# Konjac glucomannan microparticles for antitubercular drug delivery by inhalation

**Filipa Guerreiro[1,2], Ana M Rosa da Costa[3], Maria Leonor Faleiro[1] & Ana Grenha[1,2]**

[1]CBMR – Centre for Biomedical Research, Drug Delivery Laboratory, University of Algarve, Campus de Gambelas, Faro, 8005-139, Portugal

[2]CCMAR – Centre of Marine Sciences, University of Algarve, Campus de Gambelas, Faro, 8005-139, Portugal

[3]Algarve Chemistry Research Centre (CIQA) and Department of Chemistry and Pharmacy, Faculty of Sciences and Technology, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

## Summary

Tuberculosis (TB) remains a deadly disease worldwide, although effective oral antibiotherapy is available. Frequent reports on systemic toxicity associated with the therapy, as well as emergence of multidrug resistant tuberculosis (MDR-TB) may partly justify the active state of the disease. As *Mycobacterium tuberculosis* (Mtb), the infectious agent, is transmitted *via* inhalation and primarily accumulates in the alveolar macrophages, pulmonary delivery of antitubercular drugs appears as a possible solution to circumvent some of the limitations. In this work, konjac glucomannan (KGM) is proposed as matrix material of spray-dried microparticles tailored to exhibit suitable properties to reach the alveoli. The presence of mannose units in the polymer and the geometric size of microparticles (Feret's diameter of 1.39 µm) are expected to favour macrophage uptake. Isoniazid (INH) and rifabutin (RFB), two first-line antitubercular drugs, were efficiently associated in combination to KGM microcarriers (association efficiencies of 89.8% and 67.1%, respectively). The biocompatibility of KGM and KGM-based microparticles on macrophage-like THP-1 cells was evaluated by the MTT and the lactate dehydrogenase (LDH) release assays. Neither drug-loaded microparticles nor KGM itself induced significant cytotoxicity. The obtained results are encouraging to further evaluate INH/RFB-loaded KGM microparticles for an application as delivery system in TB therapy.

## Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb). Although TB incidence has been diminishing, it remains one of the most lethal infectious diseases worldwide [1]. Upon inhalation, Mtb bacilli are hosted by alveolar macrophages located in the alveoli. The phagocytosis of Mtb triggers a series of events, with consequent control of the infection (latent TB), or infection progress to active TB (5 to 10% of cases), depending on the immunological capacity of the individual [2]. Following the World Health Organization directives [1], conventional treatment of TB consists in a combination of multiple drugs for long periods of time. In some cases, this therapy is reported to induce systemic toxicity and the emergence of multidrug-resistant TB (MDR-TB) [3]. Alternative therapeutic strategies are thus demanded and, considering the pathophysiological characteristics of the disease, delivering the drugs directly to the lung could be a suitable approach [4]. Konjac glucomannan (KGM) is a natural polymer composed by glucose and mannose units [5]. The mannose content makes KGM attractive to be used for macrophage targeting, an effect that could be mediated by macrophage surface lectin receptor [6]. Moreover, particles should have suitable properties regarding morphology and size to reach the alveolar region and promote phagocytosis by macrophages [7].

In this work, KGM-based microparticles were prepared by spray-drying, associating isoniazid (INH) and rifabutin (RFB) in combination. Mannitol and leucine were tested as excipients. The amount of drugs associated to KGM microparticles was very similar to that used in other studies of inhalable carriers proposed for tuberculosis therapy [8,9]. The obtained microparticles were characterised regarding morphology, size, density and ability to associate the drugs. The cytotoxicity of the systems was evaluated in human macrophage-differentiated cells (THP-1).

## Experimental methods

### Preparation of microparticles

KGM (Chemos, Germany) was submitted to a process of acid hydrolysis before spray-drying. The hydrolysis was performed based on the protocol proposed by Cheng et al. [10]. For the spray-drying process, KGM dispersions were prepared at 1.5% (w/v) in ultrapure water at 70 ºC, under mechanical stirring, resulting in final solids content of 1.7% (w/v). INH (Sigma-Aldrich, Germany) and RFB (Chemos, Germany) were added to the KGM dispersion individually. RFB was solubilised in 0.01 M HCl at 5%, w/v and added to the previously prepared KGM dispersion. The mixture was stirred overnight. INH was readily dissolved in water at 10%, w/v and then added to the KGM/RFB dispersion 1 h before spray-drying. Therefore, the amount of each drug was calculated to obtain microparticles with a final theoretical ratio of KGM/INH/RFB = 10/1/0.5 (w/w). The dispersions were spray-dried in a Buchi B-290 laboratory mini spray-dryer (Buchi Labortechnik AG, Switzerland) equipped with a high-performance cyclone.

