travel through the system, following the guidelines outlined in Ph.Eur. 10 (105). Before each test, the air flow of 60 L/min was verified using a flow test adapter. The ACI separates the particles according to their aerodynamic diameter. The cut-offs of the ACI stages (-1 to 6) at airflow rates of 60 L/min are shown in Table 3.2. A fiberglass filter (Whatman, Italy) was placed below stage 6 to collect particles having an aerodynamic diameter smaller than 0.26 µm. The impactor plates were coated with a thin layer of a solution of 1% (w/v) glycerol in ethanol to avoid particle bounce.

Table 3.2. Cut-off aerodynamic diameter (µm) for stages of Anderson cascade used at 60 L/min.

Stages	-1	0	1	2	3	4	5	6
Cut-offs (µm)	8.60	6.50	4.40	3.20	1.90	1.20	0.55	0.26

Quantification of the microparticles in each stage was done with two different methods. The first was the gravimetric method, in which the plate of each stage of the equipment was weighed (KERN, ABT 120-5DM, Germany) before and after the assay, allowing a calculation of the amount of MP impacting and depositing in each stage. The second method was based on the determination of the amount of protein depositing in each stage, which was calculated using the BioRad protein assay described above. In this case, after the aerosolization, the plates and other parts of the ACI and inhaler were washed with ultrapure water. The determination of the amount of protein or dry powder depositing in the induction port (IP) was always based on the protein assay, as the part is too heavy to allow gravimetric determination.

The calculation of several aerodynamic parameters is possible through the quantification of the amount of drug deposited inside the equipment. The amount of drug quantified between the IP and the filter, that is, everything that enters inside the apparatus is known as the emitted dose (ED). The metered dose (MD) was calculated by adding the ED and the quantification of the drug remaining in the capsules and in the DPI. Mass median aerodynamic diameter (MMAD) was calculated by plotting the cumulative percentage of the mass that is less than the aerodynamic diameter specified for the probability compared to the aerodynamic diameter on a logarithmic scale. Using the particle size distribution equation obtained from the ACI analysis, the fine particle dose (FPD) was calculated knowing that it corresponds to drug particles with an aerodynamic diameter less than 5 µm. The fine particle fraction (FPF) is the ratio between FPD and MD.

3.6. Statistical analysis

The t-test and one-way analysis of variance (ANOVA) with the pairwise multiple comparison procedures (Holm-Sidak method) were performed to compare two or

multiple groups, respectively. All analyses were run using the GraphPad Prism (version 9.4) and differences were considered to be significant at a level of $p < 0.05$.

4. Results and Discussion

4.1. Preparation of BL-loaded microparticles by spray-drying

Mannitol is one of the molecules approved by regulatory organisms such as the Food and Drug Administration (FDA) for pulmonary administration, as it has low toxicity and displays rapid degradation after inhalation (189). Furthermore, it shows good flow properties after spray-drying, as described in the literature (190). This justifies the selection of this excipient for the preparation of inhalable microparticles of BL, aiming at an inhalable immunisation strategy. Bronchovaxom® was used as the source of BL, containing OM-85 BL which correspond to eight bacterial species, including *Haemophilus influenzae*, *Streptococcus (Diplococcus) pneumoniae*, and *Staphylococcus aureus,* responsible for some relevant respiratory diseases. Unfortunately, it was not possible to work with the isolated BL and, therefore, the inhalable formulation was prepared using the whole content of Bronchovaxom® capsules, to which mannitol was added as excipient.

Mannitol-based microparticles containing BL were successfully produced by spray-drying, with process yields varying roughly between 50 and 65% (Table 4.1.). A formulation corresponding to spray-dried Bronchovaxom®, obtained by direct processing

Table 4.1. Bacterial lysates (BL)-loaded microparticles obtained by spray-drying and resulting yields (mean ± SD; n ≥ 3)

Microparticles Man/BL (w/w)	Yield (%)
BL 100%	26.7 ± 1.4
10/0.2	61.6 ± 3.3
10/0.4	49.9*
10/0.5	59.6*
10/0.7	64.2*
10/0.9	61.4 ± 2.2

Man: mannitol. *n=1

of the capsule content and devoid of mannitol, was also prepared, which evidenced a much lower yield (27%).

