Universidade do Algarve

Departamento de Ciências Biomédicas e Medicina

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Trabalho efetuado sob a orientação de: Prof. Dra. Ana Grenha, PhD

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Declaração de autoria de trabalho:

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Filipa Raquel Horta Guerreiro

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Abbreviations

AE – Association Efficiency

ATCC - American Type Culture Collection

D_{aer} – Aerodynamic diameter

DMEM - Dulbecco's Modified Eagles's Medium

DMSO - Dimethyl Sulfoxide

DNA - Deoxyribonucleic acid

DPPC - Dipalmitoylphosphatidylcholine

FBS – Fetal Bovine Serum

HCl - Hydrochloric acid

HIV - Human Immunodeficiency Virus

INH – Isoniazid

InhA - NADH-dependent enoyl-[Acyl-Carrier-Protein] reductase

KGM – Konjac Glucomannan

LC – Loading Capacity

LDH - Lactato Desidrogenase

Leu - Leucine

Man - Mannitol

ManLAM – Mannose-Capped Lipoarabinomannan

MDR-TB - Multidrug-Resistant Tuberculosis

MIC - Minimum Inhibitory Concentration

MP(s) – Microparticle(s)

MR – Mannose Receptors

Mtb – *Mycobacterium tuberculosis*

MTT - 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide

NADH - Nicotinamide Adenine Dinucleotide

NTLR - Non-Toll-Like Receptors

PBS - Phosphate Buffer Saline

PIMs – Phosphatidyl-Myo-Inositol Mannosides

PMA - Phorbol myristate acetate

(P)XRD – (Powder) X-Ray Diffraction

RIF - Rifampicin

RFB - Rifabutin

RNA - Ribonucleic acid

Rpm – Rotations per minute

SD – Spray-dried

SDS - Sodium Dodecyl Sulphate

TB – Tuberculosis

USA – United States of America

XDR-TB - Extensively Drug-Resistant Tuberculosis

XRD - X-Ray Diffraction

WHO – World Health Organization

Abstract

Microparticles (MPs) of konjac glucomannan (KGM) are proposed in this thesis as an alternative therapeutic approach for antitubercular drug delivery to the lung, in the ambit of tuberculosis (TB) therapy. KGM is composed of mannose groups that might comprise an entry mechanism in macrophages infected with *Mycobacterium tuberculosis*, mimicking one of the input paths of this infectious agent causer of TB. For this purpose, KGM MPs need to have certain properties, in particular an aerodynamic diameter between 1 and 3 μm that enables reaching the alveolar zone. These properties might be attained by a production using spray-drying technology. The objective of this work was to produce MPs of KGM through this process and associate two first-line antitubercular drugs, isoniazid (INH) and rifabutin (RFB). Furthermore, the effect of incorporating mannitol or leucine in the microparticle formulations was evaluated, regarding MPs aerodynamic characteristics, drug association and release, and biocompatibility profile.

After an initial optimization that required a hydrolysis of the original KGM polymer to enable spray-drying, several formulations of MPs were produced and characterised. The use of mannitol or leucine has demonstrated to improve the flow properties of some formulations, as did the association of RFB. However, the incorporation of excipients brought some disadvantages, especially regarding the *in vitro* release rate of the drug, which became faster. Additionally, the incorporation of mannitol was found to affect the association of INH, which became lower, suggesting a competing effect between INH and mannitol. Cytotoxicity assays in alveolar epithelial cells revealed that RFB-loaded KGM MPs may have a considerably toxic effect when used at a concentration of 1 mg/mL, in particular if MPs are also comprised of leucine.

The obtained results indicate that KGM MPs are suitable inhalable delivery systems for antitubercular drugs. Considering that a combination of drugs is advised by WHO in TB treatment, no benefit was found to occur from the incorporation of either leucine or mannitol in the formulation of KGM MPs.

Keywords: Isoniazid, Konjac glucomannan, Leucine, Mannitol, Microparticles, Pulmonary delivery, Rifabutin, Spay-drying, Tuberculosis.

Resumo

O tratamento convencional da tuberculose (TB) pulmonar baseia-se na administração de um conjunto de fármacos durante um longo período de tempo. A duração prolongada do tratamento potencia o aparecimento de efeitos secundários relativamente severos, bem como o desenvolvimento de resistência aos fármacos usados, o que contribui para o aparecimento da tuberculose multirresistente. Com o objetivo de contornar estes dois efeitos, uma das estratégias que tem suscitado um interesse crescente na área da investigação é a entrega pulmonar de fármacos através de sistemas transportadores. Estes permitem aumentar a concentração de fármacos nos pulmões, prolongar o seu tempo de residência na região respiratória e, por último, potenciam um direcionamento para os macrófagos alveolares, local onde a *Mycobacterium tuberculosis*, causadora da infeção, está alojada. Todos estes fatores contribuem para a possibilidade de diminuir a frequência de administração dos fármacos e usar doses menores, o que possivelmente diminui os efeitos sistémicos dos tuberculostáticos. Contudo, para que a estratégia funcione, é essencial que os fármacos cheguem ao seu local de ação em segurança, efeito que se potencia por utilização de transportadores (micropartículas (MPs)) para os fármacos. Os transportadores oferecem proteção contra agressões do meio e possibilitam uma libertação mais controlada dos fármacos, aumentando a sua biodisponibilidade. No entanto, para esta aplicação específica que envolve inalação de fármacos que têm de alcançar a região alveolar, as MPs devem apresentar caraterísticas específicas, nomeadamente um diâmetro aerodinâmico entre 1 e 3 μm, que permite por um lado alcançar dita região e por outro potenciar a captura pelos macrófagos.

As MPs poliméricas são dos sistemas mais estudados para a entrega de fármacos, dada a flexibilidade dos materiais utilizados. São formadas por uma matriz derivada de polímeros naturais ou sintéticos, onde uma pequena quantidade de fármaco é incorporada. Os polímeros naturais são dos mais utilizados e, entre eles, está o glucomanano de konjac (KGM). Este polímero apresenta na sua composição manoses, as quais podem ser um fator chave na utilização do KGM como material de matriz de um sistema de transporte de fármacos para o tratamento da TB, pois uma das portas de entrada da Micobactéria nos macrófagos alveolares são os recetores de manose. Mimetizando este processo, as MPs à base deste polímero, carregadas com fármacos

tuberculostáticos, também poderiam ser fagocitadas pelos macrófagos infetados e atuar no tratamento da TB pulmonar.

Existem muitas técnicas para a produção de MPs, sendo a atomização (spray-drying) uma das mais utilizadas. Esta técnica é bastante usada na indústria farmacêutica para a produção de MPs poliméricas, pois permite produzir MPs com as caraterísticas pretendidas (tamanho, morfologia, densidade) de forma simples, rápida, uniforme e económica. Contudo, muitas vezes as partículas produzidas por atomização apresentam uma tendência de aglomeração, o que leva ao empobrecimento das propriedades de aerossolização do pó, o que afeta a deposição das MPs ao longo do trato respiratório. Para reduzir esta tendência recorre-se muitas vezes à incorporação de excipientes adjuvantes, como o manitol e alguns aminoácidos. A sua presença aumenta a rugosidade da superfície das MPs, o que diminui os espaços vazios e evita a coesão entre as mesmas, aumentando consequentemente a capacidade de dispersão do pó.

O principal objetivo desta tese consistiu em avaliar a capacidade do KGM como principal material de matriz de MPs para uma aplicação na terapêutica inalatória da TB. Verificou-se a capacidade de produzir MPs de KGM pela técnica de atomização, bem como a possibilidade de associar dois tuberculostáticos de primeira linha, a isoniazida (INH) e a rifabutina (RFB), isoladamente ou em associação. Procedeu-se também à incorporação, de forma separada, de dois excipientes adjuvantes, o manitol e a leucina, tendo-se avaliado o seu efeito sobre as formulações.

As dispersões para atomização foram preparadas após a hidrólise do KGM, dado que o seu elevado peso molecular conduzia a soluções demasiado viscosas para processamento pela técnica mencionada. Embora a formulação de MPs de KGM sem adição de qualquer outro agente ou fármaco corresponda a partículas com propriedades de fluxo consideradas macroscopicamente insatisfatórias, a incorporação de RFB melhorou bastante as caraterísticas dos pós. O manitol e a leucina foram posteriormente incluídos nas formulações, tendo-se igualmente efetuado associação dos fármacos. Para todas as formulações, com e sem fármacos, com e sem excipientes adjuvantes, foi efetuada a caraterização das MPs (morfologia, densidade e tamanho), determinadas as eficácias de associação e perfis de libertação dos fármacos, e avaliado o perfil de citotoxicidade pelos métodos 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) e libertação de lactato desidrogenase (LDH).

Os resultados demonstraram que a presença de manitol ou de leucina nas formulações contribui para a diminuição do tamanho das MPs e do seu diâmetro aerodinâmico. Este efeito foi ainda mais notório quando a RFB também estava presente, sobretudo quando foi utilizada isoladamente, sem a presença de INH. As densidades aparente e de compactação aumentaram com a introdução dos excipientes, ao contrário do que aconteceu com a densidade real. Em termos de morfologia, a incorporação do manitol ou de leucina alterou a aparência da superfície das MPs, que adquiriram maior rugosidade. A análise das formulações por difração de raios X permitiu verificar que as formulações exibem alguns picos de cristalinidade, ainda que atribuíveis apenas aos excipientes e não aos fármacos, os quais são menos intensos do que os correspondentes aos excipientes não atomizados.

Relativamente à inclusão dos excipientes adjuvantes, apenas a presença de manitol afetou a associação de fármaco às MPs, diminuindo-a, e apenas no caso da INH. Este efeito não foi visível para a RFB, tendo todas as outras formulações eficácias de associação entre os 90 e os 100%. Em relação aos perfis de libertação dos fármacos, as formulações com os excipientes registaram uma libertação mais rápida de ambos os fármacos.

A citotoxicidade foi avaliada em duas linhas celulares do pulmão, A549 e Calu-3. Algumas formulações revelaram comportamento citotóxico após 24h de exposição à concentração mais elevada (1 mg/mL). O fármaco RFB também mostrou ser tóxico após 24h de exposição, apesar de ter sido utilizado em menor concentração nas formulações em comparação com a INH.

Considerados todos os resultados, conclui-se que as MPs de KGM produzidas podem ser um sistema apropriado para a entrega pulmonar de fármacos e, a incorporação de excipientes, uma solução discutível para melhorar as propriedades aerodinâmicas das MPs, uma vez que o uso de manitol ou leucina nas MPs de KGM pode ter desvantagens, nomeadamente na libertação dos fármacos, uma vez que a libertação deixa de ser feita de maneira controlada. Embora não seja no âmbito desta tese, o passo seguinte deste trabalho consistirá em estudar a captura macrofágica destas MPs, o que será um dado essencial para o sucesso da formulação na terapêutica da TB.

Palavras-Chave: Entrega pulmonar, Isoniazida, Glucomanano de konjac, Leucina, Manitol, Micropartículas, Rifabutina, Spay-drying, Tuberculose.

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1. Introduction

1.1. Tuberculosis

1.1.1. Epidemiology

Although the number of deaths caused by tuberculosis (TB) have decreased since the early 90's, this remains the most lethal infectious disease globally, after the human immunodeficiency virus (HIV). According to the World Health Organization (WHO), in 2013 about 9 million people developed this infectious disease and 1.5 million people died of it.[1,2] Of these 9 million people infected with TB, 13% were co-infected with HIV and lived mostly in Africa. WHO estimated 480 000 new cases of MDR-TB worldwide, in 2013.[1]

The largest number of TB cases reported in 2013 occurred in Asia (56%), in countries like India, China, Pakistan and Indonesia. The African continent was also greatly affected by TB in 2013 (29%) with higher incidence in Nigeria and South Africa. Western Europe, Canada, United States of America (USA), Japan, Australia and New Zealand recorded the lowest incidence rates in relation to TB (Figure 1.1).[1]

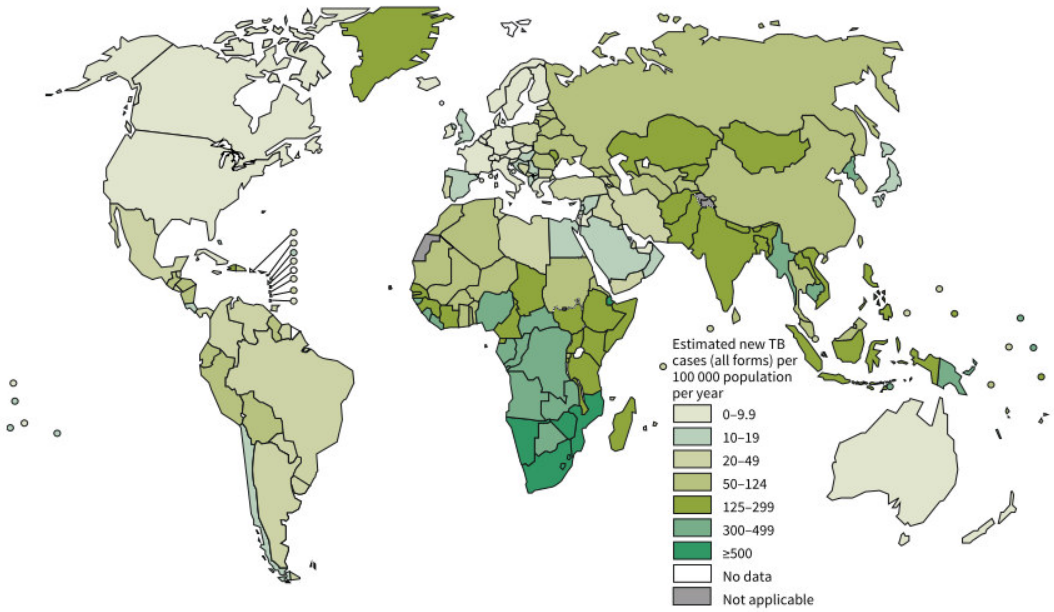

Figure 1.1. Estimated TB incidence rates, 2013.[1]

Similarly to what happens in most countries, TB in Portugal has been declining over the years (Figure 1.2a). However, the fact that it is a persistent disease and is associated with vulnerable groups requires that responses to the disease remain ensured. Currently Lisbon, Oporto and Setubal are the districts with the highest incidence of cases (Figure 1.2b). In 2013, 2393 cases of TB were reported (of which 2195 were new cases), 212 cases less than those registered in 2012.[3]

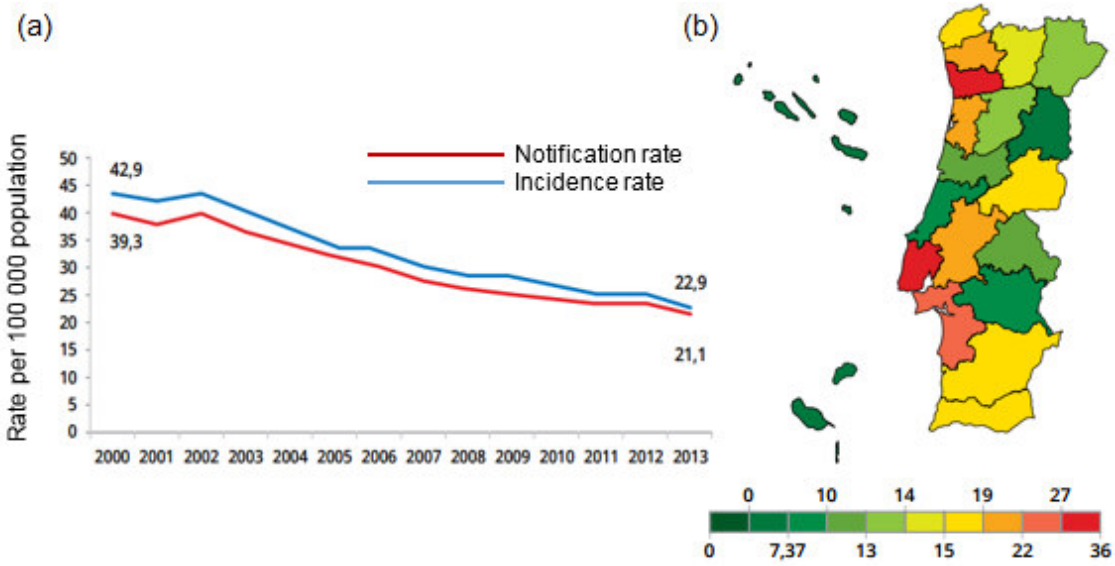

Figure 1.2. (a) Evolution of notification and incidence rate of TB in Portugal, 2000-2013. (b) Incidence rate of TB per 100,000 people per district, in 2013 (adapted from[3]).

1.1.2. The pathological agent and pathophysiology of tuberculosis

TB is a chronic and progressive infection caused by *Mycobacterium tuberculosis* (Mtb). These bacilli were discovered in 1882 by Robert Koch[4] and are aerobic acid-fast bacilli with a cell wall structure essential to survival. The bacteria cell wall is a real challenge in TB at both the physiological and therapeutic level, being in the basis of the resistance against the antibiotics and the proper host defence mechanisms. This is due to the high lipid content of components present in the cell wall (as the mycolic acid), which form a barrier.[5,6] Other important components of Mtb include the peptidoglycan for cell wall rigidity and permeability barrier,[5] and mannose-containing biomolecules (mannose-capped lipoarabinomannan (ManLAM), lipomannan, phosphatidyl-myo-inositol mannosides (PIMs), arabinomannan, mannan and mannoglycoproteins). Some of these

biomolecules play an important role in TB pathogenesis through modulation of the host immune response.[6,7]

Mtb is spread when an individual inhales Mtb-containing droplets (between 1 and 5 μm of diameter) produced by the coughing, sneezing, talking or singing of infected people with pulmonary or laryngeal TB.[5,8,9] Once inhaled, the droplets containing bacilli install throughout the airways. If they manage to overcome the mucociliary clearance, a defence mechanism consisting in synchronised action between mucus and cilia in order to eliminate foreign substances, they will possibly reach the alveoli. Once there, they will be engulfed by alveolar macrophages. These cells are part of the innate response of the host and will act to destroy Mtb in order to avoid the infection.[5] Alveolar macrophages have a number of surface receptors, some responsible for the recognition of molecules expressed on the cell wall of Mtb (Toll-like receptors) and others responsible for phagocytosis of the microorganism (Non-toll-like receptors (NTLR) and opsonic receptors).[10] Among NTLR is the C-type lectin family, to which belongs the mannose receptor (MR). This is a transmembrane protein known as one of the main routes of entry of Mtb in the alveolar macrophage, after binding of ManLAM or PIMs to MR.[7,10,11]

The phagocytosis of Mtb, mediated by the receptors, triggers a series of events that usually result on the control of the infection. If it happens as so, the infected individual will have the so-called latent TB. However, it is also possible that the infection progresses to result in active TB, particularly when individuals do not have efficient immunity. Regardless of the evolution, the initial development of the infection involves the production of proteolytic enzymes and pro-inflammatory cytokines by macrophages, with the objective of degrading the microrganism.[5,9] The release of cytokines attracts the cells responsible for cellular immunity, T-cells, to the place. In parallel, macrophages present the antigens on the surface of Mtb to T-cells, leading to their activation.[5] In people with appropriate cell immunity, activated T-cells and macrophages join together to surround TB bacilli and form a structure called the granuloma, which encloses the bacteria.[9] Activated T-cells and macrophages create a microenvironment inside the granuloma that limits the replication and dissemination of Mtb. This environment destroys the macrophages and produces necrosis in the center of the granuloma, which undergoes fibrosis and calcification. However, the bacilli are able

to adapt to survive. After 2 or 3 weeks, the necrotic environment is replaced by an environment with low oxygen, low pH and limited nutrient supply. All these conditions allow controlling the infection and the establishment of latency (latent TB) through Mtb growth restriction. The bacilli remain in a "dormant state" that can be prolonged for years.[5] In this state, which happens in 90-95% of the cases, there are no signs or symptoms of TB and no transmission of the disease occurs (Figure 1.3).[12]

Persons with a less efficient immune system will eventually develop active TB (5 to 10% of cases).[5,8] In spite of the formation of a granuloma, in these individuals this does not present the necessary features to contain the bacilli. The necrotic tissue undergoes liquefaction and the fibrous wall loses structural integrity (Figure 1.3). The semi-liquid necrotic material containing the TB bacilli may, ultimately, drain to the bronchi or near blood vessels.[5] Although most of the cases of TB are pulmonary (80%), if the dissemination of bacilli happens in the blood vessels, the occurrence of extra pulmonary TB is likely in regions such as bones, brain, kidneys, larynx, lungs, lymphatic nodes or spinal cord.[5,8]

The transmission of the microorganism to others occurs when the droplets containing Mtb are expelled from the bronchi of people with TB and infect other people, starting a new cycle of Mtb (Figure 1.3).[5]

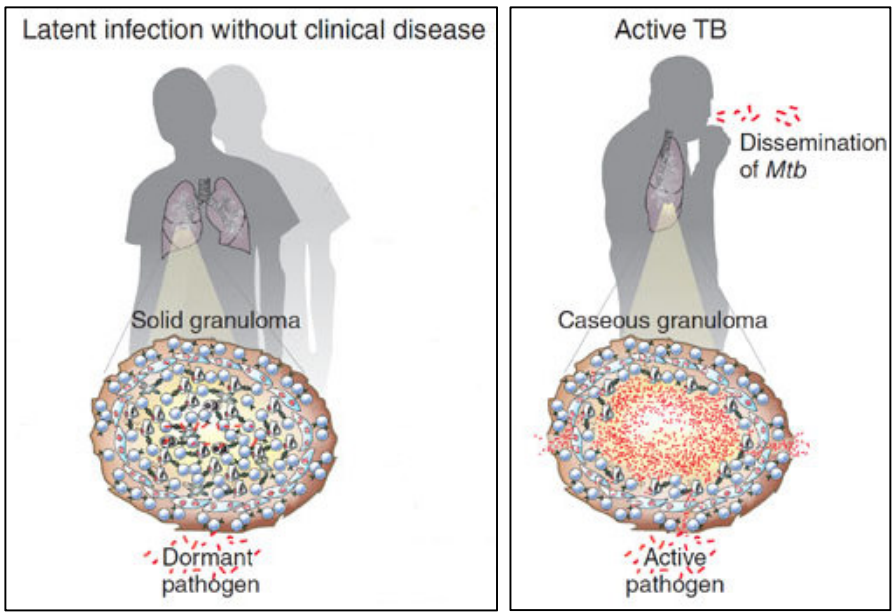

Figure 1.3. Granuloma in latent TB and active TB and dissemination of Mtb (adapted from[13]).