Characterization of microparticles

The morphology of KGM-based microparticles was characterised 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.

The size of microparticles was determined as the Feret's diameter, using an optical microscope (VWR, Belgium). For this purpose, the powder was spread between a slide and a coverslip and observed under the microscope. The measurement of 300 microparticles was considered.

To determine the apparent (tap) density, a known amount of dry powder was placed in a 10 mL graduated cylinder. The cylinder was mechanically tapped (Densipro 250410; Deyman, Spain) until a constant volume was achieved. The tap density of the KGM-based microparticles was calculated dividing the weight of dry powder by the final volume of powder.

The real density of KGM/INH/RFB microparticles was measured by helium pycnometry (Accupyc 1330, Micromeritics Instrument Corporation, Norcross, GA; n=3).

Determination of INH and RFB association efficiency (AE) was performed by dissolving a known quantity of microparticles in 0.1 M HCl, under magnetic stirring, for approximately 1 h. Samples were centrifuged (5000 rpm, 20 min, Hettich Universal Centrifuge 320; Sigma-Aldrich, Germany), filtered (0.45 µm) and the drugs quantified by HPLC (Agilent 1100 series, Germany).

Evaluation of cytotoxicity

*Metabolic activity*

The effect of KGM and KGM-based microparticles on cell viability was evaluated on human macrophage-like cells (THP-1) by the MTT assay at 0.1, 0.5 and 1 mg/mL. THP-1 cells were differentiated to acquire the macrophage phenotype by incubation with phorbolmyristate acetate (50 nM) for 48 h. After this period, medium was changed and, 24 h later, it was replaced by the samples previously suspended in fresh cell culture medium (CCM). CCM was used as negative control and sodium dodecyl sulphate (SDS; 2%, w/v) as positive control of cell death. After 24 h of exposure, 30 µL of MTT solution (0.5 mg/mL in PBS, pH 7.4) were added and incubation was allowed for further 2 h. Generated crystals were solubilised with SDS (70 µL, 10%, w/v) and the absorbance measured by spectrophotometry at 570 nm, with background correction at 650 nm. Relative cell viability (%) was calculated as follows:

$$\text{Viability (\%)} = \left(\frac{\text{A} - \text{S}}{\text{CM} - \text{S}}\right) \text{x } 100$$

where A is the absorbance obtained for each of the concentrations of the test substance, S is the absorbance obtained for positive control and CM is the absorbance obtained for untreated cells (incubated with CCM). The latter reading was assumed to correspond to 100% cell viability.

*Lactate dehydrogenase (LDH) release*

LDH was quantified on the supernatant of macrophage-like cells incubated with the same formulations mentioned above for the MTT assay. CCM and Triton X-100 (10%, w/v) were used as negative and positive control, respectively. After 24 h exposure to the highest concentration (1 mg/mL), 100 µL of cell supernatant were collected, 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. The samples were analysed by spectrophotometry at 490 nm with background correction at 690 nm. The LDH release (%) was determined by the following equation:

$$\text{LDH release (\%)} = \left(\frac{\text{A}}{\text{LB}}\right) \text{x } 100$$

Where A represents the absorbance obtained for LDH released by the cells exposed to the test substances and LB is the absorbance obtained for treated cells with Triton X-100.

**Results and Discussion**

KGM was submitted to acid hydrolysis to reduce its viscosity and, thus, facilitate the spray-drying process. KGM-based microparticles were successfully produced with the following conditions: the spray flow rate at 473 L/h, aspirator at 90% and the feed flow of 0.74 mL/min. Inlet temperature ranged between 170 - 180 ºC and outlet temperature ranged between 103 – 105 ºC.

As depicted in Figure 1, KGM-based microparticles displayed an irregular, convoluted morphology. However, their surface is suggested to be smooth.