This difference in yield was certainly attributed to the low solids content present in the Bronchovaxom®-only dispersion, as can be observed in Table 3.1. As referred before, Bronchovaxom® content includes several excipients, apart from OM-85 BL. These excipients, which include propyl gallate, sodium glutamate, corn starch, and magnesium stearate, may not be as amenable for spray-drying as mannitol is, which could also justify the difference in spray-drying yields. In fact, mannitol is reported as a very adequate excipient in spray-drying (191). The registered spray-drying yields were considered satisfactory (192), especially considering that a standard cyclone separator was used instead of the high-performance cyclone that is described to have important impact in the process yield (193). It is also important to refer that, due to time constraints, not all formulations were replicated. From the initial runs of spray-drying testing the whole set of microparticle formulations, only microparticles corresponding to BL 100%, Man/BL = 10/0.2 and Man/BL = 10/0.9 (w/w) were replicated. All the others were produced at n = 1.

4.2. Microparticles characterisation

Electronic microscopy was used to visualise the microparticles. Spray-dried microparticles corresponding to BL 100%, Man/BL = 10/0.2, Man/BL = 10/0.4, Man/BL = 10/0.9 (w/w) were characterised and results are displayed in Figure 4.1. For pharmaceutical applications where the product is to be administered by inhalation, the morphology of the microparticle is a critical issue. Formulation variables such as solvent evaporation rate and solute diffusion rate from the inner core to the outer layer of the particles have been shown to have a substantial impact on the final particle morphology (194,195). In addition to this, process parameters also affect morphology, for example, higher concentrations of mannitol solutions lead to the production of particles with a rougher surface as it corresponds to less solvent in each droplet, leading to shorter evaporation times and porous particles with lower density; or higher temperatures cause the excipient layer to develop rapidly on the outside of the droplet, trapping the solvent vapour. The increased solvent vapour pressure can result in a porous particle or the

collapse of the whole particle, depending on the type of API and used solvents and excipients (196,197).

From a macroscopic perspective, all the produced microparticles appeared to have acceptable flow properties and favourable appearance, as the obtained powders did not form agglomerates when collected. However, flow properties require experimental determination for conclusions. In general, the microparticles containing mannitol were spherical in shape and the outer layer did not evidence porosity. No significant differences were noticed among the microparticles containing mannitol, which indicates that the concentration of BL and other excipients present in Bronchovaxom® did not influence their morphology. The microparticles resulting from the direct processing of Bronchovaxom® showed a spherical shape and are suggested to have a smaller size. Additionally, the photographs suggest the presence of some aggregates and some particles evidence signs of collapse. Importantly, microparticles containing mannitol appear as individualised, non-aggregated microparticles.