1.1.3. Conventional therapeutic approach

Before the discovery of the first antitubercular drug, streptomycin, individuals suffering of TB were just admitted in sanatoriums, and treatment was limited to a good nutrition and "get fresh air".[14] Streptomycin is no longer used due to its toxicity and the development of resistance to the drug, but treatment continues to be based on antibiotherapy, usually administered through the oral route.

The WHO recommends that the treatment of active TB must be done through a combination of multiple drugs for long periods of time, in order to prevent the development of multidrug-resistant TB (MDR-TB).[8,15] The therapy begins with the first-line antitubercular drugs isoniazid (INH), rifampicin (RIF), ethambutol and pyrazinamide, administered daily for a period of 2 months to reduce the number of bacilli rapidly dividing. The therapy remains daily or three times per week for 4 more months only with INH and RIF to sterilize lesions containing fewer and slow-growing bacilli.[8,14,16] In cases where the treatment with first-line antitubercular drugs fails, mainly due to lack of patient adherence to the treatment and to MDR-TB, the solution encompasses a treatment with second-line antitubercular drugs. Ethionamide or prothionamide, kanamycin or amikacin, terizidone/cycloserin, capreomycin, viomycin and para-aminosalicylic acid are examples of second-line antitubercular drugs. These are known to be less effective and more toxic (multiple side-effects).[8,16]

Special circumstances may lead to a change in the medication usually recommended for the treatment of TB. An example of this situation is the use of Rifabutin (RFB) in people infected with HIV. In these cases, RIF is contraindicated due to its incompatibility with antiretroviral drugs.[17–20] RFB is also a first-line antitubercular drug,[18] being recommended in the treatment of MDR-TB.[17–19]

The experimental work presented herein involves the use of both INH and RFB and, therefore, the characteristics of these drugs are detailed below.

*1.1.3.1. I*soniazid

As mentioned above, INH is a first-line antitubercular drug,[8,14] which use for this end started in 1952.[21] INH is easily soluble in water (140 mg/mL)[22] and is very sensitive to degradation by light. The chemical formula of this drug corresponds to $C_6H_7N_3O$, as depicted in Figure 1.4, exhibiting a molar mass of 137.14 g/mol.[8,23]

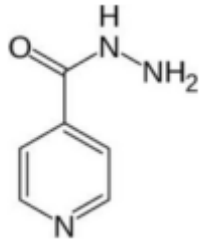

Figure 1.4. Chemical structure of INH.[8]

Its mechanism of action involves entering the *Mycobacterium* by passive diffusion.[14] INH is a prodrug activated by *Mycobacterium tuberculosis* catalase-peroxidase, an enzyme that promotes the binding of the drug to nicotinamide adenine dinucleotide (NADH). The formed NADH-INH adducts will generate, subsequently, covalent complex with NADH-dependent enoyl-[Acyl-Carrier-Protein] reductase (InhA), which inhibits the synthesis of mycolic acid, an essential component of the cell wall of *Mycobacterium*. Apart from the inhibition of InhA, INH-NADH adducts appear to inhibit β-ketoacyl-ACP synthase also involved in the synthesis of mycolic acid.[8,14,16,17,24,25] The absence of mycolic acids leads to death of the bacterium.[17] An important characteristic of INH is that it is only active on dividing bacilli, having a minimum inhibitory concentration (MIC) against Mtb of 0.125 μg/mL.[26] Those bacteria that are in latent state or under anaerobic growth, are not affected by this drug.[14]

1.1.3.2. Rifabutin

RFB was discovered in 1980 and is derived from rifamycin-S. The chemical formula of RFB corresponds to $C_{46}H_{62}N_4O_{11}$ and its structure is shown in Figure 1.5. It has a molar

mass of 847.02 g/mol and is only slightly soluble in water (0.19 mg/mL).[19,20,26–28] According to pharmacopoeia, RFB should be protected from light and excessive heat.[29]

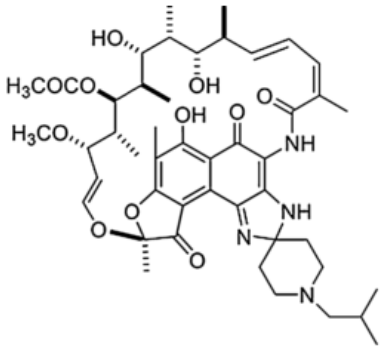

Figure 1.5. Chemical structure of RFB.[28]

RFB has a broad spectrum of microbial activity, including against Mtb. Studies with RFB have shown that this has a similar or even greater efficacy than other rifamycins.[19,20,27,28] The activity of RFB is based on the inhibition of transcription by binding to the β-subunit of the bacterial DNA-dependent RNA polymerase and subsequent inhibition of protein production.[16,19,20,28] The MIC of RFB against Mtb is 0.01 μg/mL.[26]

1.2. Pulmonary drug delivery

The conventional oral antibiotherapy applied in TB can cause toxicity in many organs, in particular the liver. The liver is responsible for first-pass metabolism of the antitubercular drugs and considering the long duration of the treatment, a failure in liver functions is admissible, as well as in other organs.[8,30] Additionally, the emergence of MDR-TB due to resistance to at least two of the first-line antitubercular drugs (INH and RIF) and of extensively drug-resistant TB (XDR-TB), characterised by resistance to at least two first-line drugs and three or more second-line drugs, are increasing issues raising severe concern.[14] Addressing these problems involves not only finding new drug molecules, but also applying therapeutic alternatives to existing drugs that allow, on one hand to shorten the treatment duration and, on the other, decrease drug resistance.[31] One of the alternative therapeutic lines might comprise finding innovative delivery

approaches that replace the conventional administration of drugs occurring by the oral and parenteral routes of administration.[16] In this regard, the use of inhalable systems could be a successful approach in the treatment of pulmonary TB. Considering the accumulation of the bacteria in the lung, the purpose of using such systems is to obtain high drug concentrations in the lungs, which could shorten the treatment and prevent MDR-TB.[8] This would possibly allow less frequent administration of lower drug doses, reducing major side effects at systemic level and increasing therapeutic efficacy.[8,16,32–35] Additionally, there is the possibility of providing alveolar macrophage targeting and control of the drug release, both contributing to therapeutic effectiveness.[16,35]

Nevertheless, successful administration of drugs to the lungs requires suitable carriers that must be rigorously designed to exhibit the adequate properties to reach a desired zone. This is highly dependent on the therapeutic objective and, in the case of TB, therapeutic carriers must reach the alveolar zone, where macrophages hosting Mtb reside.[36] The above mentioned possibilities of cell targeting and controlled drug release are also only achievable using tailor-made carriers, which will further provide drug protection.[16]

The fate of particles administered to the lungs is highly dependent on several parameters, including the deposition pattern and the mechanisms of lung clearance.[34] The deposition of aerosol particles in a particular region of the respiratory tract depends on their aerodynamic properties, namely the aerodynamic diameter (d_{aer}), and on the patient's breathing pattern. D_{aer} of a particle is equivalent to the diameter of a sphere of 1 g/cm^3 which has the same aerodynamic behaviour as the particle that is to be characterised.[33,34] Carriers with a d_{aer} higher than 5 μm are mainly deposited in the upper airways, while sizes below 5 μm can be distributed to the lower airways. This distribution pattern has, thus, a clear relation with the intended applications and the pharmacological responses to the treatment. Carriers with a d_{aer} between 1 and 3 μm are those with higher probability of depositing in the alveolar region.[37] This might be of interest for systemic delivery of drugs, as the alveoli are rich in capillaries and permit the passage to the systemic circulation, and also for local diseases with a focus on the alveolar zone, as is the case of TB. Importantly, particles with a very small size (d_{aer} < 0.5μm) face a high risk of exhalation, because their low mass prevents effective deposition in the mucosal surface (Figure 1.6). As regards to the patient breathing

pattern, a faster breathing promotes increase in the deposition of large particles in the upper airways, while a slow breathing increases the number of particles that penetrate the peripheral regions of the lungs.[33,34,38,39]

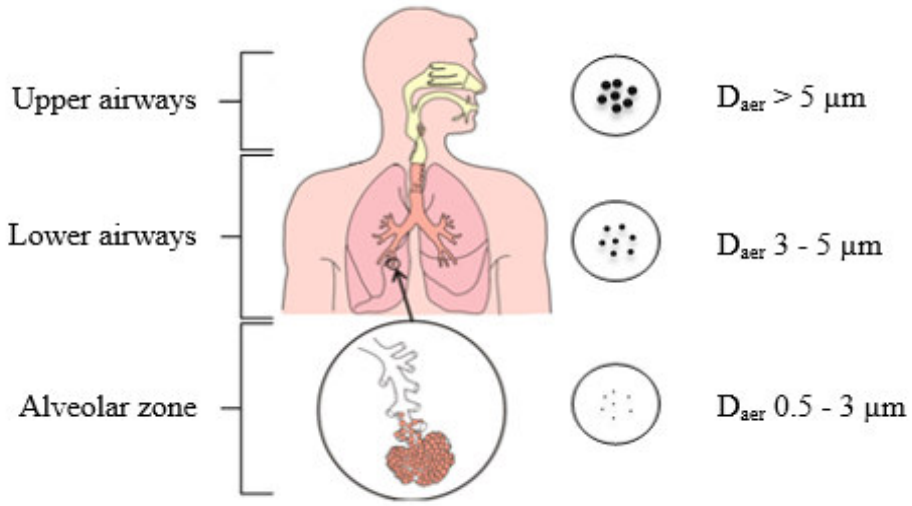

Figure 1.6. Deposition of MPs along the respiratory tract according to their d_{aer} (adapted from [39]).

The lung possesses several mechanisms to prevent the retention of aerosol particles inside the airways, in order to prevent potential harmful interactions between these particles and lung structures. The proper airway structure, with irregular and constant divisions, is an important defence barrier, as it imposes different deposition patterns, as explained above. Additionally, the removal or absorption of drugs from the lungs depends on the mucociliary clearance system, the site of deposition in the respiratory tract, biopharmaceutical factors, the drug release rate and the drug properties, such as the molecular weight.[33] Lung delivery of drugs promoted by the use of carriers has several advantages compared to the use of free drugs. Apart from increasing drug residence time and providing protection from local degradation, the carriers might promote stability against forces generated during aerosolization. Furthermore, they can be designed to enable vectorization to specific cells.[34]

In the particular case of lung TB and considering the hosting of Mtb by alveolar macrophages, a successful inhalable therapeutic strategy requires microparticles (MPs)

with a d_{aer} between 1 and 3 μm, which allows both reaching the alveolar space and a favoured phagocytosis by macrophages.[40]

1.3. Microparticle matrix materials

MPs are spherical particles with a diameter of 1 μm to 1000 μm that are composed of a continuous matrix. There are many materials reported to enable the production of MPs, but polymers have been the most used materials in drug delivery. Apart from being biocompatible and biodegradable in many cases,[38] polymeric carriers are reported frequently to have better stability, higher drug loading capacity (LC), slower drug release, longer pharmaceutical activity and better storage properties when compared to other drug delivery systems such as liposomes.[41,42] The application of polymeric MPs in pulmonary delivery has been frequent, because polymers are highly flexible and the particles might be tailored to deposit preferably in certain regions of the lung.[38,42] The polymeric matrix may be derived from natural or synthetic polymers.[43,44]

Among natural polymers are proteins (albumin, gelatin and collagen) and a long list of polysaccharides (chitosan, carrageenan, starch, hyaluronic acid, dextran, glucomannan, etc.).[44] Natural polymers are formed during the life cycle of organisms and are available in large quantities from renewable sources. They have a highly organised structure that contributes to their biocompatibility and biodegradability,[43] properties of critical concern in biomedical applications, including drug delivery.[45]

1.3.1. Konjac Glucomannan

Konjac glucomannan (KGM), extracted from the *Amorphophallus konjac tuber* C (also known as devil's tongue or, simply, konjac),[46–52] is native of Asia and grows mainly in tropical and subtropical regions of China and Japan.[47–49] Known as a natural and biocompatible polymer, KGM is structurally composed by a linear chain of monomers of D-glucose and D-mannose, in a molar ratio of 1:1.6, linked by β-1,4 glycosidic linkages. It is further branched through units of β-1,6 glucosyl for each 50-60 monomers

in C-3 position, with an acetyl group for each 19 monomers in C-6 position (Figure 1.7).[46–48,52–56]

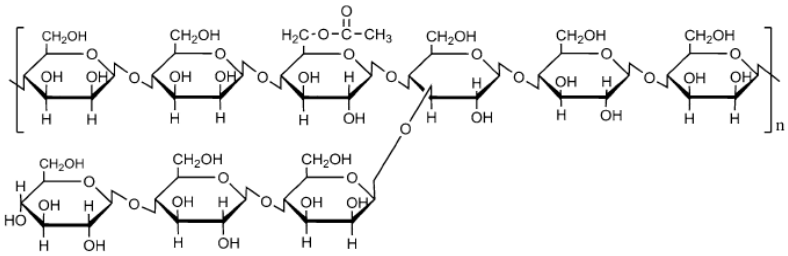

Figure 1.7. Chemical structure of KGM.[52]

Although soluble in water, a property mainly attributed to the presence of acetyl groups in the molecular chain[54], KGM is highly viscous in aqueous solutions. This is due to the presence of internal friction forces between all molecules (molecules of the structure of KGM and/or molecules of the solvent). Furthermore, this polymer exhibits a large molecular weight between 200-2000 *k*Da, depending on the source, cultivation, processing and storage time.[47,49,52,54,56]

KGM has been used in the pharmaceutical industry, especially as matrix material of delivery systems intended to provide a controlled drug release.[47,50,55] With a special relevance in the ambit of TB therapy, the presence of mannose units on the polymer chain make it an interesting material for macrophage targeting. As referred in section 1.1.2, macrophages have on their surface mannose receptors[6] that in fact mediate the recognition of Mtb. Mannose units in KGM might also mediate a recognition, favouring the access of drug-loaded particles to the intracellular compartment of macrophages, by phagocytosis.[57] Therefore, the use of KGM as matrix material of a drug carrier aimed at the pulmonary delivery of antitubercular drugs, might be an effective approach.

1.4. Spray-drying

As mentioned above, reaching a determined zone of the lung tree requires that the used carriers display very specific characteristics, in particular as regards to their density, size and morphology.[35] Spray-drying is one of the most used techniques in the

pharmaceutical industry to prepare polymeric MPs for drug delivery applications.[32,41,58,59] It is described as highly reproducible, even on an industrial scale, it is simple, fast, inexpensive and usually provides a regular distribution of particle size, as well as ensures uniformity of drug distribution even when used at lower doses.[41,58,60]

Spray-drying is a method where a solution or suspension is converted into dry particles through some fundamental steps: atomization, drying and particle formation. During the process, a liquid passes through an atomizer which will create a spray, forming droplets that are exposed to a stream of hot air, or other gas capable of promoting the rapid evaporation of the solvent present in the formed droplets. After evaporation, the resultant dried particles are separated from the gaseous stream by a cyclonic separator and accumulate in the collection vessel (Figure 1.8).[32,41,59–61]

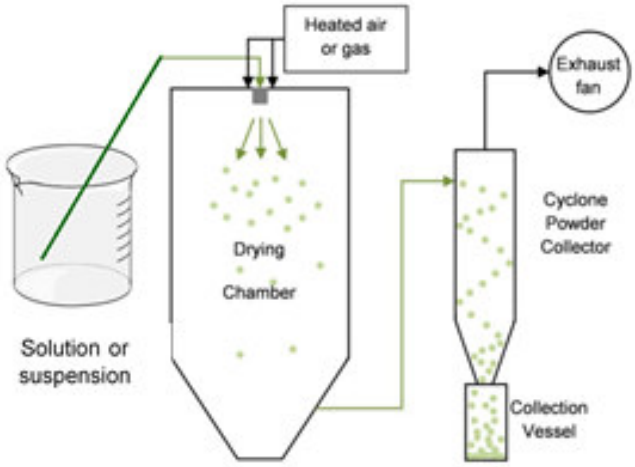

Figure 1.8. Diagram of spray-drying method (adapted from [60]).

There are many parameters involved in the whole process, most of them affecting the final properties of the MPs. Therefore, by optimising these parameters and tuning them according to the final characteristics desired for the produced powders, it is possible to obtain very different products. It is this flexibility that makes the technique so valuable. The parameters possibly undergoing optimization might be divided in two categories: 1) characteristics of the solution/suspension to be sprayed; 2) adjustable parameters in the equipment. In the former, the viscosity of the dispersion and its content in solid materials are the relevant characteristics. Regarding the proper equipment, there is the need to consider inlet and outlet temperatures, air flow rate, feed speed of the liquid to

be atomized and the aspirator rate. By varying these parameters, the size, shape and density of the MPs and the flowing properties of the obtained powder (compressibility, dispersibility) might be tuned to the desired effect.[32,58,59]

Nevertheless, MPs produced by spray-drying present a natural tendency to agglomerate due to strong cohesive forces that decrease flow properties, which not only affects its storage but also reflects on the ability to deposit in the lungs.[62] To circumvent this problem, the inclusion of other excipients in the formulations might be considered.[35] The literature reports the use of many excipients as a means to increase the physical or chemical stability of drugs, their mechanical properties and/or their final pharmaceutical properties.[32,63]

Mannitol has emerged as an alternative to lactose as adjuvant excipient in therapeutic formulations, because mannitol is a non-hygroscopic sugar and it has low humidity.[64] Furthermore, mannitol is not of animal origin, is not constituted of reducing groups, is highly crystalline even after being processed by spray-drying, and is approved for pulmonary delivery.[65] There are many works reporting the use of mannitol in inhalable formulations. It is generally referred that particles with a significant content in mannitol have improved flow properties.[66] According to Littringer *et al.*,[67] depending of the outlet temperature during atomization, mannitol particles might become smoother or have a rougher surface. Those with a rougher surface increase the density and particle mechanical stability as a result of the reduction of empty space between the particles.

