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Dual antibiotherapy of tuberculosis mediated by inhalable locust bean gum microparticles

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Abstract

Despite the existence of effective oral therapy, tuberculosis remains a deadly pathology, namely because of bacterial resistance and incompliance with treatments. Establishing alternative therapeutic approaches is urgent and inhalable therapy has a great potential in this regard. As pathogenic bacteria are hosted by alveolar macrophages, the co-localisation of antitubercular drugs and pathogens is thus potentiated by this strategy. This work proposes inhalable therapy of pulmonary tuberculosis mediated by a single locust bean gum (LBG) formulation of microparticles associating both isoniazid and rifabutin, complying with requisites of the World Health Organisation of combined therapy. Microparticles were produced by spray-drying, at LBG/INH/RFB mass ratio of 10/1/0.5. The aerodynamic characterisation of microparticles revealed emitted doses of more than 90% and fine particle fraction of 38%, thus indicating the adequacy of the system to reach the respiratory lung area, thus partially the alveolar region. Cytotoxicity results indicate moderate toxicity (cell viability around 60%), with a concentration-dependent effect. Additionally, rat alveolar macrophages evidenced preferential capture of LBG microparticles, possibly due to chemical composition comprising mannose and galactose units that are specifically recognised by macrophage surface receptors.

Keywords: inhalation, locust bean gum, microparticles, polysaccharides, spray-drying, tuberculosis therapy

1. Introduction

Tuberculosis (TB) remains a leading cause of death, with particular incidence and prevalence in developing countries (WHO, 2016). Drug resistance is a major problem, but therapeutic incompliance is also a great limitation (McBryde et al., 2017). Albeit the commercial availability of several effective oral and parenteral drugs and the existence of international treatment guidelines (NICE, 2016; Wells et al., 2009), new therapeutic approaches are demanded not only to improve compliance but also to reduce the severe side effects associated with conventional therapy (Kaur et al., 2016; Lee et al., 2015). Inhalable therapy has great potential in this context, enabling the direct administration of drugs to the alveoli, where macrophages hosting pathogenic bacteria are located (Gupta et al., 2016; Parumasivam et al., 2016).

In a previous work, we proposed the use of spray-dried microparticles based on locust bean gum (LBG) as inhalable formulation for the treatment of pulmonary tuberculosis. The individual association of first-line antitubercular drugs was effective and a preliminary assay further suggested high affinity of LBG microparticles for macrophages (Alves et al., 2016), which we explained by specific recognition of the mannose and galactose residues of microparticles by macrophage surface receptors (Ahsan et al., 2002). LBG was proposed in that work for the first time for lung delivery applications. It is a galactomannan and its biodegradability has been ascribed to the presence of β-mannosidase in the lung (Alkhayat et al., 1998).

This work intends to produce a dry powder that delivers two antitubercular drugs upon inhalation while providing improved internalisation by macrophages, mediated by LBG. Furthermore, the respirability of LBG microparticles was

studied in order to determine their inhalability when used as carriers for antitubercular drugs. The microparticle aerosolisation profile was experimentally determined. The uptake by macrophages was assessed and compared with that of microparticles composed by a polymer devoid of specific moieties recognizable by macrophage receptors. Finally, combined therapy is proposed by the association of two first-line antitubercular drugs (isoniazid and rifabutin) in a single microparticle formulation, to meet the World Health Organisation (WHO) requirements of combined tuberculosis therapy (WHO, 2014).

2. Materials and Methods

2.1. Materials

Locust bean gum (LBG, $C_{30}H_{50}O_{26}$, M_w 860 *k*Da (Pollard et al., 2008)), poly (vinyl alcohol) (PVA, $[\text{-}CH_2CHOH\text{-}]_n$, M_w 89-98 g/mol), isoniazid (INH, $C_6H_7N_3O$, M_w 137.14 g/mol), Tween 80®, phosphate buffer saline (PBS) tablets pH 7.4, Dulbecco's modified Eagle's medium (DMEM), L-glutamine solution (200 mM), non-essential amino acids solution and penicillin/streptomycin (10000 units/mL, 10000 g/mL), trypsin-EDTA solution (2.5 g/L trypsin, 0.5 g/L EDTA), trypan blue solution (0.4%), phorbol 12-myristate 13-acetate (PMA, $C_{36}H_{56}O_8$), thiazolyl blue tetrazolium bromide (MTT), *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide hydrochloride (EDAC), Triton-X 100, sodium dodecyl sulfate (SDS), dimethylformamide (DMF), dimethyl sulfoxide (DMSO) and HCl were purchased from Sigma-Aldrich (Germany). Lactate dehydrogenase (LDH) kit was purchased from Takara Bio (Tokyo, Japan). Rifabutin (RFB, $C_{46}H_{62}N_4O_{11}$, M_w 847.00 g/mol) was supplied by Chemos (Germany) and fetal bovine serum (FBS) by Gibco (Life Technologies,

97 USA). RPMI 1640 and Ham's F12 media were obtained from Lonza Group AG
98 (Switzerland). Ultrapure water (MilliQ, Millipore, UK) was used throughout. All
99 other chemicals were reagent grade.
100
101 **2.2. Cell lines**
102 A549 cells (human alveolar epithelium) and NR8383 cells (rat alveolar
103 macrophages) were obtained from the American Type Culture Collection
104 (ATCC, USA) and used in passages 27-37 and 9-18, respectively. THP-1 cells
105 (human monocytes) were obtained from the Leibniz-Institut DSMZ (Germany)
106 and used in passages 10-20. Cell cultures were grown in humidified 5%
107 CO2/95% atmospheric air incubator at 37 ºC (HerAcell 150, Heraeus,
108 Germany). Cell culture medium (CCM) for A549 cells was DMEM
109 supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine solution, 1% (v/v)
110 non-essential amino acids solution and 1% (v/v) penicillin/streptomycin. For
111 NR8383 cells, CCM consisted of Ham's F12 supplemented with 15% (v/v) FBS,
112 1% (v/v) L-glutamine and 1% (v/v) penicillin/streptomycin, while THP-1 cells
113 were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v)
114 L-glutamine and 1% (v/v) penicillin/streptomycin.
115 THP-1 cells were grown in suspension and cell culture was maintained between
116 0.2 x 10^6 and 0.8 x 10^6 cells/mL. When reaching this higher concentration, cells
117 were sub cultivated in new passage at the concentration of 0.2 x 10^6 cells/mL.
118 Differentiation of THP-1 monocytes to provide the macrophage phenotype was
119 performed using PMA (0.2 x 10^6 cells/mL, 50 nM, 48 hours exposure), after
120 which the medium was replaced by fresh medium without PMA for 24 hours
121 before the experiments. NR8383 cells grow in mixed culture (half population

keeps adherent and half suspended). Adherent cells were those used to perform the assays described below and their harvesting was made by scraping.