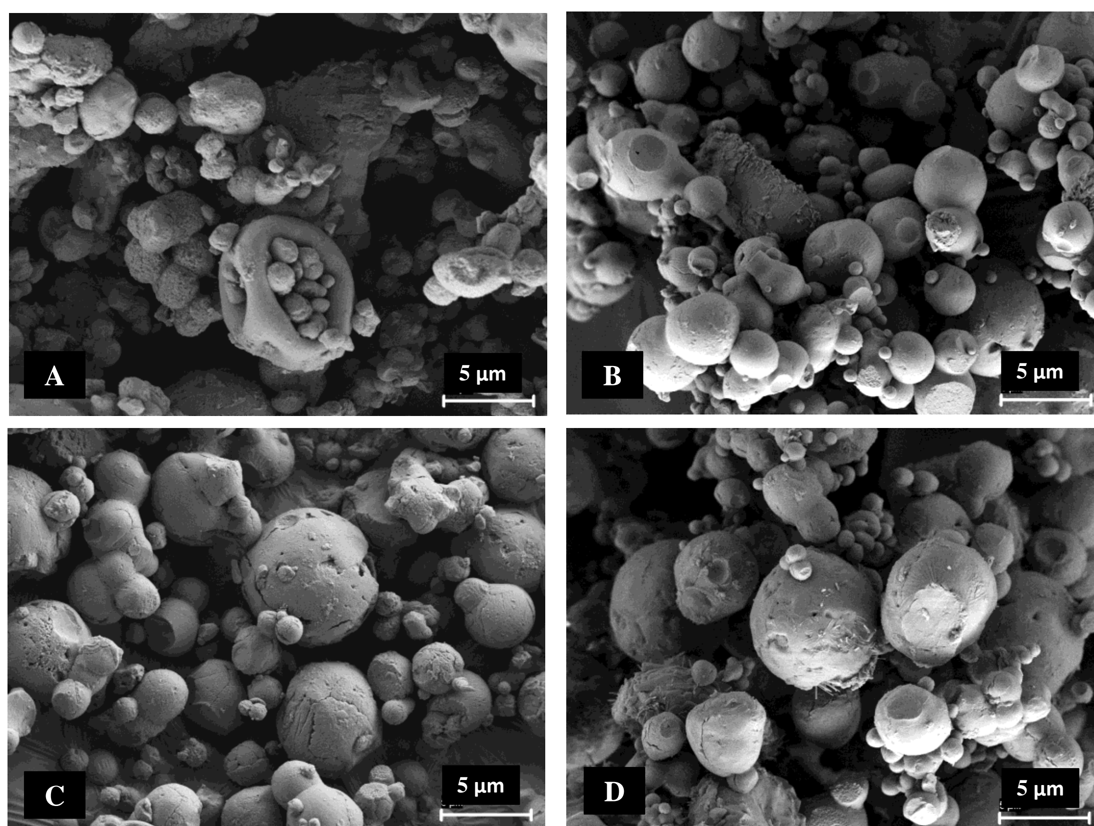

Figure 4.1. Scanning electron microphotographs of BL-loaded microparticles: (a) BL 100% microparticles (no mannitol), (b) Man/BL 10/0.2 microparticles; (c) Man/BL 10/0.4 microparticles; (d) Man/BL 10/0.9 microparticles. BL: bacterial lysates, Man: mannitol.

The technique of SEM used allowed a concomitant performance of EDX analysis. This consists of stimulating the atoms of the sample by an electron beam using SEM with an X-ray detector. When this beam interacts with the atom, an electron is forced out of its shell, leaving a gap in its place. This hole is filled by another electron from a higher energy level in the lower orbital. The amount of energy released during this process depends on how many shells the replacement electron has moved from its original location, as well as which shell it moves to. The detector transforms the energy of the X-rays that are released into voltage. The counts corresponding to the amount of X-rays in each energy level constitute the final data. While the proportional counts are related to the amount of the element, the energy level of the emitted X-rays binds to specific elements (201). EDX is a very useful approach but there are some issues with the method that limit its usefulness. Firstly, EDX is typically not a very sensitive method. The energy released by X-rays after striking a sample will not be sufficient to accurately estimate the percentage of an element if its concentration is too low. Second, for elements with a low atomic number, EDX typically does not work. Since both hydrogen and helium have only one shell, it is not necessary to remove electrons from the nucleus to produce the X-ray emission. The thickness of the sample is an additional challenge for the application of the technique. As a result of the electrons being able to migrate more easily to outer energy levels due to the thickness of the sample, the results may deviate from those expected. Furthermore, only surface layers can be accurately assessed by the method because X-rays are not particularly good at penetrating samples deeper than a few nanometres. Therefore, a mismatch between the outer and inner layers of the sample will not always be visible in EDX (198,199).

EDX analysis was performed for Man/BL = 10/0.2 and Man/BL = 10/0.9 (w/w), in both cases mannitol-based microparticles, but permitting a comparison between microparticles associating the lower and the higher amount of BL.