Other excipients referred to be added to dry powder formulations in order to improve flowability are some amino acids. Among these is leucine and some studies have shown an increased deposition of bioactive particles in the lower airways of the respiratory tract when containing this agent, due to a decrease of interparticle interactions. Leucine acts as a surfactant during the drying stage of the spray-drying process, influencing the particle surface properties by making them more wrinkled. This also decreases the empty spaces avoiding the cohesion between particles and, thereby, increasing the flowing properties.[62]

2. Objectives

Considering the need to find alternatives to the conventional therapy of TB, this thesis is aimed at developing spray-dried KGM-based microcarriers for an application in the pulmonary delivery of antitubercular drugs (INH and/or RFB). In this manner, the work was devised to:

- Produce KGM MPs by spray-drying, designing a formulation with concomitant association of INH and RFB, two first-line antitubercular drugs;
- Optimize the spray-drying process so as to obtain MPs endowed with suitable aerodynamic characteristics to reach the alveolar zone and permit macrophage capture;
- Test the effect of introducing mannitol and leucine individually as adjuvant materials in the MP formulations, regarding the flowing properties, ability to encapsulate the antibiotics and the release profile;
- Evaluate the crystallinity pattern of KGM MPs containing antitubercular drugs;
- Determine the cytotoxic effect of the developed MP formulations in the respiratory cell lines A549 (representative of alveolar epithelium) and Calu-3 (representative of bronchial epithelium).

3. Materials and methods

3.1. Konjac Glucomannan hydrolysis

KGM (Chemos, Germany) was submitted to a process of acid hydrolysis in order to decrease its molecular weight and, consequently, its viscosity (40 000 mPa·s) upon solubilization in water. The hydrolysis was performed based on the protocol proposed by Cheng *et al*.[68] Briefly, 7 g of KGM as obtained commercially were added to 50 mL of 96% ethanol (AGA, Portugal) in a two-necked round bottom flask, under stirring, to create a suspension. Then, a purge with nitrogen was performed for about 5 min to create an inert atmosphere, after which the suspension was stirred for 30 min. Afterwards, 8 mL of 37% HCl (VWR, USA) were added and the suspension was sonicated for 60 min at 45 kHz (ultrasonic bath; VWR, USA). The mixture was then filtered under vacuum through a No. 4 fritted funnel, and washed with 70% ethanol (AGA, Portugal), until neutral pH. For the evaporation of ethanol, the hydrolysed polymer was placed overnight in a fume hood and then in a vacuum oven (VD23 Vacuum Oven; Binder, Germany) for approximately 48 h at 50 °C. During this time the sample was weighed sometimes and was only removed from the vacuum oven after a constant weight was achieved. The yield (%) of the hydrolytic reaction was calculated by the following equation:

$$\text{Yield} = \left(\frac{\text{Initial amount of KGM}}{\text{Final amount of hydrolysed KGM}}\right) \times 100$$

The hydrolysed KGM was stored in a desiccator until further use.

3.2. Preparation of KGM microparticles

KGM-based MPs were prepared from KGM solutions prepared at the concentration of 1.5% or 2% (w/v). The preparation of the solutions involved the mechanical stirring of the previously weighed polymer with ultrapure water (Mili-Q Plus; Milipore Iberica, Spain), at a temperature of approximately 70 °C.

INH (Sigma-Aldrich, Germany) and RFB (Chemos, Germany) were used as model antitubercular drugs. Two different formulations containing KGM and the drugs were prepared: KGM/RFB and KGM/INH/RFB. In all cases KGM was included at the concentration of 1.5% (w/v), the only exception occurring for MPs of KGM without any other material (drug or excipient). Concerning the drugs, INH was used at a polymer/drug ratio of 10/1 (w/w) and RFB at 10/0.5 (w/w). INH was dissolved separately in water in a test tube and subsequently added to the polymeric dispersion only 1 h before spray-drying, in order to prevent any degradation. In turn, in the two formulations including RFB, a minimum volume of HCl at the concentration of 0.01M was added to enable drug dissolution. In these cases, RFB was weighed in a test tube and dissolved with 0.01M HCl. After complete dissolution, it was added dropwise to the previously prepared KGM dispersion, and the mixture was stirred for about 20 h. All dissolutions were protected from light to prevent degradation.

Formulations containing the adjuvant excipients mannitol and leucine were also prepared. Mannitol (Sigma-Aldrich, Germany) was added to the polymeric dispersion in presence or absence of drugs at a concentration of 0.75% (w/v). It was weighed and solubilized simultaneously with KGM and left to stir for about 45 min before initiating spray-drying. Leucine (Panreac AppliChem, Germany) was used at the same concentration as mannitol (0.75%, w/v) in order to allow a comparison of effects. It was previously dissolved in water and then added to the KGM dispersion under stirring. The mixture undergone stirring for about 3 h until spray-drying took place. When drugs were added to the formulations containing the adjuvant excipients (INH and RFB individually, and in association), their inclusion occurred as described above for the formulations containing only KGM, regarding the process and the polymer/drug ratios: KGM/INH = 10/1, KGM/RFB = 10/0.5 and KGM/INH/RFB = 10/1/0.5 (w/w).

Ten formulations were thus prepared, as described in Table 3.1. For KGM alone two formulations were prepared, one with RFB and another one with INH and RFB. For KGM added of each of the adjuvant excipients, a formulation with each drug incorporated individually (INH or RFB) and another one containing an association of the drugs (INH and RFB) was prepared. Three replicates were prepared for each formulation.

The dispersions were spray-dried in a Buchi B-290 laboratory mini spray-dryer (Buchi Labortechnik AG, Switzerland) equipped with a high performance cyclone. The spray flow rate was set at 473 L/h. As disclosed in Table 3.1 for each formulation, inlet and outlet temperatures ranged between 160-175 ºC and 87-107 ºC, respectively, the aspirator was set at 80-90% and the feed flow varied between 0.7 and 1.5 mL/min. The yield (%) of the spray-drying process for each MP formulation was calculated from the following equation:

$$\text{Yield} = \left(\frac{\text{Weight of collected MPs}}{\text{Initial weight of solids in the dispersion}}\right) \times 100$$

Table 3.1. Optimised parameters of spray-drying for the different formulations (mean ± SD, n = 3).

Formulation	Inlet Temperature (ºC)	Outlet Temperature (ºC)	Aspirator (%)	Flow rate (mL/min)
KGM/RFB	170 ± 1	102 ± 3	90	0.72 ± 0.05
KGM/INH/RFB	170 ± 1	102 ± 3	90	0.74 ± 0.05
Mannitol				
KGM	160 ± 1	90 ± 3	90	0.79 ± 0.02
KGM/INH	160 ± 1	87 ± 3	90	0.80 ± 0.01
KGM/RFB	160 ± 1	91 ± 3	90	0.76 ± 0.01
KGM/INH/RFB	160 ± 1	97 ± 3	90	0.75 ± 0.00
Leucine				
KGM	170 ± 1	104 ± 2	85	0.83 ± 0.01
KGM/INH	170 ± 1	105 ± 3	85	0.89 ± 0.02
KGM/RFB	175 ± 1	107 ± 2	80	1.48 ± 0.13
KGM/INH/RFB	175 ± 1	104 ± 1	80	1.41 ± 0.09

3.3. Microparticle morphological and aerodynamic characterisation

3.3.1. Morphology

The morphological characterisation of MPs was performed by field emission scanning electron microscopy (FESEM Ultra Plus, Zeiss, Germany). Dry powders were placed onto metal plates and 5 nm thick iridium film was sputter-coated (model Q150T S/E/ES, Quorum Technologies, UK) on the samples before viewing.

3.3.2. Feret's diameter

The size of MPs was measured as the Feret's diameter (distance between two parallel tangent lines on opposite sides of the particle) using an optical microscope (VWR, USA). For this purpose, the powder was spread between a slide and a coverslip and observed under the microscope. The average diameter was calculated as the mean of 300 measurements for each sample.

3.3.3. Bulk and apparent tap density

For solid materials, density is defined by the weight of a sample over its volume.[69] However, calculation of the volume of a sample may not be an easy process.[70] Powdered formulations usually have spaces between the MPs, the total volume of spaces depending on the size and morphology of MPs and their inter-individual interactions. Considering or not the powder porosity, different densities can be obtained and calculated.[70]

To determine the bulk and apparent (tap) densities, a known amount of each dry powder was gently placed in a 10 mL graduated cylinder and the initial volume registered. The cylinder was mechanically tapped (Densipro 250410; Deyman, Spain) until a constant volume was reached (n = 3). The bulk and tap densities (g/cm^3) were determined according to the following equations:

$$\text{Bulk density} = \frac{\text{Weight of dry powder}}{\text{Initial volume of powder}}$$

$$\text{Tap density} = \frac{\text{Weight of dry powder}}{\text{Final volume of powder}}$$

3.3.4. Real density

Real density represents the weight of a unit volume of a sample, without considering its porosity.[69,71] This can be determined by a gas pycnometer, in which the gas is

distributed through the solid material, filling the empty spaces. The accuracy of the pycnometer to determine the density of the sample depends on the sample material and the gas. In this regard, the sample must not contain volatile elements that might cause errors and instability, while the gas must be a pure gas or dry air.[70] Helium is one of the most frequently used gases, as it is a very small atom able to diffuse rapidly through the pores of a sample.[70,71]

The real density of KGM-based MPs was determined by helium pycnometry (Accupyc 1330, Micromeritics Instrument Corporation, Norcross, GA) (n = 3).

3.3.5. Theoretical aerodynamic diameter

D_{aer} is a very important parameter to characterise in aerosol particles, because it provides indications on their flowing abilities. It is defined as the geometric diameter of a sphere with density of 1 g/cm^3 which moves in the air with the same velocity as the particle in question. Particles that have the same d_{aer} assume the same behaviour in air, regardless of physical size, shape, density or composition.[72]

D_{aer} (μm) of the MPs was theoretically calculated based on the Feret's diameter and the real density:

$$\text{Daer} = \text{Feret's diameter} \times \sqrt{\text{real}}\,\text{density}$$

3.4. Powder X-Ray Diffraction analysis

To measure the nature and degree of crystallinity of the KGM, drugs (INH and RFB), excipients (mannitol and leucine) and the various formulations prepared using these materials, an analysis by powder x-ray diffraction (PXRD) was performed. To do so, the samples were placed in a sampleholder and analysed by a diffractometer (PANalitycal X'Pert Pro) using a CuKα radiation at a wavelength of 0.154 nm filtered by nickel. During the analysis the equipment operated at 45kV and 35 mA. PXRD patterns were obtained at 2θ angles from 5º to 70º with a step size of 0.05º and 1500 s per step, using an X'Celerator detector.

3.5. Determination of drug association efficiency and loading capacity

An initial screening of emission/absorption wavelengths was performed for each of the drugs, dissolved in 0.1M HCl (at different concentrations), in a spectrophotometer (UV-1700 Pharmaspec; Shimadazu, Japan). An absorbance peak was observed for RFB at 500 nm. It was not possible to detect a peak isolated for INH, where only this drug absorbed and there was no interference of RFB. Thus, it was found an absorption point at 268.5 nm in which occurred the intersection of INH spectrum with that of RFB and corresponding to full absorption of both drugs. Therefore, whenever needed RFB was quantified at 500 nm and INH was quantified at 268.5 nm after subtraction of the contribution of RFB at the same wavelength. A scan was also performed for KGM and the two adjuvant excipients, mannitol and leucine, to ensure an absence of interference at the same wavelengths. Association efficiency (AE), LC and drug release calculations were then determined.

3.5.1. Formulations with INH

A standard calibration curve of INH in adequate media (counting on any effect from the polymer) was prepared at 268.5 nm. To prepare the medium for INH dissolution, 1.5 mg of unloaded KGM MPs were dissolved in 10 mL of 0.1M HCl, being maintained under stirring for 1 h. The solution was then filtered (0.45 μm) and INH was dissolved in this solution. Then, dissolved INH was diluted at various concentrations. The resulting solutions were spectrophotometrically analysed at 268.5 nm.

To determine the amount of INH associated to KGM MPs, 1.5 mg of KGM MPs containing INH were solubilized in 10 mL of 0.1M HCl for 1 h, under constant stirring. After filtration (0.45 μm), the collected solutions were measured at 268.5 nm.

Based on the standard calibration curve, the AE (%) and the LC (%) of drug(s) were calculated by the following equations:

$$\mathrm{AE} = \left(\frac{\text{Real content of drug}}{\text{Theoretical content of drug}}\right) \times 100$$

$$\mathrm{LC} = \left(\frac{\text{Real content of drug}}{\text{Weight of MPs}}\right) \times 100$$

The quantification of INH present in the formulations with INH and RFB is explained in the following section.

3.5.2. Formulations with RFB (and INH/RFB)

A standard calibration curve of RFB was performed in adequate media (counting with any effect from the polymer). Thirty mg of unloaded KGM MPs (with or without excipients) were dissolved in 10 mL of 0.1M HCl. Upon 1 h stirring, the resultant solution was centrifuged at 8000 rpm for 30 min (Centrifuge 5810R; Eppendorf, Germany) and subsequently filtered (0.45 μm). This filtrate was used to solubilize RFB to prepare the calibration curve.

The determination of drug AE and LC was performed by dissolving 30 mg of unloaded KGM/RFB MPs in 10 mL of 0.1M HCl. After centrifugation and filtration in the same conditions described above, the recovered liquid (filtrate) was measured spectrophotometrically at 500 nm, when only RFB is present, and at both 500 nm and 268.5 nm for the formulation with drug association. The measurement at 500 nm was made directly and provided a direct calculation of the RFB amount present in the MPs. In turn, the measurement performed at 268.5 nm required a 1:10 dilution and represents the sum of amounts of INH and RFB. Regarding INH, its amount was calculated by subtracting the absorbance obtained at 500 nm (RFB) to that at 268.5 nm (RFB + INH).

Then, AE and LC were calculated using the equations present in section 3.5.1.

3.6. *In vitro* drug release profile

The determination of the release profile was assessed for the formulations containing the association of both drugs, with or without the adjuvant excipients. In total, three formulations were assessed: KGM/INH/RFB, KGM-Man/INH/RFB, KGM-

Leu/INH/RFB. A determined amount of each formulation was weighed (30 mg) and placed in a test tube containing 10 mL of release medium composed by Phosphate Buffer Saline (PBS; Sigma-Aldrich, Germany) pH 7.4 added of 1% (v/v) Tween® 80 (Merk, Germany), based on the protocol proposed by Chandran *et al.*[73] MPs were kept under horizontal shaking (Orbital Shaker OS-10; Biosan, Latvia) at 100 rpm and 37 °C (Dry line; VWR, USA). At pre-determined times, samples of 1 mL were collected, filtered (0.45 μm) and analysed spectrophotometrically at 500 nm to determine the amount RFB. Subsequently, a 1:10 dilution was performed and a further measurement was made at 268.5 nm in order to determine the amount representing the sum of INH and RFB. The amount of INH was obtained by subtracting the absorbance at 500 nm to that of 268.5 nm.

To know the concentration of drug released over time, three calibration curves were elaborated for the process: two for RFB, at 268.5 and 500 nm, and one for INH at 268.5 nm. These curves were prepared in a medium resulting from the incubation of unloaded KGM MPs (either containing or not adjuvant excipients) in the same medium used in the release assay (PBS pH 7.4 + 1% (v/v) Tween® 80).

The percentage (%) of drug released over time was determined using the following equation:

$$\text{Drug release} = \left(\frac{\text{Concentration of drug released}}{\text{Initial drug concentration}}\right) \times 100$$

3.7. Cell culture

In vitro assays were performed using two cell lines from different regions of the lung. A549 cells (American Type Culture Collection (ATCC, USA) are derived from a human alveolar epithelial adenocarcinoma and represent the alveolar epithelium, while Calu-3 cells (ATCC, USA) derive from human bronchial epithelial adenocarcinoma and are representative of bronchial epithelium.

Both cell lines were maintained in culture in Dulbecco's Modified Eagles's Medium (DMEM, Sigma-Aldrich, Germany), supplemented with 1% (v/v)

penicillin/streptomycin (Sigma-Aldrich, Germany), 1% (v/v) L-Glutamine 200 mM (Sigma-Aldrich, Germany), 1% (v/v) Non-Essential Amino Acid (Sigma-Aldrich, Germany) and 10% (v/v) Fetal Bovine Serum (FBS, Gibco, USA). They were cultured in 75 cm^2 flasks, in a 5% CO_2/95% humidified atmospheric air incubator at 37 ºC. Culture medium was exchanged every 2–3 days and cells were subcultured weekly in the case of A549 cells and every 10–15 days for Calu-3 cells.

3.8. Determination of cytotoxic profile of microparticles

3.8.1. MTT Assay

The 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide (MTT) assay, developed by Mossman, is a colorimetric method to determine the number of living cells. It is based on the enzymatic reduction of yellow tetrazolium salt, MTT, by enzymes present only in metabolically active cells, to form purple formazan crystals. These can be solubilized using organic solvents, thus producing a coloured solution that might be analysed spectrophotometrically. The colour intensity is dependent on formazan concentration and is directly proportional to the number of metabolically active cells.[74]

The effect of the polymer, the adjuvant excipients and the various formulations of dry powders developed in this thesis (KGM, KGM MPs, KGM/RFB, KGM/INH/RFB, mannitol, mannitol MPs, KGM-Man, KGM-Man/INH, KGM-Man/RFB, KGM-Man/INH/RFB, leucine, leucine MPs, KGM-Leu, KGM-Leu/INH, KGM-Leu/RFB, KGM-Leu/INH/RFB) on cell viability was assessed in both A549 and Calu-3 cells, at the concentrations of 0.1, 0.5 and 1 mg/mL, for a period of 3 h and 24 h. To perform the assay, A549 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and Calu-3 cells at 2×10^4 cells/well. After an incubation period of approximately 24 h (37 ºC, humidified 5% CO_2/95% atmospheric air) to enable cell adherence, the medium was removed and replaced with 100 µL of each sample suspended in pre-warmed DMEM without FBS.

The cytotoxicity of the free drugs was also tested in three concentrations corresponding to the approximate real amounts of each drug on the formulations. The theoretical

polymer/drug ratios were considered for this effect, being polymer/INH = 10/1 (w/w) and polymer/RFB = 10/0.5 (w/w). To do so, INH was solubilized in DMEM without FBS and tested at the concentrations of 0.01, 0.05 and 0.1 mg/mL. However, due to the hydrophobic character of RFB, its solubilization in culture medium is hampered. Therefore, the solubilization was performed with dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany) at a concentration of 10 mg/mL with subsequent dilution to three concentrations (0.005, 0.025 and 0.05 mg/mL) being performed with DMEM without FBS. To demonstrate the absence of toxicity of DMSO at the used concentrations, various dilutions of DMSO in DMEM were tested for 24 h. Two controls were transversely used in the assays: DMEM was considered a positive control of cell viability and sodium dodecyl sulphate (SDS, 2%, w/v) (Sigma-Aldrich, Germany) was the corresponding negative control.