2.3. Preparation of microparticles

LBG microparticles, without drug and containing an association of the antitubercular drugs INH and RFB, were prepared by spray-drying, according to a previously reported protocol (Alves et al., 2016). The preparation of unloaded LBG microparticles involved grinding LBG in a glass mortar for 10 min, after which 5 mL HCl 0.1 M were slowly added and grinding continued until complete mixture of powder and HCl solution was obtained. This was followed by the addition of purified water previously heated to 85 ºC, up to a final volume of 50 mL. The concentration of LBG in the final solution was 2% (w/v). The solution was maintained under magnetic stirring for 30 min and subsequently placed on a water bath at 85 ºC under slow stirring for additional 30 min. At the end, the solution was kept under stirring at room temperature overnight, until the moment of spray-drying.

For the production of drug-loaded microparticles, a solid dispersion of LBG and RFB was first prepared, by trituration in a mortar. After grinding, the same procedure used to prepare the LBG solution described above was applied. In parallel, INH was triturated in a mortar, being then solubilized with purified water under mild stirring for 10 minutes. The resulting solution was slowly added to the previously formed LBG/RFB solution immediately before spray-drying.

Microparticles were produced at LBG/INH/RFB mass ratio of 10/1/0.5 (final concentration of solids is 2.3%, w/v) using a laboratory mini spray dryer (Büchi

147 B-290, Büchi Labortechnik AG, Switzerland) operating in open mode and
148 equipped with a high-performance cyclone. Protection from light was ensured
149 for the whole process. The operating parameters were: inlet temperature: 160 ± 2
150 ºC; aspirator setting: 85%; feed rate: 0.7 ± 0.1 mL/min; and spray flow rate: 473
151 L/h. These conditions resulted in outlet temperature of 100 ± 2 ºC. After spray-
152 drying, microparticles were collected, placed in a dark flask and stored inside a
153 desiccator until further use.
154 The spray-drying yield was calculated by gravimetry, comparing the total
155 amount of solids initially added with the resultant weight of microspheres after
156 spray-drying (Grenha et al., 2005).
157 Fluorescent (unloaded) microparticles of LBG or PVA were also prepared, to be
158 used in the assay of macrophage capture. Covalent binding between fluorescein
159 and the polymeric molecules was performed before microparticle production. To
160 do so, LBG or PVA (1.0 g) were dissolved in HCl solution (10^{-4} M) at a
161 concentration of 1% (w/v). LBG solution was applied the same treatment
162 described above for the preparation of LBG microparticles. PVA solution was
163 maintained at 80 ºC under stirring overnight. Fluorescein (43 mg) was dissolved
164 in ethanol 96% (v/v) and added to the previously formed LBG or PVA solutions.
165 EDAC (ca. 33 mg) was dissolved in milli-Q water and added to the solution.
166 This was kept under stirring for 72 h and then dialysed (2000 Da M_w cut off)
167 against water. Light protection was ensured in the whole process. The resulting
168 suspension was frozen and freeze-dried (FreeZone Benchtop Freeze Dry System,
169 Labconco, USA). The fluorescently-labelled polymers were stored in a
170 desiccator until further use, under light protection. Fluorescent microparticles
171 were produced by spray-drying. The used conditions were the same described

above for LBG, while the following were used for PVA: inlet temperature: 155 ± 2 ºC; aspirator setting: 80%; feed rate: 1.0 ± 0.1 mL/min; and spray flow rate: 473 L/h. These conditions resulted in outlet temperature of 96 ± 2 ºC.

2.4. Characterisation of microparticles

2.4.1. Morphology

The surface morphology of LBG microparticles was characterised by field emission scanning electron microscopy (FESEM; 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.

2.4.2. Feret's diameter

Microparticle size was estimated as the Feret's diameter and was directly determined by optical microscopy (Microscope TR 500, VWR international, Belgium) from the manual measurement of 300 microparticles (n = 3).

2.4.3. Density

Real density (g/cm^3) was determined using a Helium Pycnometer (Micromeritics AccuPyc 1330, Germany) (n = 3). Tap density (g/cm^3) was determined using a tap density tester (Densipro 250410, Deyman, Spain), by measuring the volume of a known weight of powder before and after tapping, respectively (n = 3). The determination of tap density involved tapping the sample until no further reduction of powder volume was observed (average of 180 taps).

2.4.4. Drug association efficiency and loading

The determination of microparticle drug content was performed by UV-Vis spectrophotometry (Pharmaspec UV-1700, Shimadazu, Japan), at 265.5 nm for INH and 500 nm for RFB. A screening of the matrix material (LBG) revealed no interference at the selected wavelengths.

In order to determine the drug content, a certain amount of drug-loaded microparticles was incubated with HCl 0.1 M, under magnetic stirring for 60 min, which ensures complete dissolution of the carriers. Samples were then centrifuged (8000 rpm, 30 min; 5810 R, Eppendorf, Germany) and filtered (0.45 µm) before quantification. Calibration curves were performed at 265.5 nm for INH and at both this wavelength and at 500 nm for RFB, using the medium of dissolution of unloaded microparticles. The latter curve was used to determine the concentration of RFB and the former allowed the determination of the fraction of the absorbance at 265.5 nm that is due to RFB, the remainder being attributable to INH, thus allowing the determination of its concentration through the corresponding calibration curve. Drug association efficiency (AE) and microparticle (MP) loading capacity (LC) were estimated as follows (n = 3):

AE (%) = (Real amount of drug on MP / Theoretical amount of drug on MP) x 100 (Eq. 1)

LC (%) = (Real amount of drug on MP/ Weight of MP) x 100 (Eq. 2)

2.5. Aerodynamic characterisation of microparticles

HPMC size 3 capsules (Quali-V-I, Qualicaps, Spain) were filled with 22.5 mg of LBG/INH/RFB powder. The content of four capsules was discharged in each aerodynamic test using the medium resistance RS01® inhaler (Plastiape Spa,

Italy) and experiments were performed in triplicate. The device was connected to the Andersen cascade impactor (ACI, Copley Scientific, UK) operated at 60 L/min, ensuring a pressure drop of 4 kPa through the device. This was activated for 4 s in order to let 4 L of air passing through the system, thus complying with the standard procedure described by USP 38 and Ph.Eur.8 (Ph.Eur., 2014; USP, 2015).