The results are shown in Table 4.2. and evidence that carbon and oxygen are the main elements in both microparticles, with only slight compositional differences and nothing requiring special mention. Nevertheless, as mentioned before, these results can only be associated to the outer layer of the microparticles. In fact, nitrogen (N) is not detected on the surface, which was expected to be correlated with the presence of protein and, thus, with the bacterial lysates.

Table 4.2. Elemental composition of Man/BL = 10/0.2 and Man/BL = 10/0.9 (w/w) microparticles.

Elements	Man/BL = 10/0.2	Man/BL = 10/0.9
C	64.96 ± 2.84	65.89 ± 1.79
O	33.69 ± 2.77	31.43 ± 1.98
Na	0.56 ± 0.10	1.36 ± 0.26
Mg	0.10 ± 0.09	0.08 ± 0.03
Al	0.03 ± 0.01	0.03 ± 0.01
P	0.10 ± 0.10	0.05 ± 0.01
Cl	0.36 ± 0.10	0.97 ± 0.08
K	0.08 ± 0.06	0.10 ± 0.01
Cu	0.10 ± 0.00	0.09 ± 0.03

BL: bacterial lysates, Man: mannitol.

Another parameter of great interest for characterisation in the context of inhalation is particle size. Along with particle density, the particle size of an inhalable dry powder plays a key role in the success of the formulation, as both parameters will contribute to the aerodynamic diameter that instructs the dispersion pattern of the powder and its sedimentation. The microparticles produced in this work were found to have Feret's diameter ranging from 2.77 to 3.32 µm, as displayed in Table 4.3. The absence of marked differences between the formulations is coincident with the observations of SEM images. However, this effect or absence of it was surprising, due to the existing differences in the solids content of the formulations, which is considerable with variations between 3% and 7% (w/v), as shown in Table 3.1. The particle size distribution, determined through the parameters D10, D50, D90 and respective Span, confirmed the size similarities, showing only slight differences between the formulations (Table 4.3). For example, if D50 is to be focused, the formulation Man/BL = 10/0.9 shows 2.20 µm while the formulation Man/BL = 10/0.2, which strongly differs in Bronchovaxom® concentration, shows 2.80 µm.

Table 4.3. Feret's diameter (mean ± SD) and statistical parameters of particle size distribution (D10, D50, D90, Span).

Microparticles Man/BL (w/w)	Feret's Diameter (µm)	D10 (µm)	D50 (µm)	D90 (µm)	Span
BL 100%	3.15 ± 2.75	1.22	2.29	5.98	2.08
10/0.2	3.19 ± 2.00	0.98	2.80	6.10	1.83
10/0.4	3.32 ± 2.07	1.26	2.90	5.85	1.59
10/0.9	2.77 ± 1.98	0.98	2.20	5.37	2.00

BL: bacterial lysates; D10: size below which 10% of all particles are found; D50: size below which 50% of all particles are found; D90: size below which 90% of all particles are found; Man: mannitol.

4.3. Determination of BL association efficiency and microparticle loading capacity

To better understand the results of this section, it is necessary to differentiate the concepts of AE and LC. The capacity of microparticles to retain the molecule of interest, BL in this case, within the polymeric matrix refers to the AE. On the other hand, LC refers to the actual amount of drug in the microparticles. The AE and LC values for the various formulations are summarised in Table 4.4.

BL correspond to parts of bacteria and, thus, are comprised of a mixture of proteins, polysaccharides, and lipids. The direct quantification of BL is not possible, but the amount can be extrapolated if any of its specific components is quantified.

Table 4.4. Protein association efficiency (AE), corresponding microparticle loading capacity (LC) of proteins and extrapolated LC of bacterial lysates in the different formulations. (mean ± SD; n=3) Different letters represent significant differences in each parameter ($p<0.05$).