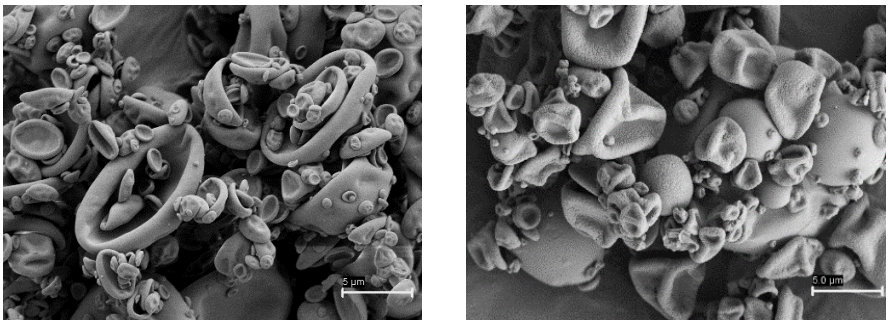

**Figure 1 - Representative microphotograph of unloaded KGM (left) and KGM/INH/RFB (right) microparticles obtained by field emission scanning electron microscopy (FESEM). Scale bar = 5 µm.**

Table 1 describes the characteristics of produced microparticles. Their size (expressed as the Feret´s diameter) was 1.77 µm for unloaded microparticles and 1.39 µm for KGM/INH/RFB microparticles, thus being much smaller than the ones reported in the literature (4.0 µm) [11]. This occurrence was probably due to the hydrolysis performed in the beginning of our work with the aim of reducing the viscosity of the polymer. KGM-based microparticles had a tap density between 0.17 and 0.22 g/cm$^3$. Real density was 1.52 g/cm$^3$ for KGM/INH/RFB microparticles (this value was not determined for unloaded KGM microparticles). The results obtained experimentally suggest the suitability of KGM/INH/RFB microparticles to achieve the alveoli after inhalation. However, an experimental validation using a cascade impactor is required to determine the aerodynamic diameter, which is being planned.

INH and RFB were successfully associated to KGM microparticles (Table 1). However, the AE was different for the two drugs, RFB showing lower AE (67%) than INH (90%). Although macroscopically RFB was fully solubilised in the spraying solution, its hydrophobic character is suggested to limit somehow the association, with RFB being lost during the spray drying process.

**Table 1 – Characterisation of KGM/INH/RFB microparticles (mean ± SD, n = 3).**

| | **Feret´s diameter (µm)** | **Tap density (g/cm$^3$)** | **Real density (g/cm$^3$)** | **AE (%)** |
|---|---|---|---|---|
| **Unloaded KGM** | 1.77 ± 1.00 | 0.17 ± 0.01 | n.d. | - |
| **KGM/INH/RFB** | 1.39 ± 0.79 | 0.22 ± 0.01 | 1.52 ± 0.01 | (INH) 89.8 ± 2.8<br>(RFB) 67.1 ± 7.1 |

AE: association efficiency; INH: isoniazid; n.d.: not determined; RFB: rifabutin

The evaluation of cytotoxicity generated by exposure to microparticles is a relevant indication in the application of drug carriers. According to ISO 10993 [12], a cytotoxic effect is considered to exist when cell viability decreases below 70%. As shown in Figure 2 (left), KGM-based microparticles do not generate significant toxicity after 24 h exposure to macrophage-like cells, either in presence or absence of drugs. In fact, cell viability remained above 70% in all cases. KGM, tested as control, revealed a similar effect. Results of the LDH release assay were consistent with those of the MTT, as drug-loaded microparticles generated similar release of the enzyme comparing with untreated cells (exposed to CCM). Moreover, all samples generated much lower level of released LDH comparing with the positive control (20-40% comparing with 100%) ($p < 0.05$).