ACI separates particles according to their aerodynamic diameter and it was assembled using the appropriate adaptor kit for the 60 L/min air flow test. Cut-offs of the stages from -1 to 6 are the following: 8.60, 6.50, 4.40, 3.20, 1.90, 1.20, 055, 0.26 μm. A glass fiber filter (Whatman, Italy) was placed right below stage 6 in order to collect particles with diameter lower than that of stage 6 cut-off.

The plates of the impactor were coated with a thin layer of ethanol containing 1% (w/v) Tween 20 to prevent particle bounce. The drugs were recovered from the apparatus with water/acetonitrile mixture (50/50, v/v) and quantified by HPLC (Agilent 1200 series, Germany). A LiChrospher® 100 RP-18 (5 μm) column of 4 mm i.d.×250 mm length with security guard cartridge was used. The eluent was a mixture of phosphate buffer pH = 7 (A) and acetonitrile (B) at a flow rate of 1 mL/min, starting with A/B = 95:5 and kept for 5 min, followed by a 7 min step gradient, until a 20:80 A/B ratio was reached, which was then kept for 16 min. Detection was performed by a diode array detector at 275 nm. The used chromatographic conditions were: gradient flow (Phosphate buffer pH = 7 (A), acetonitrile (B); A/B from 95:5 in the first 5 min it is changed to 20:80 in 7 min and kept in this 20:80 during other 16 min). A linear calibration plot for INH and RFB was obtained over the range 10-400 μg/mL (n= 3). Under these

conditions, the retention times of INH and RFB were 4.7 and 17.4 min, respectively.

The quantification of drug deposited inside the impactor allows calculating different aerodynamic parameters. The emitted dose (ED) is the amount of drug ex-device, considered the total amount of drug collected in the impactor, quantified by HPLC (induction port, stages -1 to 6 and filter). The mass median aerodynamic diameter (MMAD) was determined by plotting the cumulative percentage of mass less than the stated aerodynamic diameter on probability scale versus aerodynamic diameter on logarithmic scale. The fine particle dose (FPD) corresponds to the mass of drug particles with aerodynamic diameter lower than 5 µm calculated using the particle size distribution equation obtained from the ACI analysis. The fine particle fraction (FPF) is the ratio between the FPDs and the MD.

The drug recovery ranged between 77-91% in all the experiments, being thus coincident with the requisites of the pharmacopeia (Buttini et al., 2013).

2.6. Determination of drug release profile

The determination of drug release was performed in PBS pH 7.4 added of 1% (v/v) Tween 80®. The assay respected sink conditions, as the maximum amount of drug was always below 30% of its maximum solubility (EMA, 2014). INH solubility was considered 274 ± 4.79 mg/mL (Hiremath and Saha, 2008), while that of RFB was determined experimentally to be 0.496 mg/mL (Alves et al., 2016). A determined amount of microparticles (20 mg) was incubated with the medium (10 mL), at 37 °C, under mild shaking (100 rpm, orbital shaker OS 20, Biosan, Latvia). Samples (1 mL) were periodically collected and the amount of

each drug quantified as indicated above (n = 3). Direct quantification applied for RFB, while INH required dilution (1:10). A calibration curve was performed using the medium resulting from the incubation of unloaded LBG microparticles, after centrifugation (8000 rpm, 60 min) and filtration (0.45 μm).

2.7. *In Vitro* Biocompatibility Study

2.7.1. Cell viability evaluation by MTT test

The evaluation of cell viability upon exposure to LBG/INH/RFB microparticles was performed by the MTT assay, using two cell lines of high relevance within the scope of tuberculosis infection, A549 and macrophage-differentiated THP-1 cells. A549 cells were seeded at a density of 1 x 10^4 cells/well on 96-well plates (Orange Scientific, Belgium), in 100 μL of complete DMEM. Cells were incubated for 24 h at 37 ºC in 5% CO_2 atmosphere before use. THP-1 cells were differentiated with PMA to obtain the macrophage-phenotype before the experiments, according to the procedure described above, with the necessary adaptations. THP-1 cells were seeded on 96-well plates (0.035 x 10^6 cells/well) in 100 μL of RPMI supplemented with 50 nM of PMA and incubated for 48 h at 37 ºC in 5% CO_2 atmosphere. After that time, CCM was renewed for other 24 h, before the experiments.

Microparticles (unloaded or drug-loaded) were exposed in the form of a suspension prepared in pre-warmed CCM without FBS and evaluated at the concentrations of 0.1 and 0.5 mg/mL (includes polymers and drugs), for 3 and 24 h. INH and RFB were also tested as free drugs, at concentrations representing their loading in microparticles (0.01 and 0.05 mg/mL for INH; 0.005 and 0.025 mg/mL for RFB).

MTT solution (0.5 mg/mL in PBS, pH 7.4) was added after the exposure time (in A549 cells, samples were previously removed; in THP-1 cells no removal was applied) and incubated for 2 h, after which formazan crystals were solubilised with DMSO (A549 cells) or 10% SDS in a 1:1 mixture of DMF:water (THP-1 cells) and the absorbance measured by spectrophotometry (Infinite M200, Tecan, Austria). The viability of untreated cells was assumed to correspond to 100% of cell viability, and viability of treated cells was compared to this control. The assay was replicated at least three times, each with six replicates.

2.7.2. Determination of cell membrane integrity

The integrity of cell membrane after exposure to LBG microparticles was determined by the quantification of LDH released by cells. This was performed in A549 and macrophage-differentiated THP-1 cells upon 24 h exposure to a concentration of 0.5 mg/mL of unloaded or drug-loaded LBG microparticles. The chosen concentration corresponds to the maximum concentration tested in the MTT assay. Free INH and RFB were also tested as controls (0.05 mg/mL for INH; 0.025 mg/mL for RFB).

Cells were cultured in 96-well plates in the conditions described before for the MTT assay (the assays were performed simultaneously). Upon exposure, cell culture supernatants were collected, centrifuged (16 000 x g, 5 min, 4 ºC) and processed using a commercial kit. Absorbances were measured by spectrophotometry (Infinite M200, Tecan, Austria) at a wavelength of 490 nm (background correction at 690 nm).