Microparticles Man/BL (w/w)	AE (%)	LC $_{theoretical}$ (%)	LC $_{real}$ (%)	LC $_{lysates}$ (%)
BL 100%	16,45 ± 1,05^{a}	0,54	0,33 ± 0,02^{d}	2,13 ± 0,14^{g}
10/0.2	52,94 ± 8,04^{b}	0,18	0,17 ± 0,02^{e}	1,08 ± 0,12^{h}
10/0.5	65,12 ± 6,70^{c}	0,32	0,34 ± 0,05^{f}	2,20 ± 0,30^{i}
10/0.9	59,28 ± 6,94^{b,c}	0,38	0,33 ± 0,05^{f}	2,16 ± 0,30^{i}

BL: bacterial lysates; Man: mannitol.

In this work, the amount of proteins present in the microparticles was determined using a specific assay, the BioRad protein assay. The microparticles corresponding to BL 100%, Man/BL = 10/0.2, Man/BL = 10/0.5 and Man/BL = 10/0.9 (w/w) were assessed

regarding this parameter. As the Bronchovaxom® manufacturer indicates that each capsule contains 7 mg of BL, the determination of the amount of protein contained in the capsule permits establishing a direct relation between the amount of protein and that of BL.

The microparticles theoretical loading was expected to be higher in the BL 100% microparticles, as these are devoid of the additional mannitol. In turn, in the microparticles containing mannitol, as the concentration of this excipient remains constant, the theoretical loading increases with the amount of BL added to the formulation. The theoretical LC values are very close to the real LC values for microparticles containing mannitol, as opposed to microparticles with BL only. Protein AE range within 53% and 65% for microparticles containing mannitol, displaying significant differences between Man/BL = 10/0.2 and Man/BL = 10/0.5 ($p<0.05$).

The AE results resulted in LC variations roughly between 0.2% and 0.3%. In both parameters, the microparticles Man/BL = 10/0.5 showed the highest nominal values of AE and LC. In any case, it is important to consider that for this formulation only one batch was produced and, thus, more replications are required. The AE values for the formulations with mannitol were considered satisfactory, however the Man/BL = 10/0.2 microparticles showed lower values of both AE and LC comparing with the other two formulations ($p<0.05$). Comparing the formulations with the same concentration of BL (BL 100% and Man/BL = 10/0.2), differentiated by the absence or presence of mannitol, the LC value is much higher in the formulation with only BL ($p<0.05$), which may indicate that due to the lower amount of excipients, the protein is more concentrated. The LC above described refers to the quantified protein that is in the microparticles. The determination of protein enabled an extrapolation to calculate the corresponding amount of BL present in the microparticles. This is of relevance because the immunological response to be induced will be mediated by the BL as a whole and not only by the protein content. Therefore, it was possible to calculate LC for BL content, and this showed values between 1.1% and 2.2%. The LC of BL of formulation Man/BL = 10/0.2 was significantly lower than in the other formulations ($p<0.05$).

For applications in drug delivery to the lungs, it is important not only the aerodynamic characteristics, but also there must be a sufficient loading of drug to reach the lung in a quantity capable to have therapeutic effect. In the present work, the induction

of an immune response is expected and, so, the amount of antigen must be enough for such outcome. Although perhaps in comparison with a drug, the amount of antigen can be lower to obtain the desired effect, loading has high as possible ae desirable. The analysis of the obtained results is biased by the fact that not all formulations were sufficiently replicated. Therefore, the whole study needs to be completed but, if a formulation had to be chosen based on the current results, microparticles composed of Man/BL = 10/0.9 (w/w) would be the most appropriate to proceed.

4.4. Aerodynamic characterisation of microparticles using an Andersen cascade impactor (ACI)

ACI is one of the official instruments for assessing the particle size distribution of aerosols produced by nebulisers and inhalers. This apparatus provides a direct measurement of the aerodynamic particle size, which drives the transport of drug particles through the air (104). The characterisation of the aerodynamic diameter, along with other aerodynamic parameters, provides an indication on the quantity of particles reaching each lung zone.