After exposure to the formulations or controls (3 h or 24 h), the supernatant of cells was removed from all wells and replaced with 30 µL of MTT solution (0.5 mg/mL in PBS pH 7.4; Sigma-Aldrich, Germany). Plates were placed back in the incubator and 2 h later 50 µL of DMSO were added to dissolve the purple crystals formed during the reaction of the cells with MTT. After solubilization, the plate was read by spectrophotometry (Infinite M200; Tecan, Austria) at 540 nm and corrected for background absorbance at 640 nm, as indicated by Carmichael *et al.*[75] Relative cell viability (%) was calculated as follows:

$$\text{Cell viability} = \left(\frac{\text{A} - \text{S}}{\text{CM} - \text{S}}\right) \times 100$$

Where A is the absorbance obtained for each of the concentrations of the test substance, CM is the absorbance obtained for untreated cells (incubated with cell culture medium only) and S is the absorbance obtained for the 2% SDS. The latter reading was assumed to correspond to 100% cell viability. The assay was performed on three occasions with six replicates at each concentration of test substance in each instance.

3.8.2. Lactate Dehydrogenase release assay

Measuring the amount of lactate dehydrogenase (LDH) present in the cell culture medium of cells exposed to a potential toxicant is another method to determine cytotoxicity. LDH is a cytoplasmic enzyme which only releases from the cells if the cell membrane is damaged. The method itself is based on a colorimetric reaction and involves the reduction of NAD+ to NADH by LDH, converting lactate into pyruvate. The resultant NADH is used in the conversion of the tetrazolium salt into red formazan crystals, which might be quantified spectrophotometrically.[76]

In simultaneous experiments with MTT (described previously), the quantification of LDH is performed on the supernatant of the cells incubated with each formulation or control. The assay was performed on A549 and Calu-3 cells, using the same formulations referred above for the MTT assay. The determination of LDH activity was performed for the highest concentration tested in the MTT (1 mg/mL) and for the longest incubation time (24 h). After the exposure of the cells to the formulations/controls, 100 µL of cell supernatant were collected and placed on a 96-well plate. DMEM was used as negative control of cytotoxicity and assumed as the 100% release of LDH in terms of result presentation. Triton X-100 (Amresco, USA) was used as a positive control of cytotoxicity, as it induces the lysis of the cells and then leads to the release of the total amount of LDH. This reagent was diluted 1:10 before use. The supernatant was centrifuged (16000 x g, 5 min, Heraeus Fresco 17 centrifuge; Thermo Scientific, Germany) and processed with the LDH kit (TaKaRa, Japan) following the indications of the supplier.

At the end, the samples were analysed by spectrophotometry at 490 nm with a background correction at 690 nm. The LDH release (%) was determined by the following equation:

$$\text{LDH release} = \left(\frac{\text{A}}{\text{CM}}\right) \times 100$$

where A represents the absorbance obtained for the LDH release by the cells exposed to the test substance and CM is the absorbance obtained for untreated with cells (incubated

with cell culture medium). The assay was performed on three occasions with three replicates at each condition.

3.9. 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 6.1) and differences were considered to be significant at a level of $p < 0.05$.

4. Results and Discussion

4.1. Preparation of KGM microparticles by spray-drying

Aiming at an application in lung TB therapy, several formulations of dry powders based on KGM were developed along this thesis for the objective of inhalation.

The commercially available KGM was found to produce solutions of very high viscosity, which impeded spray-drying. A process of chemical hydrolysis was thus performed in order to reduce the molecular weight of the polymer, in an attempt to reduce the viscosity to a level that enabled the production of KGM-based MPs by spray-drying. The mean yield of the various hydrolyses performed during the whole work was 94% (rendering approximately 6.5 g of hydrolysed KGM per hydrolysis).

Several attempts were made to produce unloaded KGM MPs, but none was successful. The resultant product had poor flow properties certainly resulting from the visible aggregation. Knowing from previous experience of the group that the incorporation of the antibiotic RFB improved significantly the macroscopic flowing ability of dry powders, a formulation of KGM/RFB was produced, revealing successful after little optimization. A formulation of KGM/INH/RFB was also produced with success.

The inclusion of adjuvant excipients in spray-dried powders in order to improve the aerosolisation properties has been reported in the literature.[62] Therefore, given the difficulties in processing KGM (in absence of RFB), the excipients mannitol and leucine were selected for this end. These were included separately and for each of them, a total of four formulations was developed: one without drugs, two formulations each with each drug, and one formulation with both drugs. Considering the formulations without excipients and those containing the excipients, a total of 10 formulations were developed and characterised, an eleventh one potentially being that of unloaded KGM MP, which was not successfully obtained and thus no characterisation is available apart from morphology.

All MPs were produced with very satisfactory yields, which varied between 66 and 79%. In fact, the literature does not frequently provide yields of 75-80% and these are relevant values, because they mean a low loss of materials and indicate the effectiveness of the technique. One of the reasons mostly contributing to the high yields is certainly

the use of the high performance cyclone, instead of the conventional cyclonic separator. The improvement of the yield due to the use of this cyclone was previously reported[77] and was the reason for its use. Going back to the yields of the formulations, it was verified that, in general, the presence of adjuvant excipients increased the yield of the process (Table 4.1).

Table 4.1. MP formulations obtained by spray-drying and resulting yields (mean ± SD, n = 3)

Formulation	Yield (%)
KGM/RFB	68.3 ± 3.2
KGM/INH/RFB	68.9 ± 2.7
Mannitol	
KGM	66.3 ± 8.1
KGM/INH	66.3 ± 6.3
KGM/RFB	78.5 ± 0.6
KGM/INH/RFB	79.1 ± 1.1
Leucine	
KGM	78.8 ± 3.5
KGM/INH	77.4 ± 4.0
KGM/RFB	76.5 ± 0.0
KGM/INH/RFB	72.0 ± 0.0

4.2. Characterisation of microparticles

4.2.1. Size and density

The initial approach to produce KGM MPs used a KGM solution at 2% (w/v), but it was soon determined in drug-loaded MPs that this concentration resulted in too large sizes (> 2 μm) for the objective of macrophage targeting. The concentration of KGM was thus reduced to 1.5% (w/v), as a lower concentration of solids in the spraying dispersions is expected to result in the size decrease of the produced MPs.

As can be observed in Table 4.2, the size of MPs (expressed as the Feret's diameter) ranged between 1.11 and 1.44 μm in the three groups of formulations (KGM without excipients, KGM with mannitol, KGM with leucine). This range of sizes is much

smaller than those reported in the literature (4.0 μm),[78] probably because the KGM dispersion prepared in this thesis was less viscous due to the hydrolysis of KGM. In certain cases the presence of excipients and/or drugs was verified to reduce the size of MPs, but no trend was found for the effect. In the literature there are no data on glucomannan MPs formulated with mannitol or leucine, so it becomes impossible to make a reliable comparison with other reported results. In this study, MPs containing leucine showed the smaller sizes. Moreover, those MPs associating RFB as sole drug were also in all groups the smaller MPs. The incorporation of RFB in KGM-Leu formulation significantly reduced the size of these MPs compared to the other formulations with RFB (KGM/RFB and KGM-Man/RFB, $p < 0.05$). There was also a significant difference in the size of MPs comprised of KGM-Man/INH and KGM-Leu/INH formulations ($p < 0.05$), with the latter presenting a smaller size. Formulations produced with mannitol were the only showing significant differences between them. Within this group, the incorporation of RFB has made the size of the MPs significantly reduced when compared to MPs composed of KGM-Man and KGM-Man/INH ($p < 0.05$).

Bulk and tap densities generally increased with the incorporation of excipients. Bulk density varied between 0.10 and 0.35 g/cm^3 and tap density between 0.18 and 0.48 g/cm^3 (Table 4.2). The tap density values for the formulations without excipients (0.18 – 0.22 g/cm^3) are very similar to those reported by other authors (0.17 g/cm^3).[78] Curiously, this value was much lower when compared to that reported for chitosan MPs in the same work (0.48 g/cm^3).[78] Back to Table 4.2, comparing formulations having leucine with those containing mannitol, the former were those registering the higher bulk density values. The same statistical differences were maintained for tap density.

With respect to real density, the values ranged between 1.38 and 1.52 g/cm^3 (Table 4.2). The values were higher than in another study presenting a density of 0.99 g/cm^3 for glucomannan MPs,[78] although they are in line with real densities usually reported for polysaccharide-based spray-dried MPs.[79,80] The statistical analyses revealed that there are many statistically significant differences between the determined real densities (decreased density with incorporation of excipients in the formulation only with RFB, smaller density for leucine-containing MPs, differences among leucine-containing MPs,

etc) although the true meaning of those differences might not be as relevant regarding the aerodynamic performance of the aerosol.

Table 4.2. Feret's diameter, bulk, tap and real densities, and aerodynamic diameter (d_{aer}) of KGM-based MPs in presence and absence of mannitol or leucine (mean ± SD, n = 3).

Formulation	Feret's Diameter (μm)	Bulk density (g/cm^3)	Tap density (g/cm^3)	Real density (g/cm^3)	d_{aer} (μm)
KGM/RFB	1.33 ± 0.76	0.10 ± 0.01	0.18 ± 0.00	1.48 ± 0.04	1.61 ± 0.10
KGM/INH/RFB	1.39 ± 0.79	0.12 ± 0.01	0.22 ± 0.01	1.52 ± 0.01	1.72 ± 0.06
Mannitol					
KGM	1.44 ± 0.83	0.16 ± 0.04	0.25 ± 0.05	1.43 ± 0.00	1.72 ± 0.03
KGM/INH	1.43 ± 0.81	0.17 ± 0.00	0.25 ± 0.00	1.43 ± 0.00	1.70 ± 0.10
KGM/RFB	1.26 ± 0.74	0.19 ± 0.00	0.30 ± 0.01	1.39 ± 0.02	1.49 ± 0.03
KGM/INH/RFB	1.32 ± 0.81	0.18 ± 0.01	0.29 ± 0.01	1.45 ± 0.01	1.60 ± 0.03
Leucine					
KGM	1.18 ± 0.75	0.32 ± 0.02	0.46 ± 0.01	1.40 ± 0.00	1.40 ± 0.07
KGM/INH	1.28 ± 0.74	0.35 ± 0.02	0.48 ± 0.02	1.41 ± 0.00	1.52 ± 0.04
KGM/RFB	1.11 ± 0.73	0.22 ± 0.00	0.38 ± 0.01	1.38 ± 0.00	1.30 ± 0.04
KGM/INH/RFB	1.23 ± 0.78	0.21 ± 0.00	0.36 ± 0.00	1.38 ± 0.00	1.44 ± 0.14

The incorporation of the adjuvant excipients mannitol and leucine enabled the production of specific KGM-based MPs which production was not possible in their absence (unloaded KGM MPs and KGM/INH MPs). However, there are some results expressed in the above table that may contradict the intention to use excipients to improve aerosolisation properties of powders produced by spray-drying. Some authors argue that MPs with smaller sizes and higher tap densities are more likely to agglomerate. If this is correct, it means that MPs with leucine are more probable to agglomerate to each other, since they have smaller sizes and higher tap density.[62,78]

The characterisation of aerodynamic properties of dry powders is vital to provide an idea on their dispersibility and flowing pattern upon *in vivo* administration by inhalation. As previously mentioned, in order to enable the deposition of aerosol particles in the alveolar region (where macrophages infected with Mtb are located), these should have a d_{aer} between 1 and 3 μm. Despite the importance of the parameters described before, related with geometric size and density of MPs, the aerodynamic diameter is the parameter giving the most important indication on the aerodynamic

behaviour of a formulation. In the formulations developed in this thesis, the values of d_{aer} ranged between 1.30 and 1.72 μm (Table 4.2), which indicates the theoretical appropriateness of all KGM-based MPs to deposit in the alveoli after inhalation. Glucomannan MPs produced by spray-drying in another study had a d_{aer} of 1.49 μm,[78] which is within the range of values obtained in this work. Although statistical significant differences were found in other cases if analysed point by point, the general indication is that MPs containing leucine showed the smaller d_{aer} values (between 1.30 and 1.52 μm) ($p < 0.05$). Additionally, MPs containing RFB as sole drug were those presenting the lower d_{aer} in each group of formulations. Considering that this is a theoretical calculation (methods, section 3.3.5), the final value is greatly affected by the geometric size (Feret's diameter in this case) and the real density of the dry powder. As commented below, real densities were quite similar between formulations and, therefore, the greatest contribution to the final value comes from the Feret's diameter. As this was smaller for MPs having leucine and RFB, it is logical that also these were the formulations with lower d_{aer}.

4.2.2. Morphology

SEM observation allowed the determination of the morphology of MP formulations produced by spray-drying and the effect of mannitol and leucine in morphological characteristics of MPs. Previous studies suggested that rougher surfaces increase dry powder dispersion properties. The presence of roughness on the surface of particles prevents agglomeration through van der Waals forces promoted by contact between particles.[78]

As depicted in Figure 4.1, MPs without excipients presented an irregular morphology with several concavities, but with a smooth surface. MPs of KGM only (no drugs) shown in the Figure 4.1a were produced at a KGM concentration of 2% (w/v). Although this is not suggested by the microphotographs, this formulation presents very poor flowing properties and its optimization did not succeed. The same morphologic effect was observed for MPs prepared at 1.5% (w/v), which correspond to microphotographs

of MPs loaded with drugs (Figures 4.1b and 4.1c). The inclusion of drugs was observed to not have an effect on particle morphology, as the same concavities are still observed.

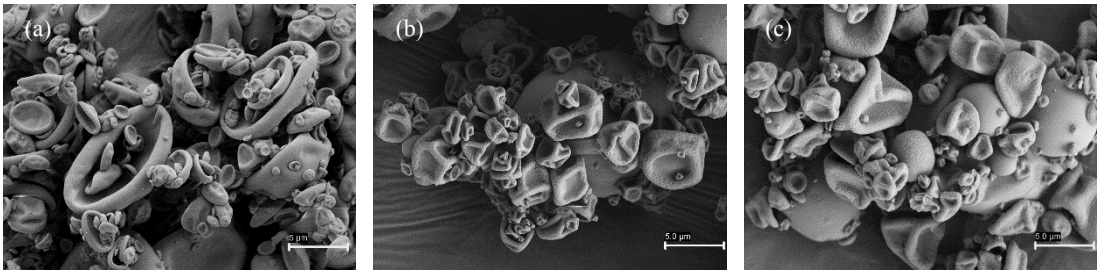

Figure 4.1. SEM microphotographs of KGM-based MPs without adjuvant excipients (mass ratios of KGM/drug indicated in parenthesis, KGM included at 2% (w/v) when used alone and 1.5% (w/v) when used with drugs). (a) Unloaded KGM MPs; (b) KGM/RFB (10/0.5); (c) KGM/INH/RFB (10/1/0.5). Scale bars = 5 µm.

The incorporation of mannitol (0.75%, w/v) in KGM MPs has produced very significant and visible changes in the morphology of MPs. As can be observed in Figure 4.2, mannitol has apparently deposited around the entire surface of the MPs in structures of acicular form. Curiously, the presence of RFB seems to prevent this effect. In fact, KGM-Man/RFB MPs are devoid of the acicular structures (Figure 4.2c) and KGM-Man/INH/RFB MPs have a much lower amount of these (Figure 4.2d).

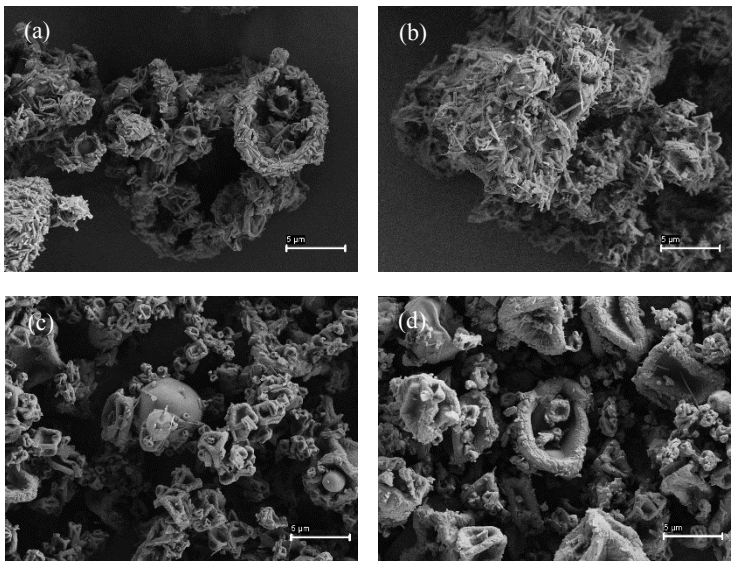

Figure 4.2. SEM microphotographs of KGM-based MPs containing mannitol (mass ratios of KGM/drug indicated in parenthesis, KGM included at 1.5% (w/v) and mannitol (Man) at 0.75% w/v). (a) Unloaded KGM-Man MPs; (b) KGM-Man/INH (10/1); (c) KGM-Man/RFB (10/0.5); (d) KGM-Man/INH/RFB (10/1/0.5). Scale bars = 5 µm.

The presence of acicular forms covering the whole surface of MPs containing mannitol endows the particles with higher surface roughness. This might possibly reduce the contact between the MPs and eventually have an impact on the aerosol performance based on that assumption. However, no experimental data are available to ensure that effect.

The inclusion of leucine on KGM MPs was also found to have an effect on particle morphology. In fact, comparing Figure 4.1a with 4.3a, it is observed that the addition of leucine makes concavities more pronounced and surfaces more irregular, in some cases even having roughness (Figure 4.3d). According to Osorio, a rougher surface improves the dispersion of MPs, since it makes more difficult the contact between the MPs,[78] although this is not reflected in the determinations of Feret's diameter and tap densities.

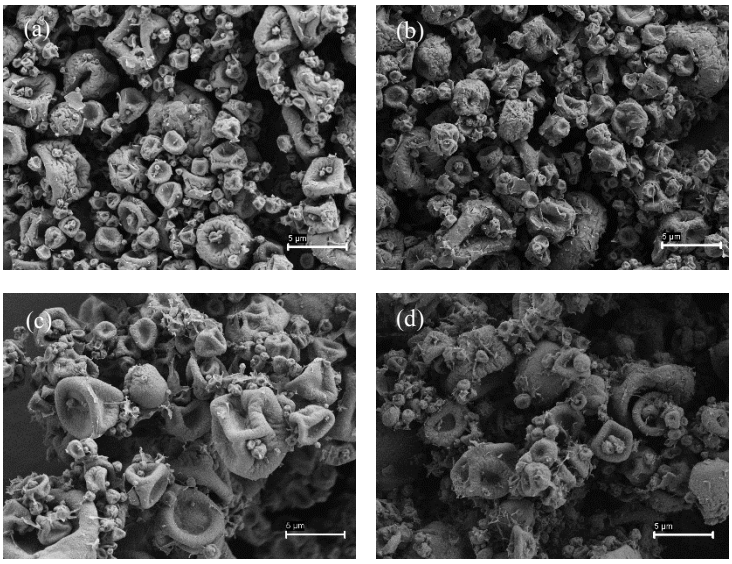

Figure 4.3. SEM microphotographs of KGM-based MPs containing leucine (mass ratios of KGM/drug indicated in parenthesis, KGM included at 1.5% (w/v) and leucine (Leu) at 0.75%, w/v). (a) Unloaded KGM-Man MPs; (b) KGM-Leu/INH (10/1); (c) KGM-Leu/RFB (10/0.5); (d) KGM-Leu/INH/RFB (10/1/0.5). Scale bars = 5 μm.

Apart from the composition of spraying suspensions, the morphology of MPs may also be affected by the conditions of the spray-drying process itself, in particular by the used inlet and outlet temperatures. According to Maas *et al.* increased outlet temperatures during spray-drying process increase the surface roughness of particles.[65] Although different inlet temperatures were used in this work and different outlet temperatures

naturally registered, no sufficient data are available to conclude on the occurrence of this effect.