A negative control of LDH release was performed incubating cells with CCM only and Triton-X 100 (10%) was used as positive control, being assumed as

100% LDH release. Released LDH upon incubation with each sample was determined by comparison with the positive control. All measurements were performed in triplicate.

2.8. Macrophage capture of LBG microparticles

The determination of macrophage ability to capture microparticles was determined *in vitro* in two cell lines, differentiated THP-1 cells and NR8383 cells. The latter were seeded (1.0×10^6 cells per well) in 6-well plates for adhered cells, with 2 mL of Ham's F12 medium. This procedure was performed 24 h before the test to ensure the adhesion of 50 to 75% of the original population. THP-1 cells (2.0×10^5 cells/mL) were suspended in RPMI medium supplemented with 50 nM PMA and seeded at 5 mL/well in 6-well plates for cells in suspension.

The evaluation of microparticle uptake by macrophages was performed by flow cytometry (FacScalibur cell analyser, BD Biosciences, Belgium) upon exposure to LBG and PVA microparticles (50 µg/cm^2), both fluorescently-labelled. PVA microparticles were used as control. Microparticles were aerosolised onto the macrophage layer using the Dry powder Insufflator™ (Model DP-4, Penn-Century™, USA) and 2 hours incubation at 37 ºC was allowed, without CCM (only a residual amount of medium was kept to ensure the hydration of cell surface). The phagocytic process was stopped by the addition of a cold solution of PBS.3% FBS (5 mL, two applications), which also provided washing. Cells were scraped and centrifuged (1500 rpm, 2 min, room temperature, centrifuge MPW – 223e, MedInstruments, Poland) in 2 mL of PBS.3% FBS. The cycle of resuspension in PBS.3% FBS and centrifugation was repeated thrice. At the end,

cells were re-suspended in 1 mL of PBS.3% FBS, transferred to cytometry tubes (BD Biosciences, Belgium) and maintained at 4 ºC until the analysis.

In flow cytometry, FSC-H and SSC-H channels were used, respectively, to measure size and granularity of cells, while side scatter light was used to identify cell viable population. The amount of cells exhibiting a fluorescent signal was considered to have phagocytosed microparticles. The assay for each dose was replicated at least three times.

2.9. Statistical analysis

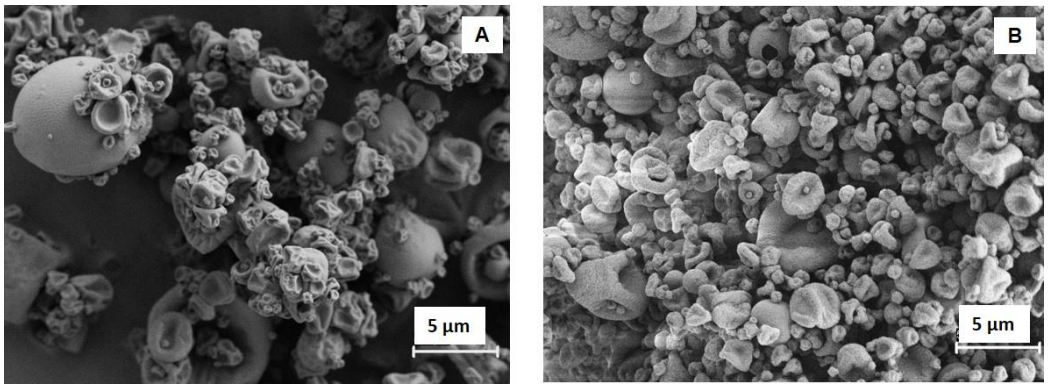
The student t-test and the one-way analysis of variance (ANOVA) with the pairwise multiple comparison procedures (Holm-Sidak method) were performed to compare two or multiple groups. For the analysis of results of in vitro release assay, a two-way ANOVA with Bonferroni's method for multiple comparison test was used. All analysis were run using the GraphPad Prism (version 6.07) and differences were considered to be significant at a level of $p < 0.05$.

3. Results and Discussion

3.1. Preparation and characterisation of LBG/INH/RFB microparticles

LBG microparticles loaded with a combination of the first-line antitubercular drugs INH and RFB were successfully obtained by spray-drying, with yields of 60-70%. The used concentration of LBG (2%, w/v) was chosen from previous experiments (unpublished data) in order to provide suitable microparticle size for the purpose of lung delivery, along with acceptable spray-drying yield. Theoretical drug loadings of 8.70% (INH) and 4.35% (RFB) were selected because they provide microparticles without the formation of aggregates, in

372 micron-size range, which are capable to deliver the two drugs in combination.
373 LGB was selected because of its potential to enhance macrophage uptake and the
374 amount of polymer was kept purposely high in order to favour internalisation.
375 The morphological observation of LBG-based microparticles, unloaded or drug-
376 loaded, revealed irregular shapes with convoluted surface (Figure 1), without
377 any evident effect resulting from drug association. The latter was actually
378 expected because loadings are relatively low. Similar observations on the
379 morphology and the absence of effect of drug association were reported in a
380 previous work with LBG microparticles associating either INH or RFB
381 separately (Alves et al., 2016).



382

383 **Figure 1.** Microphotographs of LBG-based microparticles viewed by scanning
384 electron microscopy: A) Unloaded LBG microparticles; B) LBG/INH/RFB
385 (10/1/0.5, w/w) microparticles. INH: isoniazid, LBG: locust bean gum, RFB:
386 rifabutin.

387

388 The determined Feret's diameters were rather low, 1.35 ± 0.7 µm for unloaded
389 LBG microparticles and 1.14 ± 0.51 µm for drug-loaded microparticles. Real
390 densities were around 1.4 g/cm^3 and tap densities varied between 0.2 and 0.37
391 g/cm^3. These values are in the same range of others reported for spray-dried

polysaccharide microparticles (Dalpiaz et al., 2015; Pai et al., 2015; Rassu et al., 2015).

The encapsulation of both antibiotics was very high, 94% for INH and 102% for RFB, as indicated in Table 1. The resulting loading capacities were, thus, close to theoretical maximum, being of 8.2% and 4.4% for INH and RFB, respectively. Spray-drying is a method usually reported to lead to high efficiency of drug association (Peltonen et al., 2010), as also corroborated in the present work. Moreover, the obtained results are in line with the high association efficiencies reported for each drug when associated individually (Alves et al., 2016).

Table 1. Drug association efficiency and loading capacity of LBG/INH/RFB (10/1/0.5, w/w) microparticles (mean ± SD, n = 3).