Two methods were used herein to quantify the aerodynamics: one method is based on a colorimetric quantification of the protein content of microparticles - the Bradford method; the other method uses gravimetry. Pharmacopeial determination only considers direct quantification of drugs (105) but the gravimetric method is reported in many studies available in the literature (200–202), and although this method is not the most suitable to determine aerodynamic proprieties, it is the most practical. Through the first, it was possible to quantify the protein of the microparticles in the different stages, inhaler, and capsules. The other method consisted in weighing all the plates of each stage, the filter, the inhaler, and the capsules. The IP could not be weighed, so the amount of microparticles depositing in this part was always obtained by extrapolation upon using the Bradford method to quantify the amount of deposited protein.

Man/BL = 10/0.9 (w/w) microparticles were those selected to be tested regarding the aerodynamic properties. The assay involved the discharge of 10 capsules with 30 mg of microparticles each in order to deposit enough protein to enable quantification through the Bradford method. Figure 4.2. demonstrates the stage-to-stage deposition profile of the

microparticles as determined for each method. Despite some statistically significant differences in the comparison between both methods for each stage ($p<0.05$), a relative similarity in the overall deposition profile was observed. One of the most remarkable observations is that, for both methods, the IP showed the highest deposition of microparticles (around 50%), showing that a significant portion of the dry powders correspond to large particles (> 8.60 μm), not reaching the lung.

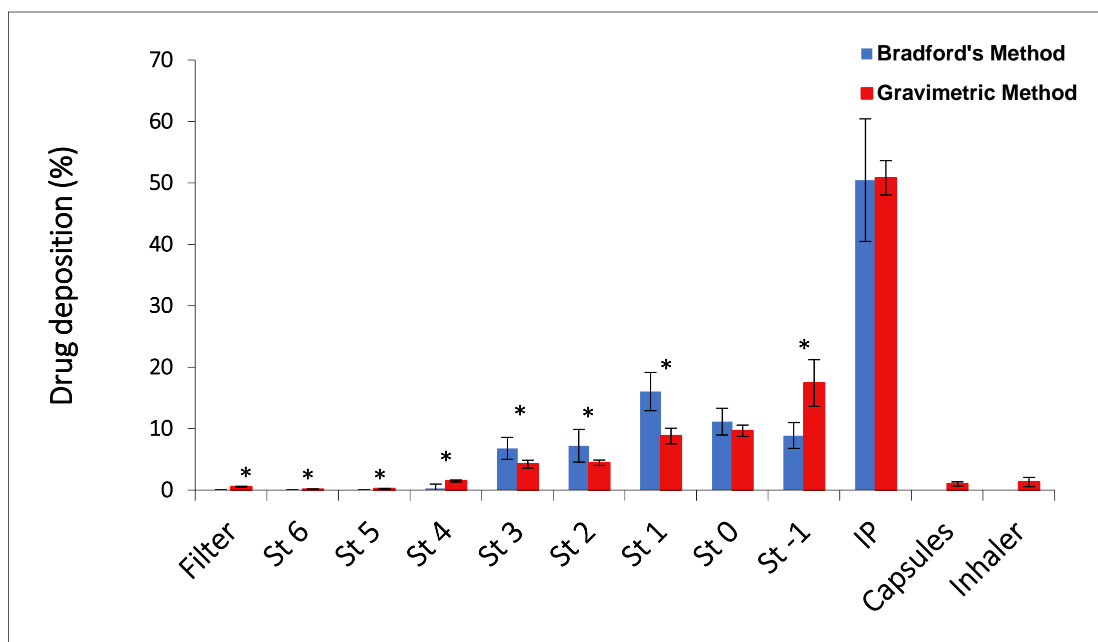

Figure 4.2. Stage-by-stage deposition profiles of Man/BL = 10/0.9 (w/w) microparticles as determined by Bradford method (blue) and gravimetric method (red) the Andersen cascade impactor after aerosolization with inhaler operated at 60 L/min (values are mean ± SD, n = 3). IP: induction port; St: stage; *Statistical differences ($p<0.05$)

The other relevant observation is the fact that the lower stages (5, 6 and F) did not receive enough amount of microparticles to enable protein quantification. When using the gravimetric method, it is important to mention that in 2 of the 5 replicates performed, the analytical balance lost 1 decimal place when weighing the plates due to overweight. The occasions corresponded to the initial approaches before the procedure was totally optimised.