Overall, considering the adequate aerodynamic diameter of the developed MPs in absence and presence of either mannitol or leucine and taking into account the improved surface roughness of MPs having excipients, which might provide better dispersing properties, these MPs (containing excipients) are apparently suitable for the objective of the work. However, the perception that one can have in practical terms when handling the different powders produced by spray-drying, might change the notion of what is the best formulation for the therapeutic purpose. The incorporation of leucine in KGM MPs was proposed to improve the flow properties of powders. On the one hand, its use allowed the production of unloaded KGM MPs and KGM MPs with INH, prevented in its absence. On the other hand, the formulations of KGM-Leu/RFB and KGM-Leu/INH/RFB showed the greatest tendency to agglomerate among all the produced formulations. This is in agreement with results obtained by other authors and already mentioned above. MPs comprised of KGM-Leu/RFB and KGM-Leu/INH/RFB were very small and had higher tap densities which promoted agglomeration. This agglomeration tendency was not so visible in MPs with mannitol, but when handling mannitol MPs and MPs without excipients, it was found that these latter showed better flow properties. This suggests that the presence of RFB had a higher ability to improve the flow properties of powders than the excipients themselves, mannitol or leucine. Thus, the formulations showing better flowing properties as observed macroscopically were KGM/RFB and KGM/INH/RFB.

4.3. PXRD pattern of KGM-based microparticles

XRD is a method used to evaluate the basic information about the crystalline structure of a material.[81] When X-ray radiation focuses on a crystal, a characteristic diffraction pattern is obtained. If a material has a perfectly crystalline structure it will display a well-defined XRD pattern, with narrow and sharp peaks. On the other hand, if the material has an amorphous structure, the pattern displayed will be drabber without peaks with significant intensity. For multiphase samples and/or small crystals, powder

XRD analysis is often more simple and convenient than the diffraction from a single crystal.[82] However, this technique has some limitations with regard to the detection limit (low amount of the crystalline component in the sample and/or crystals of reduced size to enable detection) and some degree of uncertainty in the relative intensity of the peaks caused by the preferential orientation of the samples.

All the samples and formulations produced by spray-drying were studied through this method. KGM polymer was studied in three different forms, non-hydrolysed polymer, hydrolysed polymer and MPs of hydrolysed KGM. They all exhibited the same XRD pattern without significant crystallinity peaks to be detected by the equipment (Figure 4.4). Although this might suggest that KGM is an amorphous polymer, it might also happen that this is not the case, but instead very small crystalline domains exist which cannot be detected by this technique.

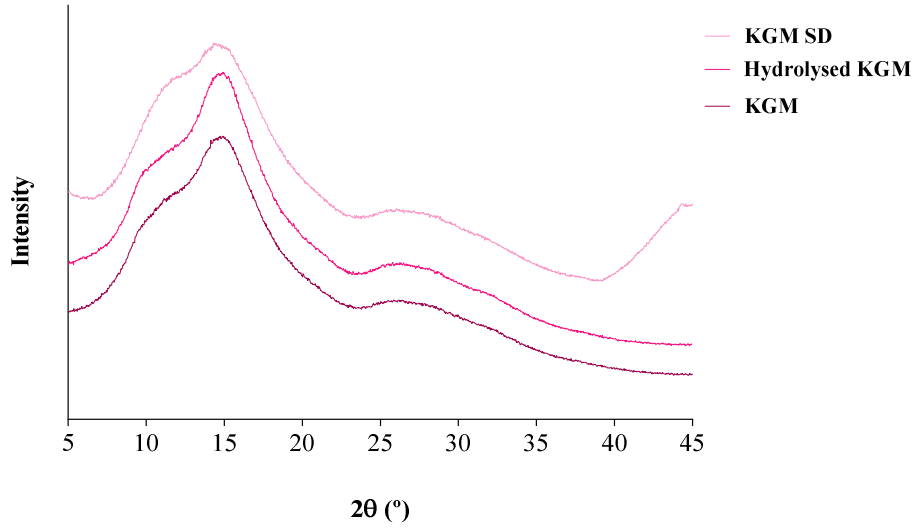

Figure 4.4. XRD patterns of KGM in three different forms: non-hydrolysed KGM, hydrolysed KGM and spray-dried (SD) hydrolysed KGM.

Regarding mannitol and leucine, these showed very intense peaks in the non-atomized form. However, this intensity decreased after spray-drying, with a more evident decrease in the case of leucine (Figure 4.5 and 4.7).

Mannitol exists in at least three polymorphic forms: α, β, and δ according to Walter-Levy's classification.[81] Each polymorphic form has different physicochemical properties that can affect the behaviour of mannitol as part of a drug delivery system.[64]

Furthermore, there may be a transition from one polymorph to another when mannitol is subject to certain conditions (high temperature or changes mediated-solution).[83] Thus, during the spray-drying process of the dispersions with mannitol, the formation of products with different polymorphic forms or a mixture of polymorphs may occur, as well as the formation of amorphous material. Therefore, it is of paramount importance to characterise these products and their polymorphic behaviour, before being used in pharmaceutical applications, since the presence of certain polymorphic forms may be less desirable in the final product.[81] According to the literature, the β polymorph the mannitol is thermodynamically more stable. Followed by the polymorph α and, finally the δ polymorph.[84] So the ideal is that the formulations with mannitol produced by spray-drying have the β polymorphic form.

The XRD pattern seen for the non-atomized mannitol indicated the β polymorphic form. After spray-drying, mannitol continued to exhibit the same polymorph, however the relative intensity of peaks decreased (Figure 4.5). The decrease in the intensity of the peaks may be due to the decrease in the size of the crystals.

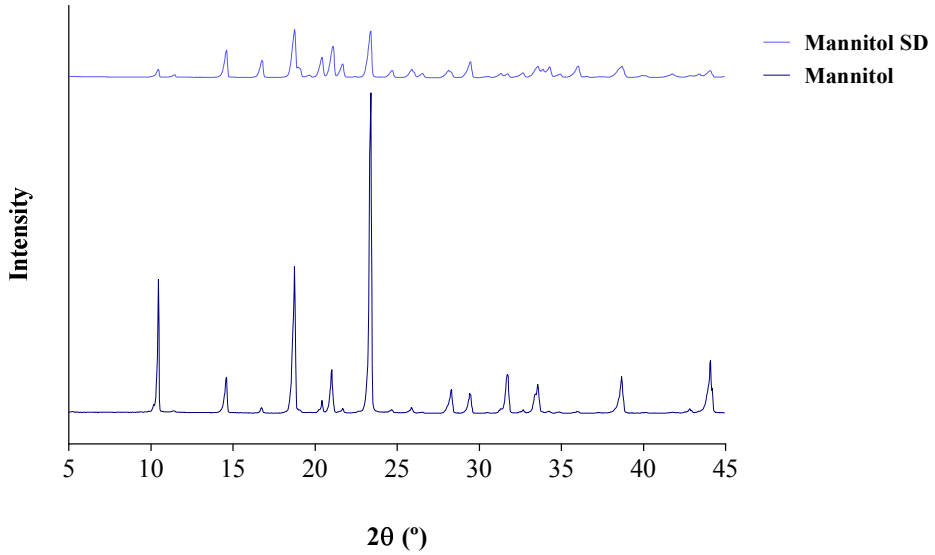

Figure 4.5. XRD patterns of mannitol as commercially obtained and spray-dried (SD) mannitol.

After mannitol spray-drying with KGM, this sample exhibited a XRD pattern where the peaks of β polymorph almost completely vanish. The peaks of δ polymorph[85] was easily detectable, suggesting that the transformation of the polymorphic form of mannitol

occurred in a large extension (Figure 4.6). This change of polymorph can be harmful to thermodynamic stability of the formulations with mannitol because, as mentioned above, the δ polymorphic form is less stable at this level.

The base of the transition from one polymorph to another is a different packaging of the molecules giving rise to changes in the crystalline structure of a material. In this case, the change of mannitol polymorph may have occurred due to the high temperatures to which mannitol was subjected during atomization or possible interactions that may have occurred between mannitol and KGM.

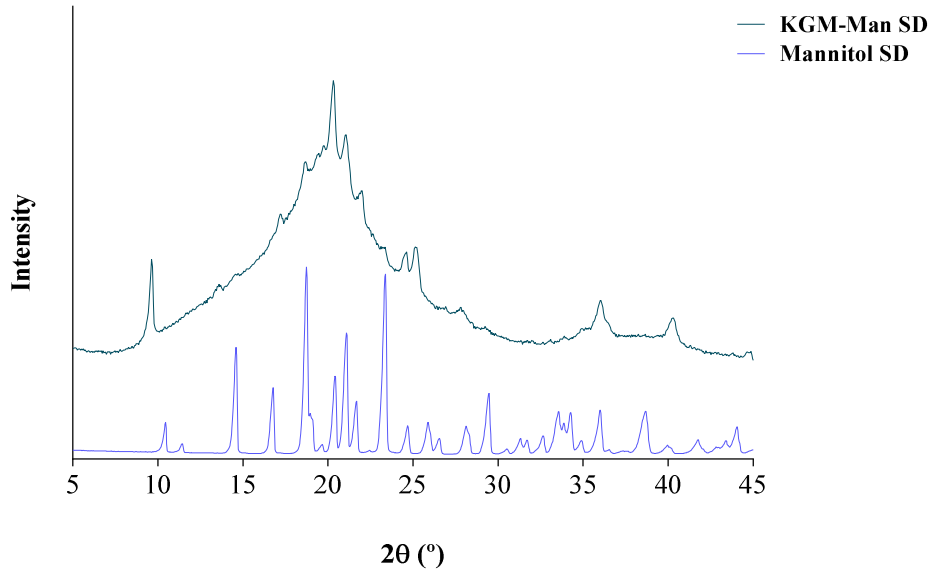

Figure 4.6. XRD patterns of spray-dried (SD) mannitol (Man) and KGM-Man MPs.

Contrary to mannitol, leucine does not show polymorphism. The decrease in peak intensity after the spray-drying process is much more pronounced than that found for mannitol. Moreover, the peaks are broader and poorly defined and in some cases show different relative intensity (Figure 4.7). These data suggest that the crystals present in the leucine produced by spray-drying are much smaller compared those of non-atomized leucine.

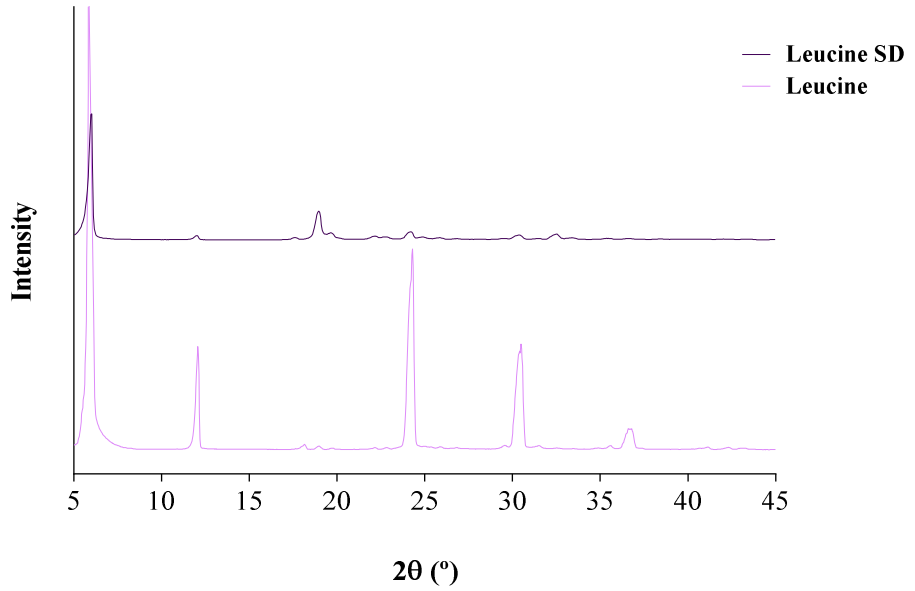

Figure 4.7. XRD patterns of leucine as commercially obtained and spray-dried (SD) leucine.

When leucine was spray-dried with KGM, the presence of leucine diffraction peaks was still verified, with intensity similar to that exhibited by spray-dried leucine by itself (Figure 4.8).

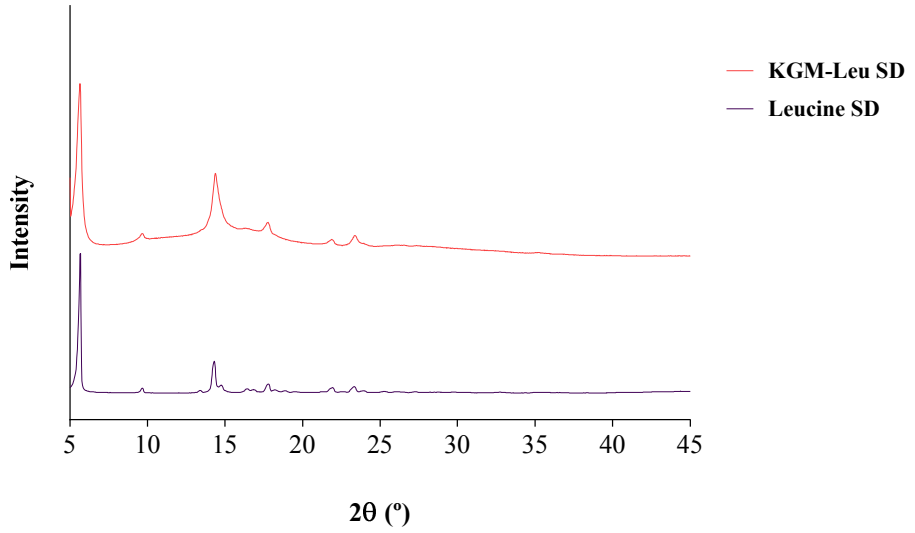

Figure 4.8. XRD patterns of spray-dried (SD) leucine (Leu) and KGM-Leu MPs.

RFB before undergoing the process of spray-drying showed a significant number of diffraction peaks, but after being atomized the peaks disappeared from the XRD pattern (Figure 4.9). This does not prove that RFB lost its crystalline profile and became an amorphous sample. As referred above in other case, this may be caused by the formation of small RFB crystals during spray-drying in such a way that prevents their detection by the XRD equipment. This is in line with the two broad peaks that are seen in the 2θ region where the majority of the peaks of RFB appear.

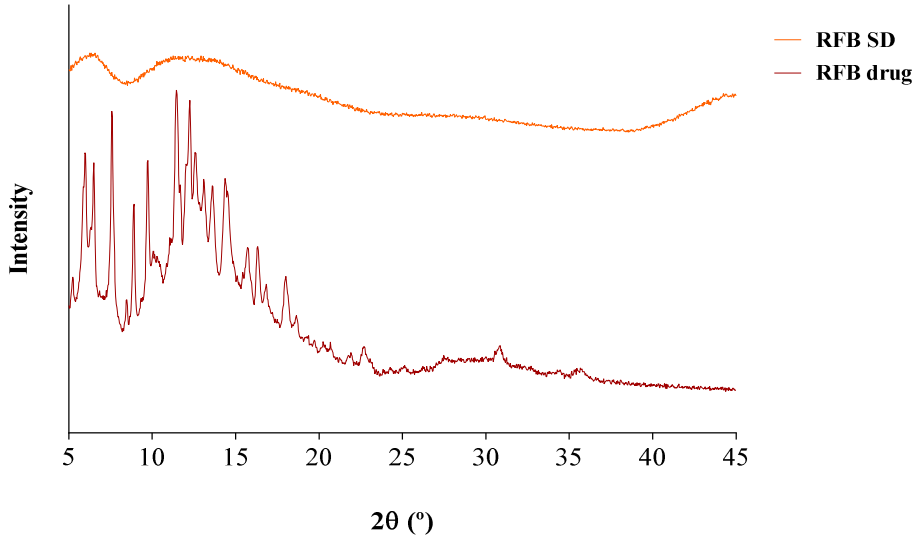

Figure 4.9. XRD patterns of RFB and spray-dried (SD) RFB.

The formulation of KGM with RFB showed no significant peak intensity. However, the presence of mannitol or leucine has changed the XRD pattern. As seen in the Figure 4.10, the only visible diffraction peaks are those corresponding to the presence of excipients in the formulations.

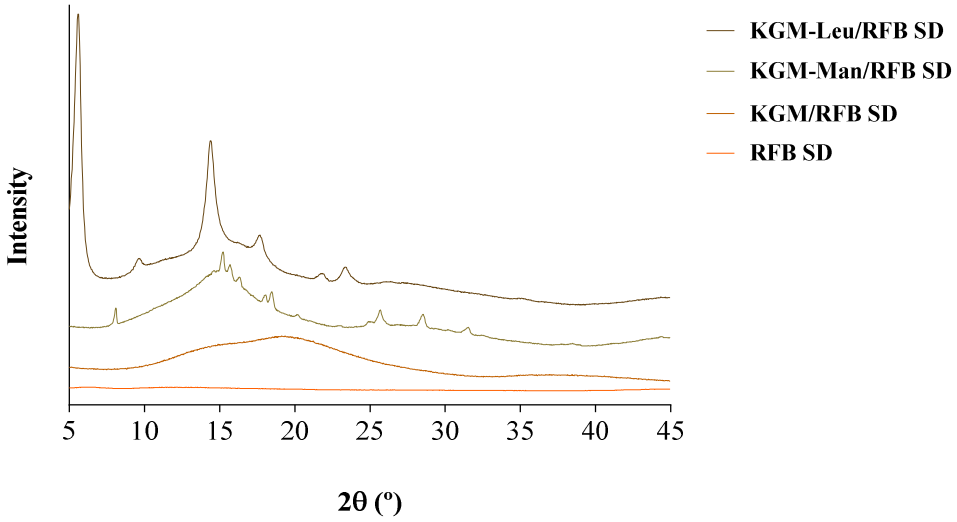

Figure 4.10. XRD patterns of spray-dried (SD) RFB and formulations with RFB. Man, mannitol; Leu, leucine.

In contrast to what happened with RFB, INH kept the presence of diffraction peaks with significant intensities after being submitted to the spray-drying process. However, this intensity was much lower comparing to the intensity of the peaks displayed in the XRD pattern of non-atomized INH (Figure 4.11). The reason for this decrease may have been the same as suggested for RFB regarding the formation of smaller crystals during the process of INH spray-drying.

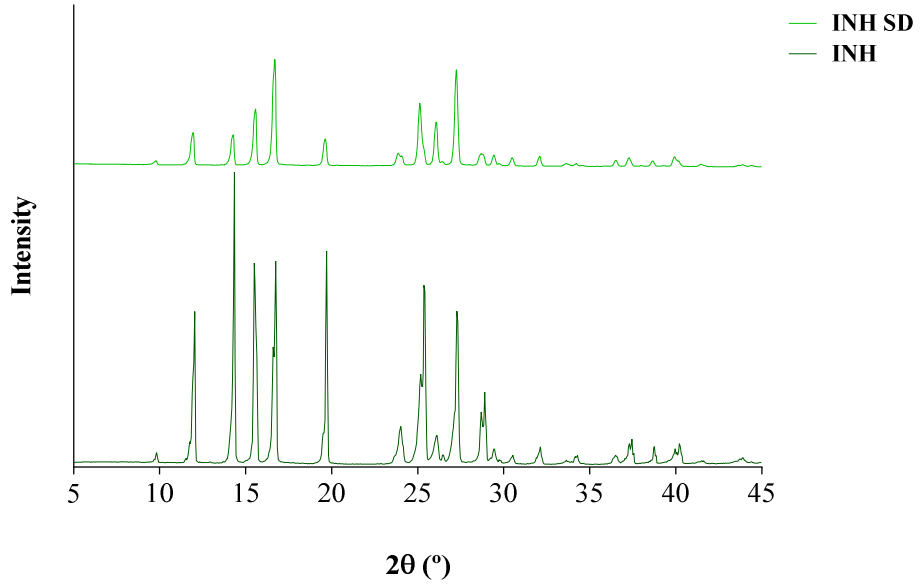

Figure 4.11. XRD patterns of INH and spray-dried (SD) INH.