Drug	Association efficiency (%)	Loading capacity (%)
INH	94.4 ± 3.3	8.2 ± 0.3
RFB	102.1 ± 1.1	4.4 ± 0.1

INH: isoniazid; RFB: rifabutin

3.2. Aerodynamic behaviour of LBG/INH/RFB microparticles

Considering the intended application in lung delivery, the determination of aerosolisation properties stands as the most important aspect in the design of inhalable dry powders. The *in vitro* aerosol performance was determined using a RS01® dry powder inhaler and the aerodynamic properties determined upon assessment in the ACI are displayed in Table 2.

Table 2. Aerodynamic characteristics of LBG/INH/RFB (10/1/0.5, w/w) microparticles (mean ± SD, n = 3). Loaded powder amount = 22.5 mg, containing 7.8 mg INH and 3.9 mg RFB, respectively.

Drug	Emitted dose (%)	MMAD (μm)	GSD (μm)	FPD (mg)	FPF (%)
INH	92.6 ± 0.9	6.2 ± 0.6	2.4 ± 0.7	2.7 ± 0.6	38.0 ± 1.6
RFB	92.0 ± 0.5	5.8 ± 0.3	2.8 ± 0.2	1.3 ± 0.1	38.1 ± 1.8

FPD: fine particle dose; FPF: fine particle fraction; GSD: geometric standard deviation; INH: isoniazid; MMAD: mass median aerodynamic diameter; RFB: rifabutin

The dose emitted from the inhaler was very satisfactory, reaching 92%. This is indicative of the favourable properties of the material LBG to produce microparticles by spray-drying with good flowing capacity. However, a consistent amount of powder impacted on the high stages of the impactor, leading to MMAD value equal to 5.8 and 6.2 μm for RFB and INH, respectively. This is due to incomplete deaggregation of microparticle clusters during the product aerosolisation. It is well known that the aerodynamic performance of a dry powder inhaler (DPI) is strongly affected by both the device and the formulation characteristics. However, the spinning movement of the capsule inside the inhaler used in this study has demonstrated to be the most efficient in powder deaggregation in comparison with other capsule-based DPI (Martinelli et al., 2015). Hence, the optimisation of microparticles, with size, shape and density promoting their aerodynamic behavior will be addressed in the future, in order to increase the amount of LBG/INH/RFB fine particles capable of reaching the target site of alveoli. Nevertheless, LBG/INH/RFB microparticles showed a FPF of 38%, indicating that 38% of the microparticles have aerodynamic diameter below 5 μm, thus having the necessary conditions to reach the respiratory zone. This value is in agreement with those usually determined for

high doses antibiotic powder formulated without lactose as carrier (Belotti et al., 2015; Maretti et al., 2016).

Figure 2 shows the stage-by-stage deposition profiles of both drugs encapsulated in the tested microparticles. The similarity of the profiles indicates that the two drugs were equally co-deposited on the different stages. This supports the decision of developing a carrier with drug combination, as the microparticles demonstrate to have homogeneous composition, leading to a co-deposition of drugs.

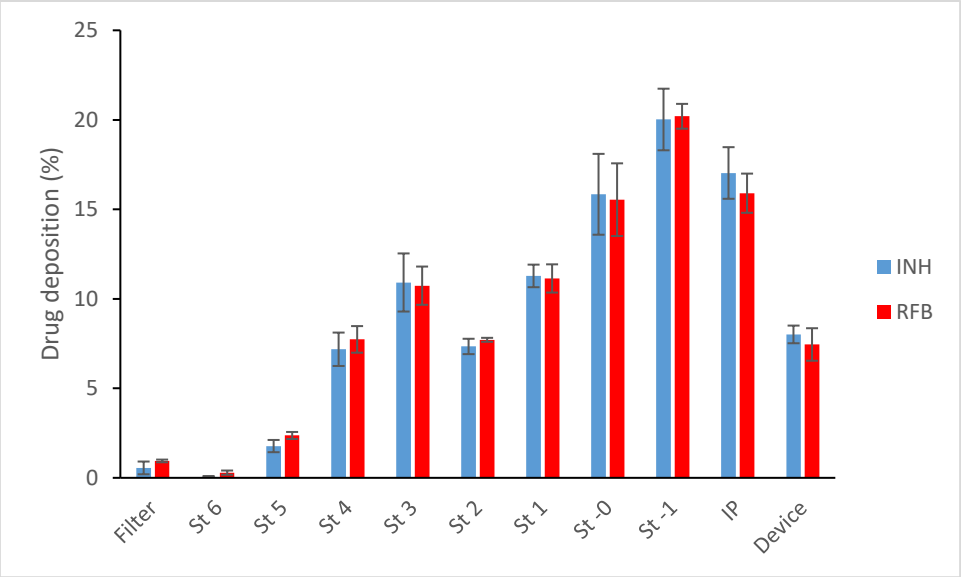


Figure 2. Stage-by-stage deposition profiles of isoniazid and rifabutin inside the Andersen cascade impactor after RS01 aerosolisation at 60 L/min, inhalation 4L (values are mean ±SD, n = 3).

3.3. *In vitro* drug release from LBG microparticles

Release studies were performed in PBS pH 7.4 added of 1% Tween 80®. In this way, the local pH of the alveolar zone is resembled, along with the content of surfactant (Kyle et al., 1990), and the dissolution of RFB is ensured (Alves et al., 2016). The release profile determined for each drug is depicted in Figure 3.