The calculated aerodynamic characteristics are shown in Table 4.5. The emitted dose was very satisfactory with almost 100% in both methods. In fact, while in the gravimetric method it was possible to quantify a small amount of microparticles remaining in the device and, thus, not being emitted, when the Bradford method was used,

no protein was quantified, resulting in virtual emission of 100%. MMAD values were 5.9 µm and 7.3 µm for the Bradford method and the gravimetric method, respectively ($p<0.05$). As the Bradford method is that providing a real quantification of the drug, as advised by the pharmacopeia, the obtained results suggest that its replacement by the gravimetric method is not adequate, as an overestimation of MMAD was found in that case. In any case, the determined aerodynamic diameters slightly overpass the range considered adequate for lung delivery (1-5 µm). This means that the great majority of the particles will remain in the upper bronchial area. Having a better distribution of the microparticles would be desirable for the overall objective, in order to better protect the lung mucosa and to generate a systemic immune response. The determined FPF was relatively low (14-19%), reflecting the high MMAD and meaning that only small amounts of particles would reach the deep lung. Other studies using mannitol as excipient have reported higher FPF values (40% - 60%) (203–206), but it is important to recall that in the present work several other excipients are present in the formulation because the isolation of BL was not possible. Nevertheless, it has been shown that currently marketed DPIs generate FPFs in the range 20-50%, indicating that the performance of the dry powder developed in this work is acceptable, but an optimisation would be desirable to improve the aerodynamic parameters (113,207).

Table 4.5. Aerodynamic properties of microparticles composed of Man/BL = 10/0.9 (w/w), as determined by the gravimetric method and by protein quantification by Bradford method (mean ± SD, n = 3). Different letters represent significant differences in each parameter (p<0.05).

Method	Emitted dose (%)	MMAD (μm)	GSD (μm)	FPD	FPF (%)
Bradford	100 ± 0.00[a]	5.86 ± 0.42[b]	1.59 ± 0.06[d]	16.15 μg ± 5.53[f]	19.17 ± 5.17[h]
Gravimetric	98.00 ± 0.33[a]	7.33 ± 0.65[c]	1.75 ± 0.05[e]	3.17 mg ± 0.50[g]	13.73 ± 1.60[i]

BL: bacterial lysates; FPD: fine particle dose; FPF: fine particle fraction; GSD: geometric standard diameter; Man: mannitol; MMAD: mass medium aerodynamic diameter.

5. Conclusion and future lines of work

Prevention plays an important role in the therapeutic approach to respiratory infections and as the lung comprises the first site of contact with the infectious agent, local immunisation together with systemic immunisation seems appropriate. For this reason, the aim of this thesis was to develop a dry powder formulation suitable for the delivery of BL, using spray-drying as production technique, and to evaluate the aerodynamic and morphologic characteristics of the developed product, as well as the ability to associate the antigens.

The aim of simply converting a commercial BL formulation into an inhalable formulation was not successful, essentially due to low production yields. Therefore, mannitol was selected as excipient and microparticles with different concentrations of BL were successfully produced by spray-drying, showing no major differences among them. Feret diameters between 2.77 µm and 3.32 µm were obtained and BL incorporated in the microparticles with satisfactory AE between 50% and 65%. The selected formulation revealed MMAD of 5.9 µm and FPF of 19%, denoting the need for further improvement of the formulation as the next step of the work. It was also noticed that, although more practical, the method of gravimetry is not adequate for the determination of aerodynamic properties, as an overestimation of the aerodynamics was observed. The use of other excipients or other solvents to improve the spray-drying process are possible variables of optimisation for the next phase.

6. References

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