When INH was atomized together with KGM (in the presence or absence of excipients) the situation has significantly changed (Figure 4.12). No peak with significant intensity could be found in the pattern of the formulation without excipients. In formulations with mannitol or leucine, the patterns display several peaks that can be assigned to the excipients and not to INH. Again, it does not mean that these formulations had no INH crystals. When the dispersion for these formulations was made, INH was used at a polymer/drug ratio of 10/1 (w/w). Therefore, INH was highly dispersed among the polymer (and the excipients) and its crystallinity was difficult to be detected by the equipment. The other reason for this has already been mentioned earlier, i.e. INH crystals might be very small and impossible to be detected.

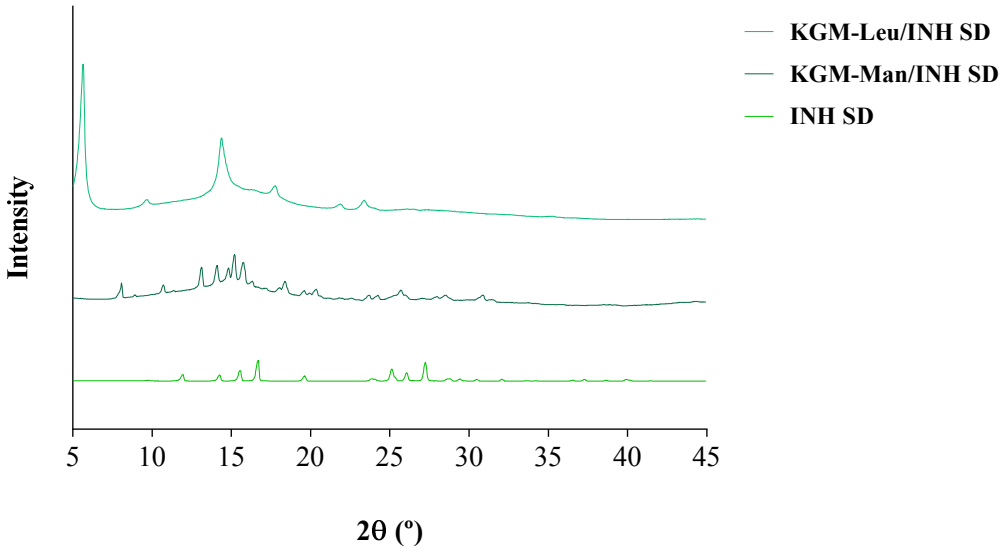

Figure 4.12. XRD patterns of spray-dried (SD) INH and formulations with INH. Man, mannitol; Leu, leucine.

The formulations of the association of two drugs, in the presence or absence of excipients, exhibited XRD patterns very similar to formulations with only one drug. Thus, in a general way, the only peaks detected in the XRD equipment in all formulations produced by spray-drying correspond to the presence of excipients, mannitol or leucine, regardless of which of the drugs was present.

4.4. Association efficiency and loading capacity

Two model antitubercular drugs were associated to the MPs, INH and RFB. Considering that the most used drugs in TB treatment are INH and RIF, selecting those for this work would have been logical, but there was an experimental impediment. In fact, some studies indicate that there is a chemical incompatibility between these two drugs when combined in solution,[86] forming adducts that are not soluble and could perhaps interfere in spray-drying processing. The incorporation of mannitol and leucine in KGM MPs formulations aimed at improving the properties of powders produced by spray-drying. Nevertheless, the incorporation of excipients in therapeutic formulations may influence other aspects, particularly the AE and the release profile of the drug.

To understand the results obtained in this section is important to differentiate between AE and LC. AE refers to the ability of the drug to be retained within the polymer matrix while LC is the capacity of the polymer to load the drug.[87] The AE and LC values for the various formulations obtained by spray-drying are summarised in Table 4.3.

AE obtained for the various formulations ranged from 40% to 100%. The lowest AE was obtained for INH in KGM-Man/RFB/INH formulation ($p < 0.05$). INH association value in this formulation was significantly lower when compared with the values of INH association obtained for the KGM/INH/RFB and KGM-Leu/INH/RFB formulations ($p < 0.05$), which were in the range of 87% - 98%. Also the association of INH in KGM-Man/INH formulation was relatively low (46.2%) when compared with KGM-Leu/INH formulation (around 100%, $p < 0.05$). This indicates that the incorporation of mannitol interferes with AE of INH and there seems to be some competition between INH and mannitol to be retained within KGM matrix. Another possibility may be the degradation of INH due to some kind of interaction with mannitol that can be damaging for this drug, although no information addressing this possibility was found on the literature. Work performed in the laboratory with other polymers, such as locust bean gum and partially hydrolysed guar gum, experienced the same problem in formulations with mannitol and INH. This is not the case for RFB, as the AE of this drug was much better, 100% for KGM-Man/RFB formulation and 98% for KGM-Man/INH/RFB formulation. This distinct behaviour of RFB comparing with

INH in presence of mannitol was coincident with that observed in other works of the team (unpublished data).

LC varied between 2.5 and 7.6%, with KGM MPs without excipients generally showing higher capacity to carry the drugs ($p < 0.05$) (Table 4.3). It is noteworthy that the LC of MPs was much lower in formulations containing mannitol than those containing leucine ($p < 0.05$), in line with the lower INH association efficiency observed in the former. In spite of what happened with INH in mannitol formulations, the LC results in this study were better than those found in some previously reported works.[88]

Table 4.3. Association efficiency (AE) and loading capacity (LC) of INH and RFB in different formulations based on KGM (mean ± SD, n = 3).

Formulation	**AE (%)**	**LC (%)**
KGM/RFB	90.3 ± 6.2	4.3 ± 0.3
KGM/INH/RFB	(INH) 87.4 ± 2.9	(INH) 7.6 ± 0.3
	(RFB) 92.4 ± 4.4	(RFB) 4.0 ± 0.2
Mannitol		
KGM/INH	46.2 ± 2.6	2.9 ± 0.2
KGM/RFB	100.1 ± 2.4	3.2 ± 0.1
KGM/INH/RFB	(INH) 40.8 ± 3.1	(INH) 2.5 ± 0.2
	(RFB) 98.0 ± 5.1	(RFB) 3.2 ± 0.2
Leucine		
KGM/INH	102.7 ± 3.4	6.4 ± 0.2
KGM/RFB	95.9 ± 3.3	3.1 ± 0.1
KGM/INH/RFB	(INH) 98.2 ± 1.7	(INH) 6.0 ± 0.1
	(RFB) 96.5 ± 2.0	(RFB) 2.9 ± 0.1

The suitability of a formulation for an application in drug delivery to the lungs is dependent on an appropriate association to the carrier, with effective loading. Thus, analysing the values obtained for the AE and LC, the formulation without excipients seems to be the best choice. Although leucine has slightly improved the association of drugs, in some cases the resultant LC is lower. Mannitol, in turn, has a negative effect on the association of INH and therefore, for this drug, the formulation containing leucine is the most appropriate approach.

4.5. Drugs release profile

The lung is coated by 10 to 30 mL of aqueous fluids, including surfactants. Numerous studies indicate that the composition and pH of lung fluids differ for the several regions of the lung and between healthy people and people with lung diseases.[89] This study focuses the design of a drug carrier which therapeutic objective requires reaching the alveolar zone. Therefore, it is important to use a release medium that resembles the alveolar lung lining fluid, in terms of pH and composition. Release studies of pulmonary formulations have been done using simple media (water or PBS) supplemented with certain components to adapt the solubilization capacity of a solution, such as surfactants (SDS and Tween 80®), phospholipids such as dipalmitoylphosphatidylcholine (DPPC) or co-solvents such as alcohol. These agents significantly influence the rate of drug dissolution due to the increased solubility of the drug and are generally used to replicate the effect that the pulmonary surfactant would have *in vivo*. Pulmonary surfactant products, Curosurf® or Survanta®, are licensed for the treatment of respiratory distress disorders and have been used as release media in some works. However, these are very expensive and are described to interfere with analytical techniques in some cases.[89] Thus, in this work it was decided to use a release medium comprising 1% (v/v) Tween® 80 in PBS pH 7.4 to study the release profile of the formulations containing the two drugs RFB and INH. Tween® is a tensioactive and thus is present in substitution of the surfactant. It is known to not affect the pH of the release medium.[90] The pH of 7.4 closely resembles that of lung lining fluid, which is reported to be around.[91]

Because of time constraints it was decided to only determine the release profile of formulations containing drugs in combination. As WHO indications on TB are clear regarding the need to have combined therapy, this seemed an adequate decision to make. Results of the release of RFB and INH from the MPs are depicted in Figures 4.13 and 4.14, respectively.

In general lines, a strong difference between profiles was found depending on the presence of any of the adjuvant excipients ($p < 0.05$). Moreover, the release of RFB was different from that of INH ($p < 0.05$).

Figure 4.13 shows that the absence of excipients in the formulation KGM/INH/RFB allowed a more sustained release of RFB, the 100% release being achieved only at 450 min. The introduction of any of the excipients strongly accelerated the release, the formulation having mannitol completing the release of RFB in 120 min, while that comprising leucine reached its maximum at 180 min. When KGM MPs (without further excipients) were incubated with the release medium, and considering that the polymer has some swelling ability, an initial swelling might have occurred, leading to a dissolution of the drug and consequent release. This swelling may have delayed the release of the drugs from the formulation KGM/INH/RFB. INH release was also faster from adjuvant-containing formulations (Figure 4.14), but the effect was not as strong as for RFB. This is due to the fact that INH presents higher solubility, which already contributes to a faster dissolution and, therefore, the acceleration was not as noticed.

Although the difference was not very marked, the release provided by KGM-Man/INH/RFB was generally faster than KGM-Leu/INH/RFB ($p < 0.05$).

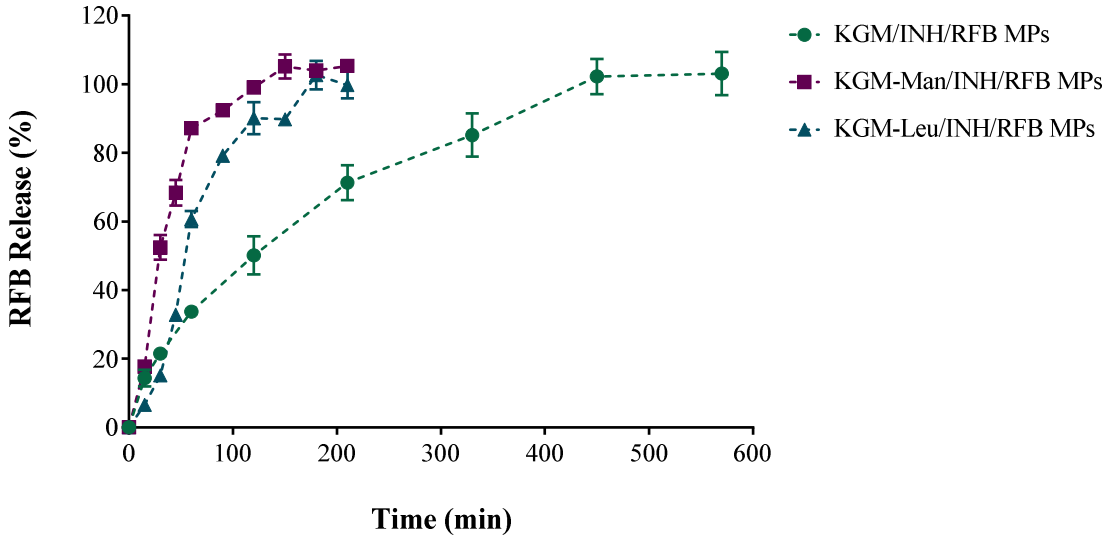

Figure 4.13. *In vitro* drug release profile of RFB from the formulations KGM/INH/RFB, KGM-Mannitol (Man)/INH/RFB and KGM-Leucine (Leu)/INH/RFB. Data represented as mean ± SD (n > 3).

Release profiles registered for INH had a pattern very similar to those of RFB when the three formulations are compared among each other (Figure 4.14). In this manner, a more sustained release of INH happened in the formulation having no excipients, while a

rapid release was observed for MPs containing any of the excipients ($p < 0.05$). In turn, the formulation of mannitol provided faster release of INH (100% in 90 min) than that having leucine (100% in 120 min) ($p < 0.05$).

Comparing the release of RFB with that of INH, RFB showed a slower release in all cases ($p < 0.05$). From the observation of both figures it becomes clear that the main property affecting drug release if possibly the drug itself. In fact, and as said before, KGM is a soluble polymer and the MPs will present a high dissolution rate. Therefore, drug properties are those most affecting the release rate and the hydrophobicity of RFB is certainly one of the reasons for its slower release. Additionally, the molecular weight of drugs might also have influenced the process. RFB has a much higher molecular weight than INH (847.02 g/mol *versus* 137.14 g/mol, respectively) and thus, might take longer to dissolve, contributing to the final pattern.

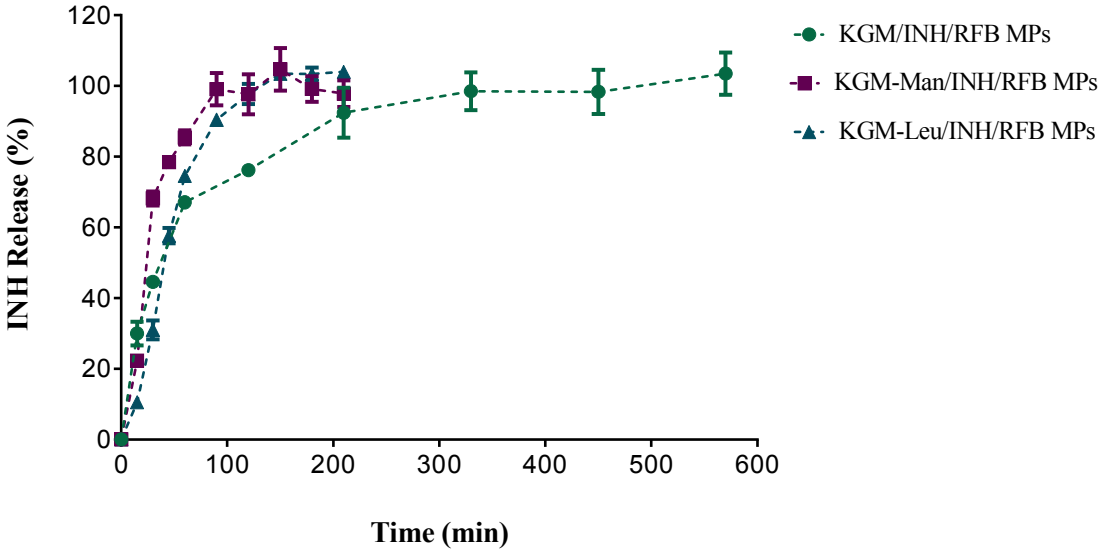

Figure 4.14. *In vitro* drug release profile of INH from the formulations KGM/INH/RFB, KGM-Mannitol (Man)/INH/RFB and KGM-Leucine (Leu)/INH/RFB. Data represented as mean ± SD ($n > 3$).

Another aspect suggested to contribute to the observed release profiles concerns the morphology of MPs. As mentioned in the discussion of characterisation, the incorporation of mannitol or leucine in formulations has resulted in increased surface wrinkling, which increases the contact surface area. Thus, MPs containing excipients

have improved contact with the release medium as compared with KGM MPs, which might have caused an increased release rate.

4.6. Cytotoxicity evaluation

The cytotoxic evaluation of the developed formulations of KGM-based MPs was performed by two different assays, MTT and LDH release, in a cell line representative of the alveolar epithelium (A549 cells).[92] Selected assay times for MTT assay were 3 h and 24 h. The former refers to a short-term contact, while the latter is considered a prolonged time, as by then the lung defence mechanisms are expected to have provided the elimination of toxicants. The LDH release assay was performed only for exposure times of 24 h and for the higher concentrations used in the MTT assay (0.05 mg/mL for RFB, 0.1 mg/mL for INH and 1 mg/mL for polymer, adjuvants excipients and formulations produced by spray-drying), as these are the conditions considered adequate for an eventual observation of cell membrane damage. The Calu-3 cell line, a model of the bronchial epithelium,[78] was also tested for some formulations, but due to time constraints, the studies were not performed for the whole set of formulations to permit their inclusion in the thesis. Nevertheless, the obtained results are available in Appendix A.

4.6.1. MTT assay

The free drugs were tested at concentrations corresponding to the percentages that were incorporated into MPs. INH was associated at a polymer/drug mass ratio of 10:1, while RFB at 10/0.5. Therefore, the amount of INH and RFB is approximately 10% and 5%, respectively, that of the total mass. INH was thus tested at 0.01, 0.05 and 0.1 mg/mL, while RFB concentrations were 0.005, 0.025 and 0.05 mg/mL. According to ISO 10993,[93] the minimum acceptable level of cell viability is 70% for cytotoxicity tests and the interpretation of data was performed based on that assumption. INH demonstrated an absence of toxicity in A549 cells, as none of the tested conditions induced cell viabilities below 70% (Figure 4.15).

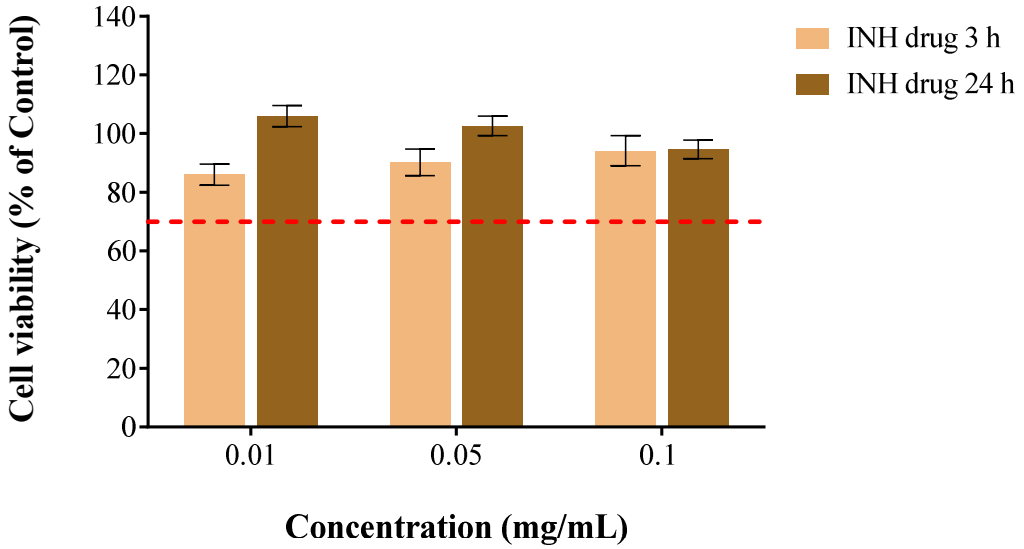

Figure 4.15. A549 cell viability as determined by the MTT assay upon 3 h and 24 h exposure to INH as free drug. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

In turn, RFB has demonstrated a certain level of toxicity (cell viability of 64%) in these cells at the highest tested concentration (0.05 mg/mL) and upon a prolonged exposure (Figure 4.16). As a whole, it is seen that RFB induces a decrease of cell viability that is both time- and concentration- dependent ($p < 0.05$).