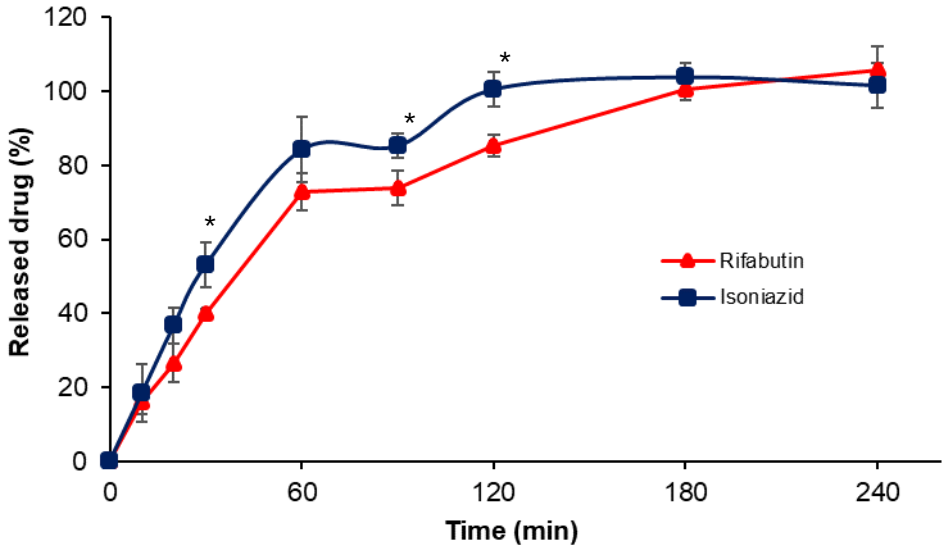


Figure 3. *In vitro* release of isoniazid (INH) and rifabutin (RFB) from LBG/INH/RFB (10/1/0.5, w/w) microparticles, in PBS pH 7.4 - 1% Tween 80® at 37 ºC (LBG: locust bean gum; mean ± SD, n = 3). *p < 0.05 comparing release of two drugs.

As can be observed, the release of the drugs is rapid, at 30 min 40% (RFB) – 50% (INH) of the antibiotics being already available. At 60 min, the values reach 73% for RFB and 84% for INH. Although the profile is very similar for both antibiotics, RFB release is somewhat slower than that of INH, with statistically significant differences at some time-points (30, 90 and 120 min, $p < 0.05$). The higher release of INH is a consequence of its higher solubility in aqueous media (O'Neil, 2006). Considering the conditions of the assay, the rapid release was expected, as LBG is a hydrophilic polymer and rapidly dissolved, releasing the associated drugs. Despite this observation, slower drug release is expected to occur *in vivo*, as has been reported (Bur et al., 2010; Haghi et al., 2014). When reaching the alveoli, microparticles will deposit on an epithelium

481 covered by the lung lining fluid (Fröhlich et al., 2016), which is estimated to
482 have 0.01 – 0.1 μm. In this manner, deposited particles will not be immersed,
483 only a small part being in direct contact with fluid instead and, therefore, erosion
484 and dissolution will initiate from underneath the microparticles (Bur et al., 2010;
485 Haghi et al., 2014). Ultimately, this will result in slower release and is also a
486 relevant observation towards the objective of having particle uptake by
487 macrophages before complete dissolution and drug release.
488
489 **3.4. *In vitro* cytotoxicity of LBG microparticles**
490 Two complementary cell viability assays were used to test the effect of
491 LBG/INH/RFB microparticles, the metabolic assay MTT and the LDH release
492 assay, which assesses cell membrane integrity. Considering the environment
493 underlying tuberculosis pathogenesis, alveolar epithelial cells (A549) and
494 macrophage-like cells (macrophage-differentiated THP-1 cells) were used.
495 Microparticle concentrations of 0.1 and 0.5 mg/mL were tested. These are
496 concentrations typically reported in the assessment of lung drug carriers, despite
497 being possibly overestimated if an alveolar area of 100 m^2 is considered
498 (Fröhlich et al., 2016). Unloaded microparticles and free drugs were tested as
499 controls.
500 Regarding the MTT assay, as can be observed in Figure 4A and 4B, the
501 exposure of A549 cells to drug-loaded microparticles induced very similar
502 results after 3 h or 24 h, evidencing an absence of time-dependent effect.
503 However, there is a clear concentration-dependent effect ($p < 0.05$), visible at
504 both time-points, as the resulting cell viability decreases from 72% to 57% (at 24
505 h) as the concentration of microparticles increases from 0.1 to 0.5 mg/mL.

Similar observations regarding the effect of time and concentration resulted from the incubation with unloaded LBG microparticles, although in that case cell viabilities remained well above the 70% considered the threshold beyond which a toxic effect is occurring (ISO, 2009).

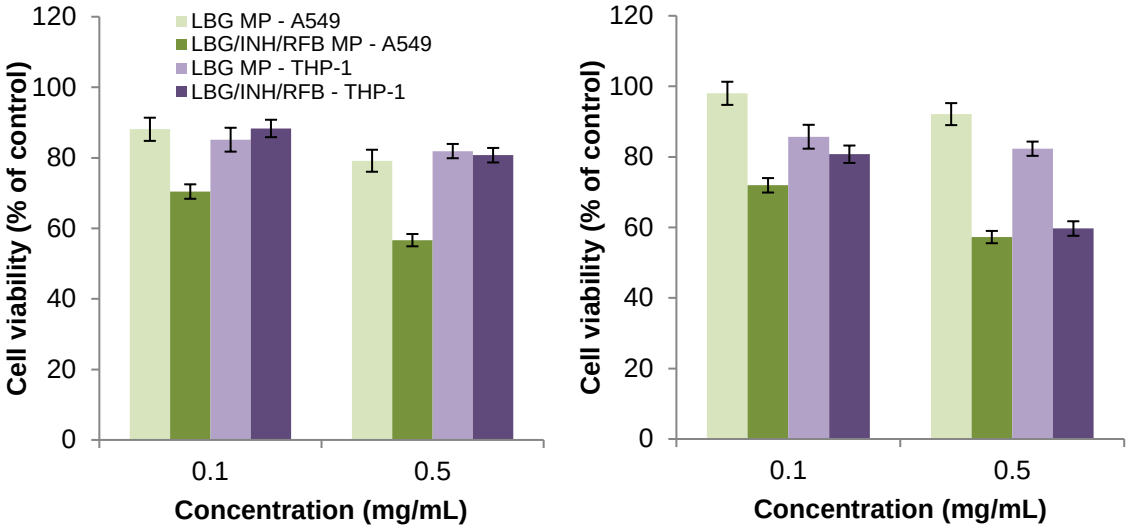


Figure 4. A549 and macrophage-like THP-1 cell viability after A) 3 h and B) 24 h exposure to unloaded LBG and LBG/INH/RFB (10/1/0.5, w/w) microparticles (MP); C) 24 h exposure to INH as free drug and D) 24 h exposure to RFB as free drug. Data represent mean ± SEM (n = 3, six replicates per experiment at each concentration). Dashed line represents 70% cell viability (INH: isoniazid; LBG: locust bean gum; MP: microparticles; RFB: rifabutin).

Macrophage-differentiated THP-1 cells showed a somewhat different behaviour, apparently being less sensitive to the contact with drug-loaded microparticles after short-time exposure (3 h, Figure 4A). Indeed, at that time-point, while A549 cells registered 57% cell viability for the concentration of 0.5 mg/mL, macrophage-like THP-1 cells remained at 81%. Nevertheless, the prolongation

of the exposure to 24 h (Figure 4B) decreased cell viability to 60%, which is similar to the 57% registered for A549 cells.