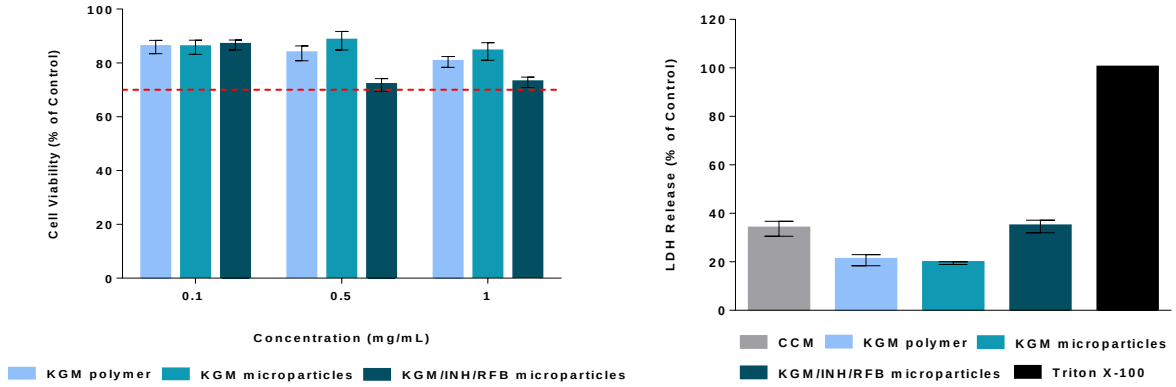


**Figure 2 - Cytotoxic evaluation.** Cell viability of macrophage-like THP-1 cells upon 24 h exposure to KGM and microparticles as determined by the MTT assay. Dotted line represents 70 % cell viability (left). LDH release from macrophage-like THP-1 cells upon 24 h exposure to 1 mg/mL KGM and microparticles (right). Data are represented as mean ± SEM (n = 3 x 6 replicates).

## Conclusion

KGM/INH/RFB microparticles were produced successfully by spray-drying, displaying theoretically suitable properties to reach the alveolar zone upon inhalation. Isoniazid and rifabutin were effectively associated to microparticles in combination. The developed formulation of microparticles showed to not generate significant cell toxicity. As a whole, these results are encouraging to further study KGM-based microparticles as inhalable antitubercular drug carriers in tuberculosis treatment.

## Acknowledgements

Funding from the Portuguese Foundation for Science and Technology (PTDC/DTP-FTO/0094/2012, UID/Multi/04326/2013, UID/BIM/04773/2013, Pest-OE/EQB/LA0023/2013, PEst-OE/QUI/UI4023/2014), and the PhD scholarship to Filipa Guerreiro (SFRH/BD/115628/2016) are acknowledged.

## References

1 World Health Organization Global Tuberculosis Report 2016; Geneva, Switzarland, 2016.
2 Knechel N A: *Tuberculosis: Pathophysiology, clinical features, and diagnosis.* Critical Care Nurse 2009, 29, 34–43.
3 Pham D-D, Fattal E, Tsapis N: *Pulmonary drug delivery systems for the treatment of tuberculosis.* International Journal of Pharmaceutics 2015, 478, 517–529.
4 Ahsan F, Rivas I P, Khan M A, Torres Suárez A I: *Targeting to macrophages: Role of physicochemical properties of particulate carriers - Liposomes and microspheres - On the phagocytosis by macrophages*. Journal of Controlled Release 2002, 79, 29–40.
5 Yao-ling L, Rong-hua D, Ni C, Juan P, Jie P: *Review of Konjac Glucomannan : Isolation , Structure , Chain Conformation and Bioactivities.* Journal Single Molecule Research 2013, 1, 7–14.
6 Azad A K, Rajaram M V S, Schlesinger L S: *Exploitation of the Macrophage Mannose Receptor (CD206) in Infectious Disease Diagnostics and Therapeutics.* Journal of Cytology & Molecular Biology 2014, 1, 1–9.
7 Hirota K, Terada H: *Endocytosis of particle formulations by macrophages and its application to clinical treatment*. In: B Ceresa (ed): *Molecular Regulation of Endocytosis pinocytosis.* InTech; pp. 413–428, 2012.
8 Rojanarat W, Changsan N, Tawithong E, Pinsuwan S, Chan H K, Srichana T: *Isoniazid proliposome powders for inhalation-preparation, characterization and cell culture studies.* International Journal of Molecular Sciences 2011, 12, 4414–4434.
9 Gaspar D P, Faria V, Gonçalves L M D, Taboada P, Remuñán-López C, Almeida A J: *Rifabutin-loaded solid lipid nanoparticles for inhaled antitubercular therapy: Physicochemical and in vitro studies.* International Journal of Pharmaceutics 2016, 497, 199–209.
10 Cheng L H, Nur Halawiah H, Lai B N, Yong H M, Ang S L: *Ultrasound mediated acid hydrolysis of konjac glucomannan.* International Food Research Journal 2010, 17, 1043–1050.
11 Osorio D T: *Desarrollo de micro-y nanopartículas de polisacáridos para la administración pulmonar y nasal de macromoléculas terapéuticas*, Universidade de Santiago de Compostela, 2007.
12 International Organization for Standardization ISO 10993-1 Biological Evaluation of Medical Devices—Part 5: Tests for in Vitro Cytotoxicity; Geneva, Switzerland, 2009.