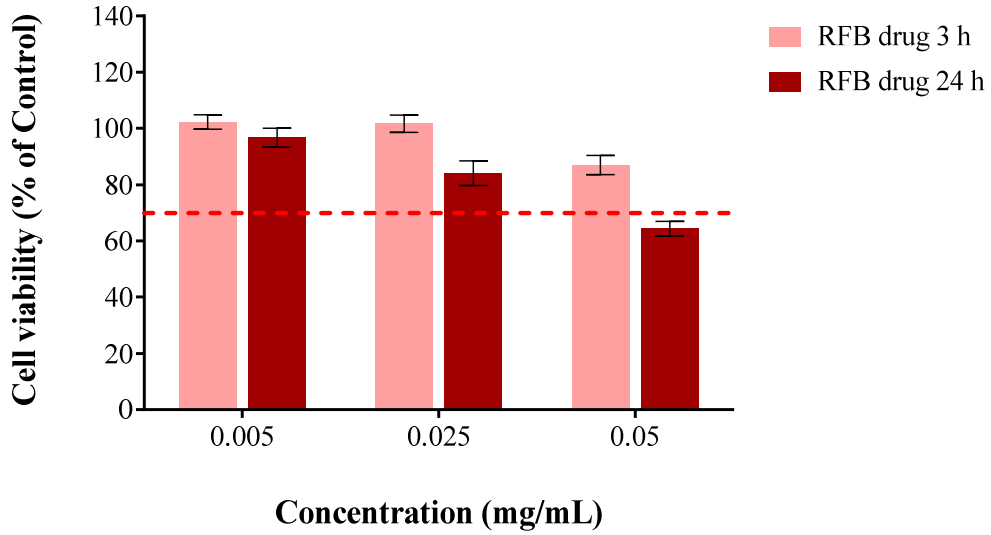

Figure 4.16. A549 cell viability as determined by the MTT assay upon 3 h and 24 h exposure to RFB as free drug. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

Considering that the final MP formulations comprise not only KGM and the drugs, but also other excipients like mannitol and leucine, it was necessary to test the effect of all these controls (KGM as polymer, spray-dried KGM, spray-dried mannitol and spray-dried leucine). MP formulations produced by spray-drying, in presence and absence of drugs, were also tested. In all cases the used concentrations were 0.1, 0.5 and 1 mg/mL, which are considered to cover the whole range of doses possibly administered.

The assessment of several formulations devoid of excipients, with or without drugs, and again considering the threshold of 70% mentioned in ISO 10993, revealed no toxicity when the exposure lasted 3 h (Figure 4.17a). This profile was changed when the exposure time increase to 24 h, as shown in Figure 4.17b. Under that condition, but only for the concentration of 1 mg/mL, all tested samples resulted in a cell viability close to 70%. Interestingly, no differences were seen between the various MP formulations, which means that the presence of RFB when associated alone or in combination with INH, did not have a significant effect on cell viability. It is interest to notice that a certain trend exists for both 3 h and 24 h incubation that spray-dried KGM induces higher cell viability than the polymer itself and the incorporation of drugs further improves the viability. However, upon 24 h incubation of 1 mg/mL the effect is no longer observed, as described above.

Regarding the exposure time, there was a significant decrease in cell viability for almost all formulations when this was extended from 3 h to 24 h, indicating a time-dependent effect ($p < 0.05$). The 24 h incubation also revealed a concentration-dependent effect (Figure 4.17b), particularly when comparing the concentration of 1 mg/mL with any of the others ($p < 0.05$).

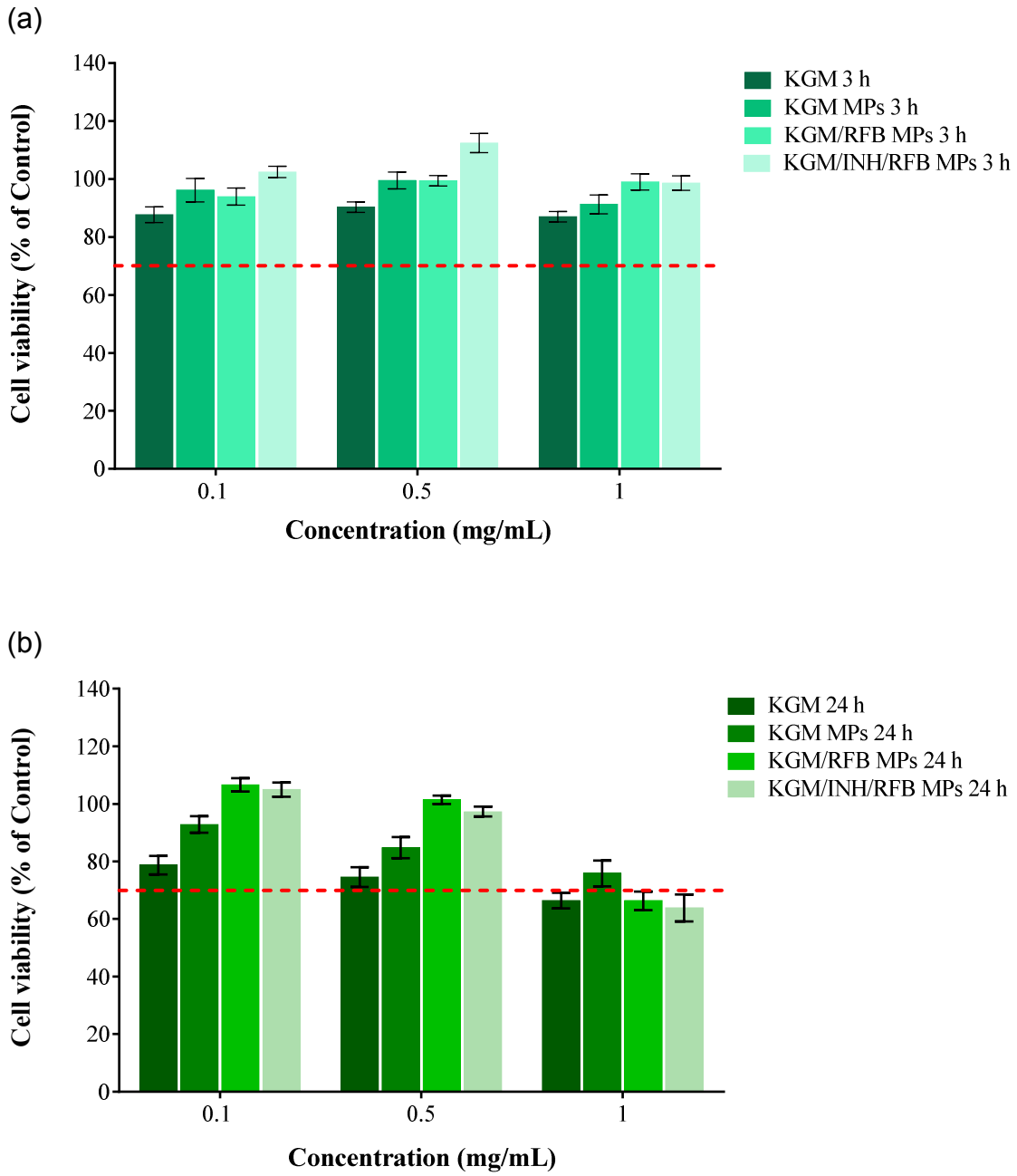

Figure 4.17. A549 cell viability as determined by the MTT assay upon a) 3 h and b) 24 h exposure to KGM polymer, KGM MPs, KGM/RFB MPs and KGM/INH/RFB MPs. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

Mannitol and leucine showed no cell toxicity under the tested conditions. Referring to Figure 4.18, mannitol SD was only close to 70% when the cells were exposed for a period of 24 h at the concentration of 1 mg/mL. In this condition there was a significant decrease in cell viability compared to 3 h exposure of mannitol SD ($p < 0.05$).

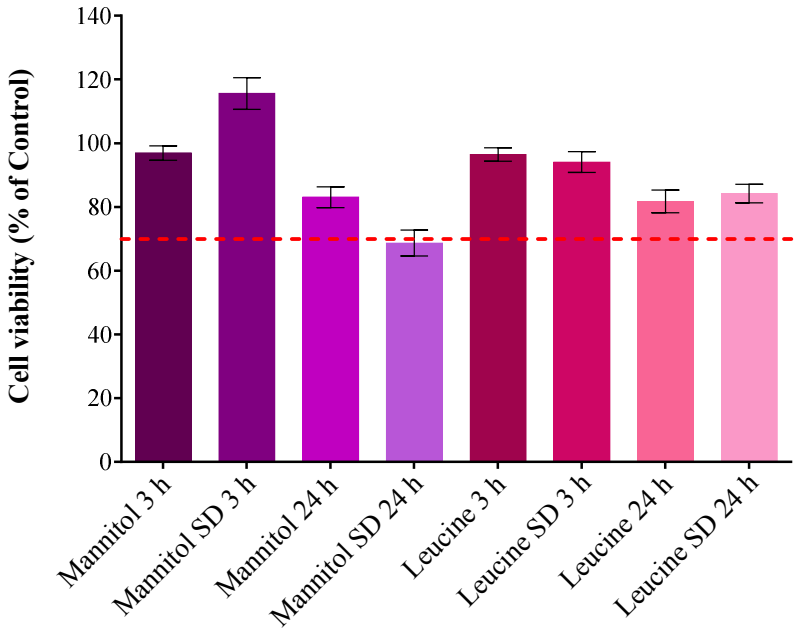

Figure 4.18. A549 cell viability as determined by the MTT assay upon 3 h and 24 h exposure to 1 mg/mL of mannitol and leucine as commercially obtained and spray-dried (SD) mannitol and leucine. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

The assessment of mannitol-containing MPs demonstrated an absence of toxicity after 3 h of exposure (Figure 4.19a), as cell viability remained above 80% in all cases. However, after an exposure of 24 h cell viabilities have shown a tendency to decrease, overcoming the 70% threshold in some formulations and demonstrating a time-dependent effect ($p < 0.05$). As can be seen in Figure 4.19b, the incubation with the higher concentration of some MPs (KGM-Man, Man-KGM/RFB and KGM-Man/INH/RFB) resulted in cell viabilities slightly below 70%, which is significant in terms of concentration and duration of exposure ($p < 0.05$).

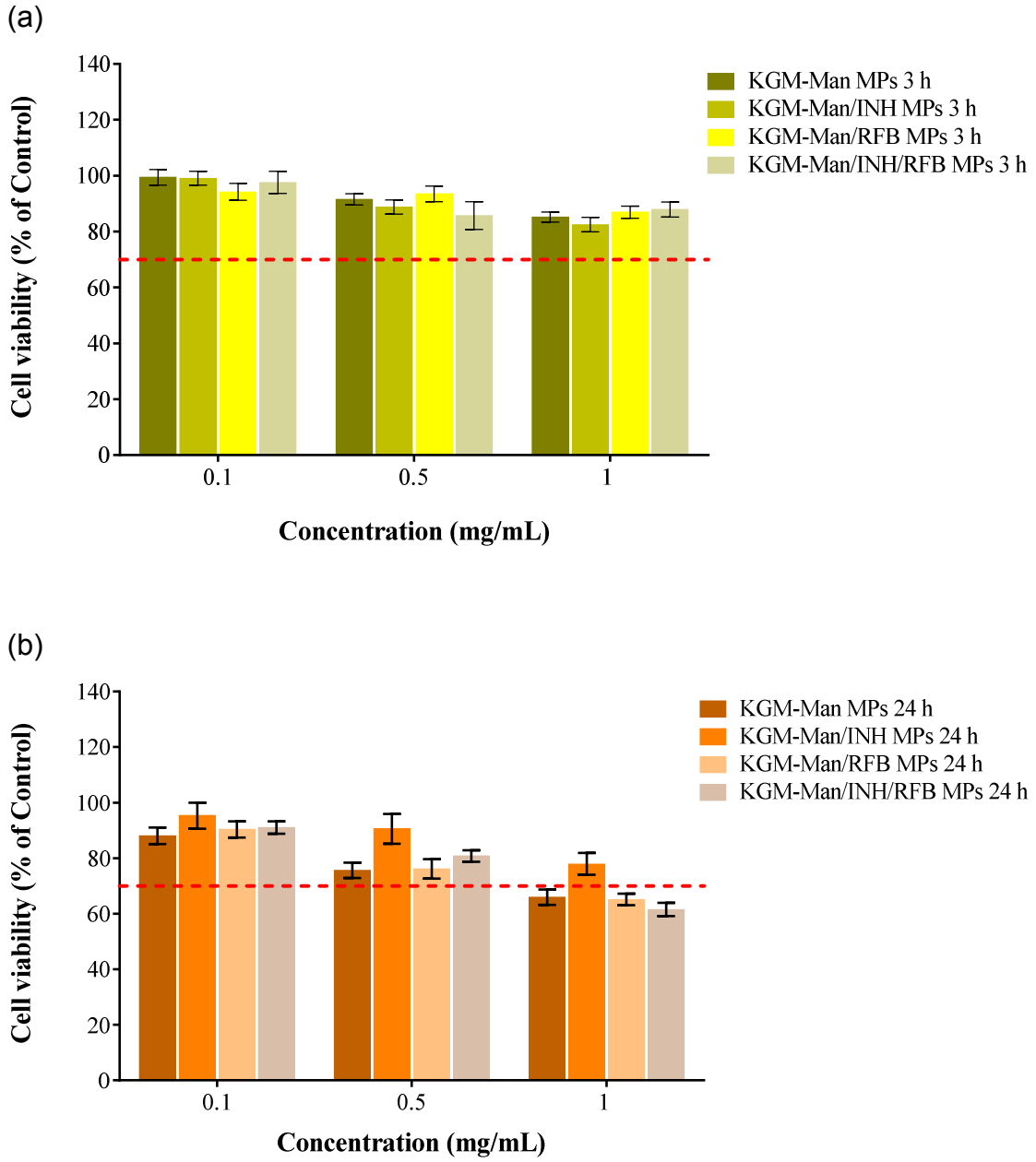

Figure 4.19. A549 cell viability as determined by the MTT assay upon a) 3 h and b) 24 h exposure to KGM-Mannitol (Man) MPs, KGM-Man/INH MPs, KGM-Man/RFB MPs and KGM-Man/INH/RFB MPs. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

The assessment of leucine-containing formulations resulted in a similar pattern comparing with those containing mannitol for an exposure of 3 h. In that case, cell viabilities were above 70% in all cases and usually well above 80% (Figure 4.20a). Again, a time-dependent effect was observed ($p < 0.05$), which is clearly visible for the

concentrations of 0.5 and 1 mg/mL. In fact, after 24 h exposure (Figure 4.20b) cell viabilities generally decreased and remained below 70% in all drug-loaded MPs at 1 mg/mL ($p < 0.05$). The concentration of 0.5 mg/mL also resulted in low viability (67%) for KGM-Leu/RFB MPs. A concentration-dependent effect was determined for KGM-Leu/RFB and KGM-Leu/INH/RFB MPs to be statistically significant ($p < 0.05$).

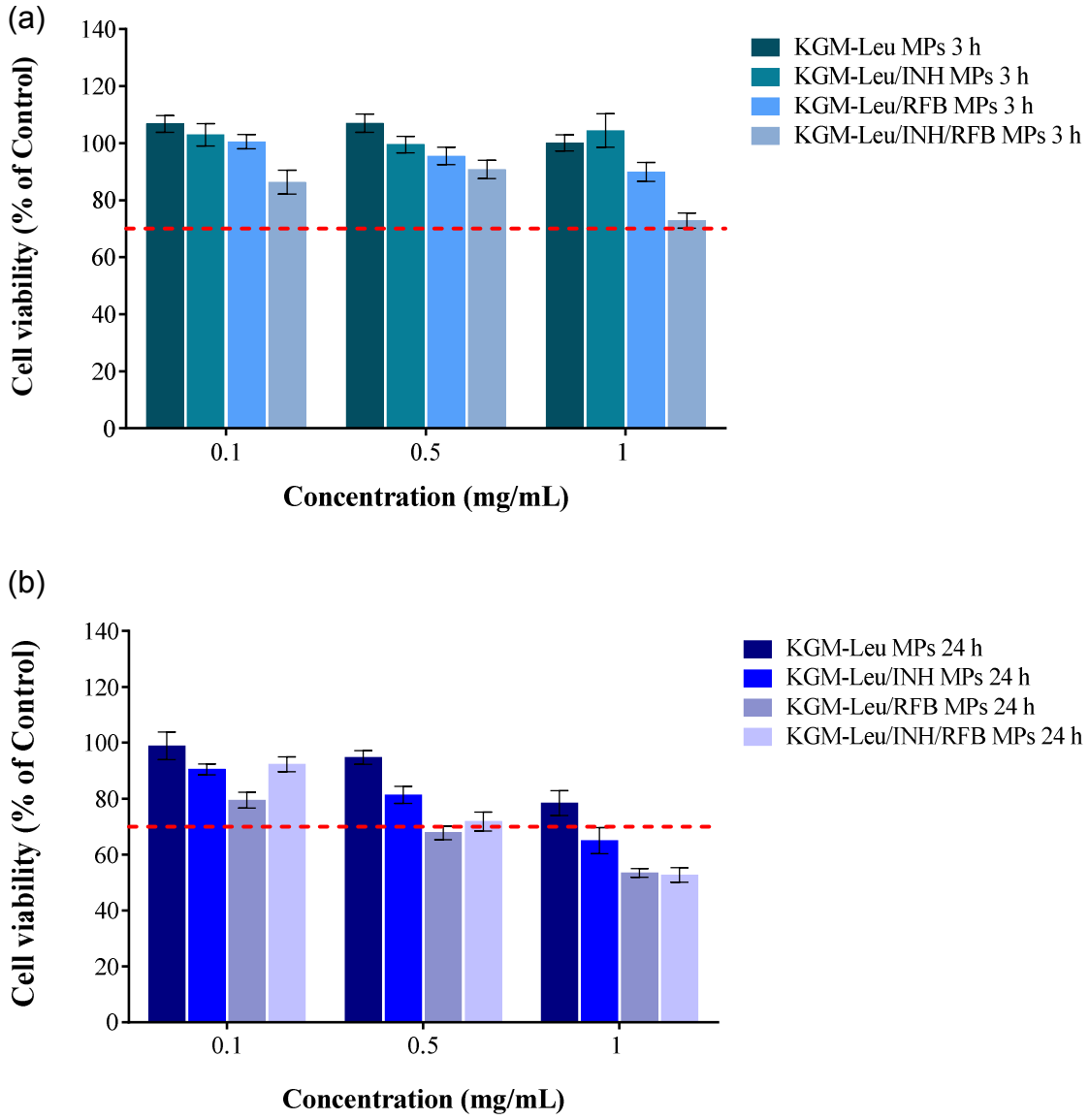

Figure 4.20. A549 cell viability as determined by the MTT assay upon a) 3 h and b) 24 h exposure to KGM-Leucine (Leu) MPs, KGM-Leu/INH MPs, KGM-Leu/RFB MPs and KGM-Leu/INH/RFB MPs. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

Doing a more global analysis of the MTT assays performed in A549 cells, the increased concentration of the samples and exposure duration was found to generally decrease cell viability. The presence of RFB in the formulations appears to be the factor deserving further discussion. As it was described at the beginning of the section of MTT assay, free RFB was toxic to A549 cells when tested at a maximum concentration for 24 h. Some formulations also showed a toxic effect at a concentration of 1 mg/mL after 24 h of exposure. KGM-Man, KGM-Man/RFB, KGM-Man/INH/RFB, KGM-Leu/RFB and KGM-Leu/INH/RFB MPs have shown a percent cell viability below 70%, although in most cases cell viabilities were close to the acceptable threshold. The toxic effect, although relatively mild, is apparently related with RFB content is most cases and, therefore, this is the issue requiring further attention.