The results obtained on both cell lines are comparable to those reported for RFB-loaded LBG microparticles in a previous work and the opposite of those obtained for INH-loaded microparticles (Alves et al., 2016). This suggests that the negative effect of drug-loaded microparticles on cell viability is certainly due, at least in part, to the RFB content. The pH of microparticle suspension in cell culture medium that is incubated with cells is around 7.2, so that is not expected to have a negative contribution. As shown in Figure 4C, INH has no effect on cell viability in any of the tested conditions (concentrations, cell lines) at 24 h when exposed as free drug. An exposure of 3 h similarly generated viabilities around 90-100%, not only for INH, but also for RFB (data not shown). In turn, free RFB (Figure 4D) was observed to induce a decrease of A549 cell viability to 65% after 24 h exposure to the higher concentration, which correlates well with the results observed in the same cell line for drug-loaded microparticles, although in that case viability was even lower (57%). However, a different behaviour was observed in THP-1 cells, which viability remained at 85% upon 24 h exposure to the same concentration of free RFB. Several reports on the literature indicate higher susceptibility of A549 cells comparing with THP-1 cells (Lankoff et al., 2012; Singh et al., 2015). This is generally observed in our results, where THP-1 cells frequently show higher cell viability in the same testing conditions. The fact that THP-1 cells have 85% cell viability when exposed to 0.5 mg/mL RFB and then register 60% when exposed to the same amount of antibiotic encapsulated in LBG microparticles, is possibly attributed to the phagocytic capacity of these cells (Lankoff et al., 2012). This

549 characteristic certainly mediates a more intense contact of the cells with RFB,
550 leading to a reduction of cell viability (Lanone et al., 2009). As referred above
551 for A549 cells, unloaded LBG microparticles were also tested as control in
552 macrophage-differentiated THP-1 cells (Figure 4A and B), resulting in cell
553 viabilities above 80% in all cases. Overall, this is a general indication on the
554 absence of any deleterious effect of the polysaccharide on cell viability under the
555 tested conditions.
556 The amount of the cytoplasmic enzyme LDH released after 24 h contact with the
557 higher concentration of microparticles and free drugs was also determined
558 (Figure 5). The results essentially corroborate those of the MTT, with
559 LBG/INH/RFB microparticles having the more intense effect on released LDH,
560 which is similar in both cell lines. The incubation with CCM generated 21%
561 LDH release in A549 cells and 38% in macrophage-differentiated THP-1 cells.
562 The free drugs (INH and RFB) and LBG MP revealed an effect similar to that of
563 the CCM in each cell line, with no significant differences in released LDH. On
564 the contrary, LBG/INH/RFB microparticles induced significantly higher release
565 of LDH in both cell lines, 58% in A549 cells and 60% in THP-1 cells ($p < 0.05$).

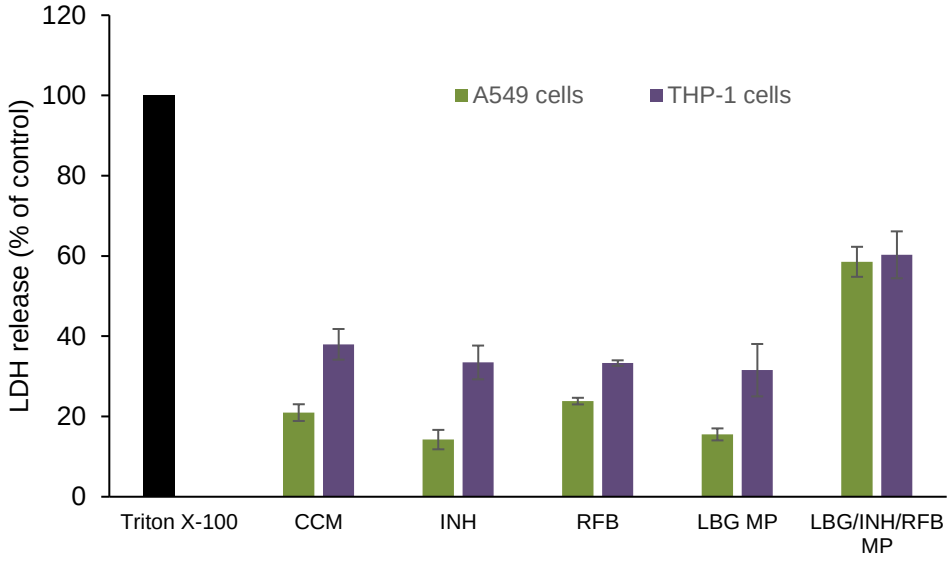


Figure 5. LDH released from A549 and macrophage-like THP-1 cells after 24 h exposure to 0.05 mg/mL isoniazid (INH), 0.025 mg/mL rifabutin (RFB), 0.5 mg/mL of unloaded LBG and LBG/INH/RFB (10/1/0.5, w/w) microparticles (MP). Cells incubated with cell culture medium (CCM) are the negative control and Triton-X 100 is the positive control. Data represent mean ± SEM (n = 3, six replicates per experiment at each concentration). *p < 0.05 compared to 10% Triton X-100

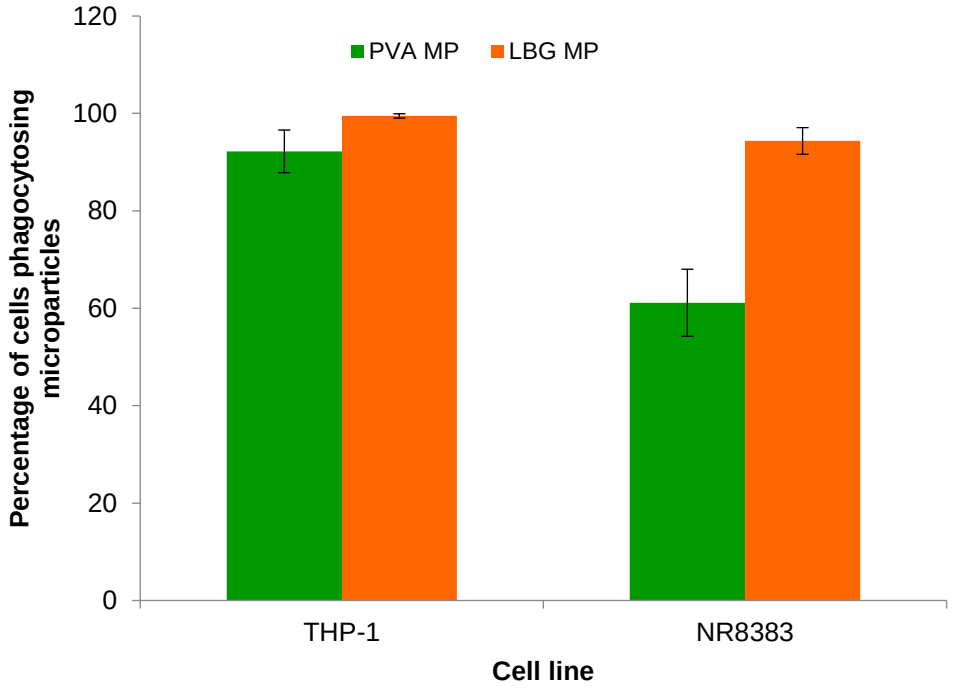
A comparison of results obtained from the MTT and LDH assays shows that the latter was more sensitive than the former. Drug-loaded LBG microparticles had stronger impact in released LDH (Figure 5) compared with the effect on mitochondrial dehydrogenase activity assessed in MTT assay (Figure 4B). Other studies report similar observations (Braz et al., 2017; Wang et al., 2009) and several justifications may apply. In fact, the two assays evaluate different aspects of the interaction between cells and particles. A possible explanation is that microparticles act as metabolic enhancers (Braz et al., 2017), thus accelerating MTT conversion into formazan in spite of the lower number of cells (as

584 indicated by the LDH assay), resulting in the overestimation of cell viability. It
585 may also happen that the interaction occurs mostly at the plasma membrane
586 level, causing cell lysis but without reaching intracellular mitochondria (Wang et
587 al., 2009). The latter is however possibly not applicable at least to THP-1 cells,
588 given the phagocytic capacity of these cells.
589 The overall observation of cell viability results indicates that the formulation still
590 needs some refinement to improve its toxicological profile. The results are no
591 longer severe as those exhibited with higher amount of rifabutin (Alves et al.,
592 2016), but there is still room for improvement, perhaps by working out a
593 solution that eliminates the use of HCl in the preparation of microparticles.
594 Additionally, the differences observed in the two assays reinforce the need to
595 diversify the range of tests used to obtain a more realistic view of the true impact
596 of the envisioned application.
597

598 **3.5. Uptake of LBG microparticles by macrophage-like cells**

599 Considering the aim of this work, it is very important to evaluate the ability of
600 alveolar macrophages to uptake the produced microparticles. A preliminary
601 study evidenced an uptake around 100% of LBG microparticles independently
602 of the tested dose (50 and 220 μg/cm^2) and used cells (Alves et al., 2016).
603 Considering this high affinity of LBG microparticles for macrophages, the
604 lowest dose (50 μg/cm^2) was selected to provide a comparison of behaviour
605 between LBG and PVA microparticles. The latter were used as control because
606 PVA is not reported to undergo specific recognition by macrophages. Moreover,
607 in order to avoid interference of microparticle size in the uptake, PVA

608 microparticles were tailored to have size similar to LBG microparticles (Feret's
609 diameter was calculated as 1.5 ± 1.0 μm).
610 As depicted in Figure 6, very high macrophage uptake (95-100%) was observed
611 for LBG microparticles in both cell lines. In turn, PVA microparticles induced
612 high uptake (92%) in macrophage differentiated THP-1 cells, but this value
613 decreased significantly to 60% ($p < 0.05$) in rat alveolar macrophages (NR 8383
614 cells). In that case, the uptake was significantly lower than that induced by LBG
615 microparticles ($p < 0.05$), showing a higher affinity of the cells for LBG.



616

617 **Figure 6.** Uptake of fluorescently-labelled locust bean gum (LBG) and
618 polyvinyl alcohol (PVA) microparticles by macrophage-differentiated THP-1
619 cells and NR8383 cells upon 2 h exposure to 50 μg microparticles/cm^2, at 37 ºC.
620 Results are expressed as mean ± SEM ($n \geq 3$).

621

622 Macrophages have a natural ability to uptake particulate matter (Pacheco et al.,
623 2013; Patel et al., 2015) and, thus, the uptake of a certain amount of particles

624 was expected in any case, independently of the particle composition. LBG is,
625 however, a galactomannan, being composed of mannose and galactose units.
626 These are reported to mediate favourable recognition by macrophage surface C-
627 type lectin receptors (Chavez-Santoscoy et al., 2012; Coombs et al., 2006; East
628 and Isacke, 2002).
629 NR8383 cells are reported to naturally express a functional mannose receptor in
630 culture (Vigerust et al., 2012). Therefore, the different response of these cells to
631 the two formulations of microparticles is possibly due to a higher affinity for
632 LBG, mediated by the specific receptor recognition of LBG residues. On the
633 contrary, THP-1 cells differentiated by PMA adopt an activation state of M0
634 which has been reported to not express the mannose receptor (Daigneault et al.,
635 2010). The inability to differentiate between both polymers is, therefore, the
636 possible reason for the similar capture of the two microparticle types.
637

638 **4. Conclusions**

639 In this work, LBG microparticles loaded with a combination of the first-line
640 antitubercular drugs isoniazid and rifabutin were proposed as inhalable carriers
641 for tuberculosis therapy. The co-encapsulation of the drugs in a single carrier
642 meets WHO requirements regarding combined tuberculosis therapy. Drug
643 release from microparticles was fast, but this is expected to be counterbalanced
644 by the reduced amount of fluid in the alveolar zone in *in vivo* conditions. The
645 experimental assessment of aerosolisation properties of LBG microparticles
646 demonstrated a favorable respirable dose lower than 5 µm, albeit the extrafine
647 dose potentially capable of reaching the target alveolar zone should be enhanced
648 in order to maximise macrophage uptake. A preferential ability of rat

649 macrophages to uptake LBG microparticles in comparison with a control was
650 observed *in vitro*, an effect attributed to the presence of mannose and galactose
651 units in LBG. The cytotoxic evaluation of these microparticles demonstrated
652 moderate decrease of cell viability to around 60%, indicating the need to
653 improve this aspect. Overall, the proposed strategy of dual antibiotherapy of
654 tuberculosis mediated by inhalable LBG microparticles is believed to be a
655 promising approach in the treatment of the disease.
656

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666

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