4.6.2. LDH release assay

The LDH assay is an indicator of disruption of the cell membrane which leads to lack of the cytoplasmic enzyme. The release of LDH into the culture medium is an indicator of irreversible cell death mediated by cell membrane disruption. The LDH release assay evaluates the integrity of the cell membrane and is considered to complement the results of the MTT assay.[94]

One of the most important information provided by the MTT assay was that only when A549 cells were exposed to higher concentrations of MP (1 mg/mL) for 24 h there was a risk for cell viability. Thus, the amount of LDH released by A549 cells has been assessed only in this situation. For this purpose two controls were used, the culture medium was used as negative control (taken as 100%) and to lysis buffer as a positive control. The positive control corresponded to the minimum amount of cytoplasmic enzyme that can be released, while the positive control corresponded to the maximum amount. Observing Figures 4.21 and 4.22, it is seen that only the RFB drug (Figure 4.21) and two formulations containing mannitol, KGM-Man/RFB and KGM-Man/INH/RFB (Figure 4.22), had a significantly higher LDH release compared to the negative control ($p < 0.05$). In other formulations no significant differences were found and the release of the cytoplasmic enzyme was similar to that induced by culture medium. As expected, treatment with lysis buffer resulted in a significant increase in the

amount of LDH released due to the complete disruption of A549 cells ($p < 0.05$) (Figures 4.21 and 4.22).

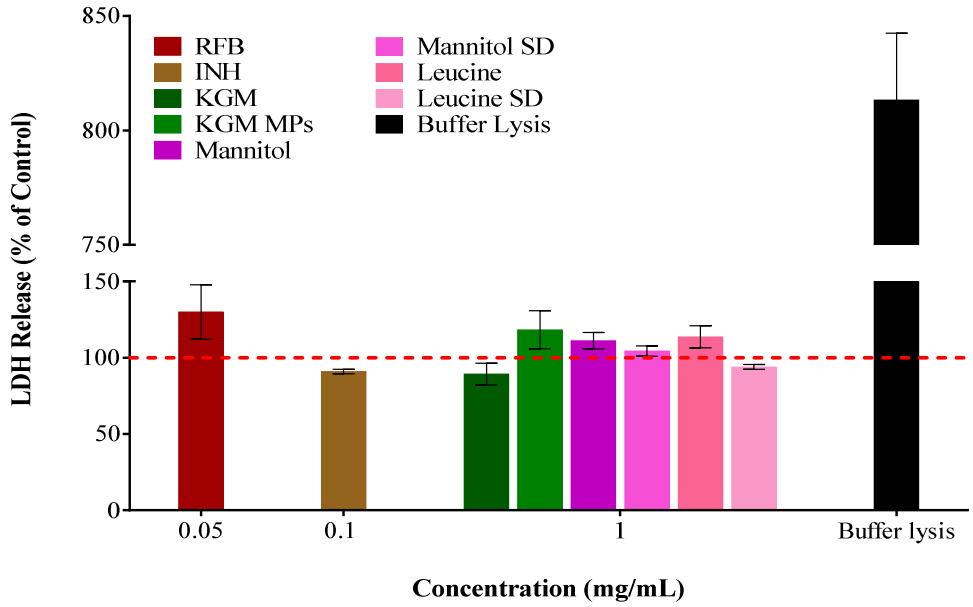

Figure 4.21. LDH release from A549 cells after 24 h exposure to drugs, KGM polymer and KGM MPs and excipients as commercially obtained and spray-dried (SD) at higher concentration tested in MTT assay. The data represented are mean ± SEM (n = 3). Dotted line (100% LDH) represents the result of incubation with cell culture medium, used as control.

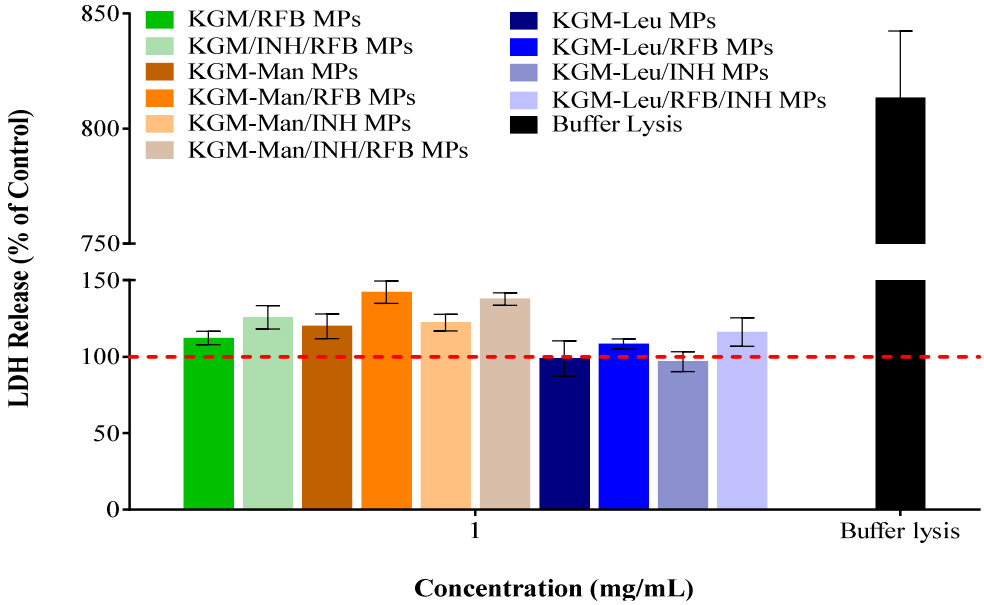

Figure 4.22. LDH release from A549 cells after 24 h exposure to MPs produced by spray-drying at higher concentration tested in MTT assay. The data represented are mean ± SEM (n = 3). Dotted line (100% LDH) represents the result of incubation with cell culture medium, used as control.

These results suggest that only RFB drug, KGM-Man/RFB and KGM-Man/INH/RFB MPs may cause cell membrane disruption. These MP formulations were also observed in the MTT assay as inducing a certain level of toxicity. However, in that assay other formulations exhibited a lower level of cell viability, which did not find parallel in the LDH release assay.

Taking all the cytotoxicity results as a whole, although cell viabilities determined in the MTT assay and the amounts of released LDH were not dramatic, there is in fact a certain degree of cytotoxicity for some formulations, with a particular focus on those associating RFB. This effect was mainly verified for the higher tested concentration of MPs (1 mg/mL), while the concentration of 0.5 mg/mL presented a much milder effect. Therefore, one of the approaches to circumvent the RFB effect relies on decreasing RFB loading in MPs thus moving the final concentration of contact towards that of 0.5 mg/mL. *In vitro* assays testing the response of macrophages infected with mycobacteria to these drug-loaded MPs would elucidate the possibilities of this approach.

5. Conclusions

The objective of this study was to develop spray-dried KGM MPs for an application in TB therapy. KGM was the basic matrix component, which comprised also RFB and/or INH, chosen as model antitubercular drugs, and eventually mannitol or leucine, used as adjuvant excipients.

KGM MPs produced in this work have shown important features regarding the envisaged application, particularly regarding the presented aerodynamic properties. The incorporation of mannitol and leucine aimed to improve the flow properties of dry powders produced by spray-drying. Although this objective has been met in some formulations, it was not verified in others. Furthermore, in certain cases the use of excipients inclusively affected negatively other relevant properties of MP formulations. In this regard, it was observed that the presence of excipients accelerated drug release *in vitro*, particularly that of RFB and the incorporation of mannitol decreased the ability of MPs to associate INH. The cytotoxic effect of formulations was also evaluated. While INH-loaded formulations did not generally induce any cytotoxicity, a mild cytotoxic effect was verified for formulations containing RFB, either alone or in combination with INH. Although this situation was only verified for the higher concentration tested, this may prevent the use of the formulations, at least at the concentration of RFB that was used (5% of total polymer, w/w). This suggests the need to reduce the amount of RFB present in the formulations.

As a whole, considering results of characterisation of MPs, the drug association and release pattern, and cytotoxicity, it is concluded that KGM MPs without the association of any excipient are those of election. Possibly, decreasing the amount of RFB loaded in the MPs is the strategy to reduce the observed cytotoxicity, although a parallel study to determine the effectiveness of the selected dose would be relevant to determine the potential of the approach.

6. Future Work of Interest

Unfortunately, it was impossible to complete the cytotoxicity assays in Calu-3 cells, as was initially designated. Therefore, that is a priority. However, in addition it would be important to perform the same assays (MTT and LDH) on macrophages. To this end, a human monocytic cell line that might be differentiated into macrophage-like cells is usually described.

Due to the toxicity promoted by RFB, it would be relevant to produce and test MPs having a lower concentration of this drug. Perhaps a reduction to 2% (w/w) would be adequate, with the expectation of reducing cytotoxicity.

It would also be interesting to evaluate whether KGM MPs have the capacity of being phagocytosed by macrophages. To this end, the polymer would have to be marked with a dye, such as fluorescein, and new MPs produced with fluorescently-labelled KGM. After incubation of fluorescent KGM MPs with macrophage-like cells, an analysis by cytometry would indicate the percentage of macrophages taking up MPs.

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Appendix A: Cytotoxicity assays in Calu-3 cells

INH was tested on a range of concentration, while RFB was tested only at the highest concentration (0.05 mg/mL) tested for A549 cells, representing the higher concentration present in MPs formulations. Similarly to what occurred in A549 cells, Figure A.1 evidences an absence of toxicity of INH. Although in this cell line the exposure to RFB for 24 h did not decrease cell viability beyond 70%, as occurred for A549 cells, the corresponding value of cell viability is still the lowest of those observed for the assessment of free drugs.

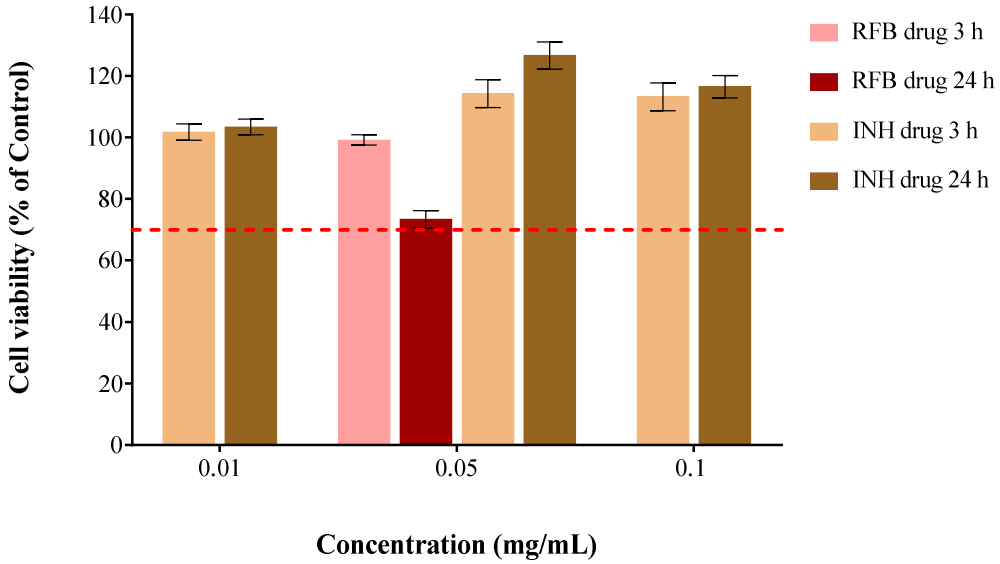

Figure A.1. Calu-3 cell viability as determined by the MTT assay upon 3 h and 24 h exposure to INH and RFB as free drug. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

Regarding KGM MPs without mannitol or leucine, the formulation of unloaded KGM MPs was not tested. In tested formulations, no toxicity was observed at 3 h, but 60% cell viability resulted from the 24 h exposure to 1 mg/mL KGM/RFB MPs (Figure A.2). A concentration- and time-dependent effect were clearly identified for this formulation ($p < 0.05$).

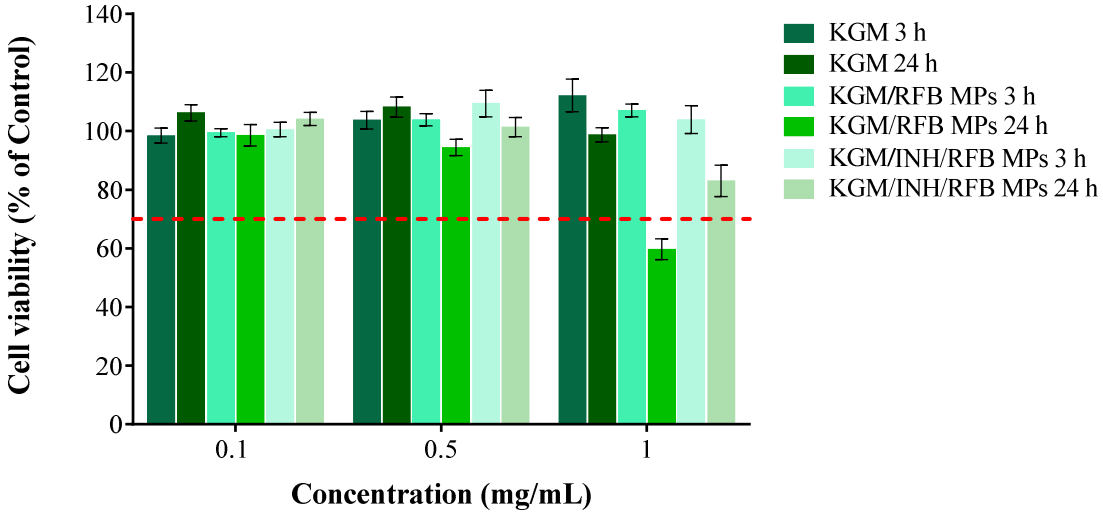

Figure A.2. Calu-3 cell viability as determined by the MTT assay upon 3 h and 24 h exposure to KGM MPs, KGM/RFB MPs and KGM/INH/RFB MPs. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

Neither of the mannitol formulations was studied in the Calu-3 cell line. On the contrary, some tests were carried out for leucine-containing formulations. MTT assays performed with A549 cells revealed that formulations containing leucine were those exhibiting the stronger cytotoxic effect. Despite KGM-Leu/INH/RFB MPs were not tested in Calu-3 cells, these cells did not show the same sensitivity to formulations with leucine, as none demonstrated a cytotoxic effect in the tested conditions. Only KGM-Leu/RFB formulation was close to 70%, with a significantly lower cell viability as compared to the lower concentration (0.1 mg/mL) and exposure time (3 h) ($p < 0.05$) (Figure A.3), but still above 70% cell viability.

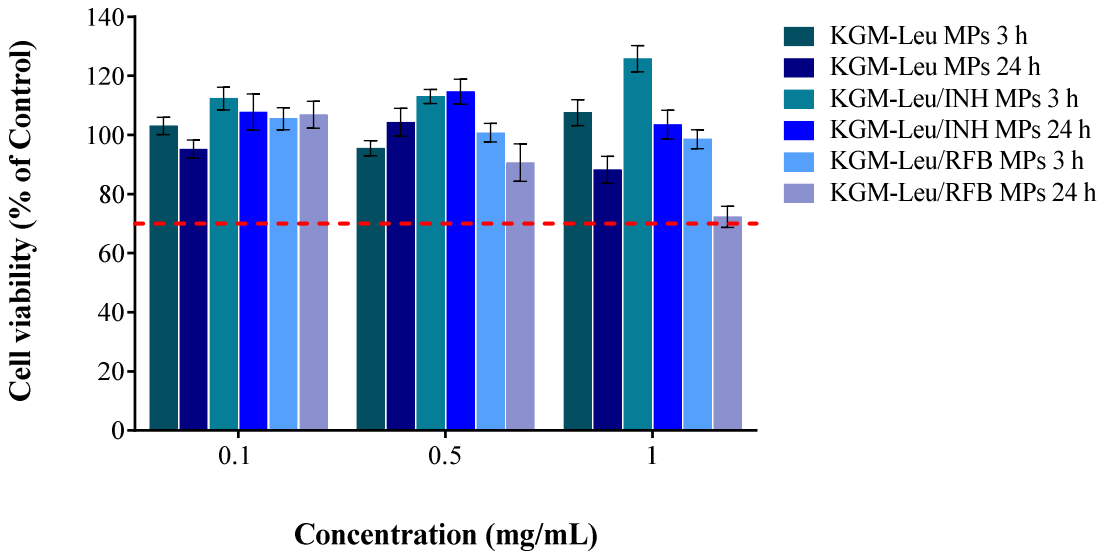

Figure A.3. Calu-3 cell viability as determined by the MTT assay upon 3 h and 24 h exposure to KGM-Leucine (Leu) MPs, KGM-Leu/INH MPs and KGM-Leu/RFB MPs. Data are represented as mean ± SEM (n = 3). Dotted line represents 70% cell viability.

The LDH release assay was performed in samples tested in the MTT at higher concentrations, as commented for A549 cells. The results obtained in Calu-3 cells are presented in Figure A.4. As can be seen, only KGM/RFB formulation had a significantly higher LDH release compared to the control (cell culture medium, $p < 0.05$), an effect that was not observed in A549 cells. As expected, drugs and formulations tested had a significantly lower LDH release compared to the lysis buffer ($p < 0.05$).

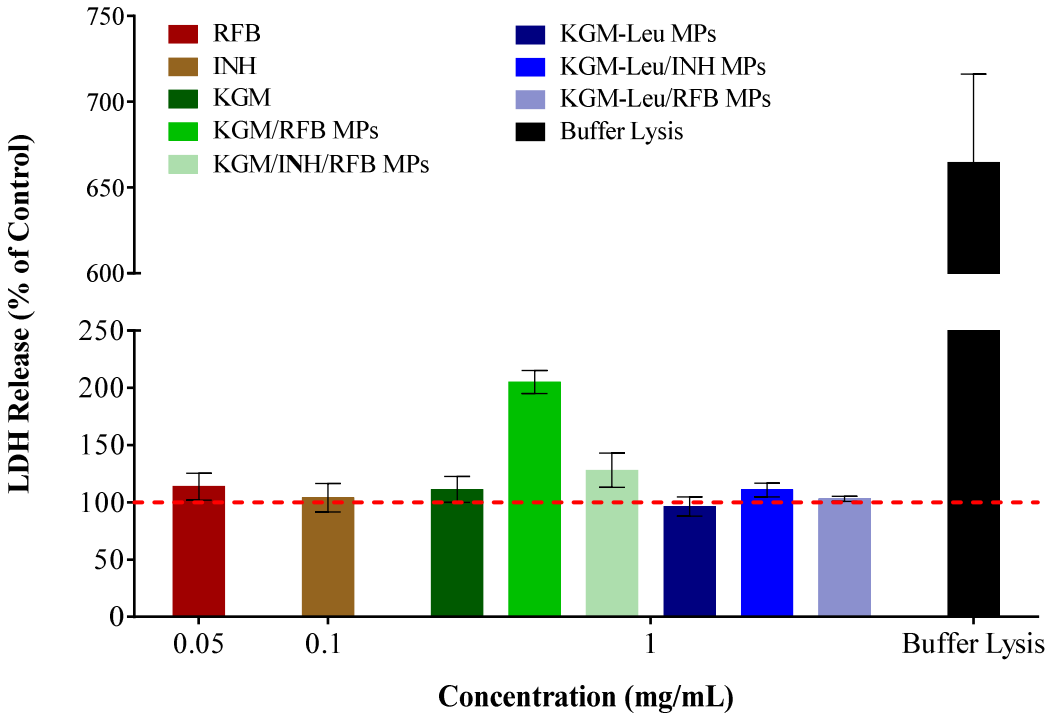

Figure A.4. LDH release from Calu-3 cells after 24 h exposure to drugs, KGM polymer and MPs produced by spray-drying at higher concentration tested in MTT assay. The data represented are mean ± SEM (n = 3). Dotted line (100% LDH) represents the result of incubation with cell culture medium, used as control.

Although the general message is that overt toxicity of formulations was not observed, perhaps with the exception of the LDH release result of KGM/RFB MPs, that deserves further investigation, it seems that a different sensitivity of the cells to the exposed samples was also a relevant factor.