Carrageenan from red algae: a tool in mediation of interaction with macrophages

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Abstract

Macrophages have unique surface receptors that might recognize preferentially several moieties present on the surface of infecting organisms, including in the bacterial cell wall. Benefiting from a similar composition regarding the referred moieties, polysaccharides might be good candidates to compose the matrix of drug carriers aimed at macrophage targeting, as they can use the same recognition pathways of the infecting organisms. Carrageenan (CRG), a polysaccharide extracted from red edible seaweed, is an interesting possibility for the approach of directly targeting alveolar macrophages, as its composition is reported to be recognized by several macrophage lectin receptors. Inhalable starch/CRG microparticles were successfully produced, effectively associating isoniazid (96%) and rifabutin (74%) simultaneously. Furthermore, the produced microparticles presented adequate aerodynamic properties for pulmonary delivery with potential to reach the respiratory zone, with a mass median aerodynamic diameter (MMAD) between 3.3–3.9 μm. It was further demonstrated that the antitubercular activity of the drugs remained unchanged after encapsulation. The formulation evidenced no cytotoxic effects on lung epithelial cells (A549), although mild toxicity was observed on macrophage-differentiated THP-1 cells for the drug loaded formulation. Starch/CRG microparticles also exhibited a propensity to be captured by macrophages in a dose-dependent manner, as well as an ability to activate the target cells. This work provides indications on the potential of the starch/CRG carriers to interact with macrophages, thus providing a platform for drug delivery in the context of macrophage intracellular diseases. Additionally, if tuberculosis is focused, these microparticles can be used as inhalable drug carriers.

Keywords: alveolar macrophages, carrageenan, lung deposition, macrophage activation

Introduction

Macrophages are the subset of cells responsible for the maintenance of tissue homeostasis and defense from infections. However, several bacterial strains have evolved to acquire specialized strategies to evade the

common elimination mechanisms of macrophages and survive inside these cells, leading to illness. Examples of this are *Listeria monocytogenes*, *Mycobaterium tuberculosis, Legionella pneumophila* and *Coxiella burnetti* [1]. From the listed pathogens, *M. tuberculosis* is the one raising more concerns and tuberculosis was thus chosen as model disease in this work. Tuberculosis remains a leading cause of death; therapeutic failure being mainly due to non-compliance with prolonged treatments, often associated with severe side-effects. New therapeutic strategies are demanded and, considering that the lung is the primary site of infection, direct lung delivery of antibiotics is an interesting and, possibly, effective approach. This requires, however, suitable carriers that reach the alveoli where Mycobacterium hosts (macrophages) reside, while promoting macrophage capture and the intracellular accumulation of drugs. The narrow list of works exploring the application of carriers for macrophage targeting in tuberculosis therapy mostly reports synthetic polymers. The proposal of carriers based on natural materials is scarce, despite the higher probability of biocompatibility and biodegradability. To improve macrophage internalization, some works report particle surface functionalization with targeting ligands recognized by macrophage lectin receptors. Several natural polymers present an important advantage in this respect, being composed of basic units undergoing direct recognition by macrophage lectin-type and seven-transmembrane receptors [1, 2]. In this regard, polysaccharides like carrageenan (CRG) are an interesting possibility for the approach of directly targeting alveolar macrophages, as the material is reported as macrophage activator [3]. CRG is extracted from red edible seaweed, being mainly used as thickener in food industry (EU approved additive - E407). The prevalent polysaccharides in carrageenan are designated as kappa-, iota-, and lambda-carrageenan. k-CRG, used in the present work, is composed of alternating α-1,3 and β-1,4 linked units of galactose-4-sulphate and anhydrogalactose units. Galactose moieties are reported to be recognized by several macrophage lectin receptors [4]. However, reports on the inflammatory properties of CRG [5] have raised concern, although it is apparently well accepted for an application in certain areas. Different pharmacopoeias (British, United States and European) include CRG as pharmaceutical excipient. Its use is reported in a variety of non-parenteral dosage forms, including suspensions, emulsions, gels, creams, lotions, eye drops, suppositories, tablets, and capsules [6]. Several publications report its potential application in drug delivery [7]. Antiviral and antibacterial properties have also been reported for this polysaccharide [8, 9], while some ongoing clinical trials report its ability for interaction with viruses [10, 11]. Despite all the reported applications, the fact that the lung is a privileged site for immunological responses, made us to deem adequate approaching CRG formulation using another material as diluent. The rationale of this approach was to keep an amount of CRG that could still provide a targeting benefit, while trying to reduce the possibility of inflammation. Starch was selected for the effect, as it is a low cost polymer, abundantly available, degradable and approved as an excipient in several pharmaceutical applications [12, 13].

This work thus reports spray-dried starch/CRG microparticles, which were tested regarding their ability to interact with macrophages, namely concerning the capacity for targeting and activating these cells. Additionally, assuming tuberculosis as model disease, the ability of microparticles to associate first line antitubercular drugs was assessed, as well as their potential for inhalation purposes.

Materials and methods

Materials

k-CRG was obtained from FMC Biopolymer (Norway). Starch (C*pharmGel 12012) was a kind gift from Cargill (USA). Dimethylformamide (DMF), dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle's medium (DMEM), HCl, isoniazid (INH), L-glutamine solution (200 mM), non-essential amino acids solution and penicillin/streptomycin (10000 units/mL, 10000 g/mL), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), phorbol 12-myristate 13-acetate (PMA), phosphate buffer saline (PBS) tablets pH 7.4, sodium dodecyl sulfate (SDS), trypan blue solution (0.4%), trypsin-EDTA solution (2.5 g/L trypsin, 0.5 g/L EDTA), Tween 80® and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Germany). APC-CD11b antihuman antibody was obtained from BioLegend (USA). Rifabutin (RFB) was supplied by Chemos (Germany) and fetal bovine serum (FBS) by Gibco (Life Technologies, USA). RPMI 1640 was obtained from Lonza Group AG (Switzerland). Lactate dehydrogenase (LDH) kit was supplied by Takara Bio (Tokyo, Japan). Middlebrook 7H9 (M7H9) and oleic acid, albumin, dextrose and catalase (OADC) supplement were purchased from Remel (Lenexa, KS, USA). Ultrapure water (MilliQ, Millipore, UK) was used throughout. All other chemicals were reagent grade.

Cell lines

A549 cells (human alveolar epithelium) were obtained from the American Type Culture Collection (ATCC, USA) and used in passages 27-37. THP-1 cells (human monocytes) were obtained from the Leibniz-Institut DSMZ (Germany) and used in passages 10-20. Cell cultures were grown in humidified 5% CO_2/95% atmospheric air incubator at 37 ºC (HerAcell 150, Heraeus, Germany). Cell culture medium (CCM) for A549 cells was DMEM supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine solution, 1% (v/v) non-essential amino acids solution and 1% (v/v) penicillin/streptomycin. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS, 1% (v/v) L-glutamine and 1% (v/v) penicillin/streptomycin. These cells were grown in suspension and cell culture was maintained between 2 x 10^5 and 8 x 10^5 cells/mL.

Bacterial strain

Mycobacterium bovis bacillus Calmette-Guérin (BCG) (DSMZ 43990) was provided, as a gift, by Maria Chasqueira, Centro de Estudos de Doenças Crónicas da Faculdade de Ciências Médicas da Universidade Nova de Lisboa (CEDOC/FCM-UNL). Mycobacteria were cultivated for 5 to 7 days in M7H9 broth, supplemented with 10% OADC and 0.05% Tween 80®. Mycobacteria were handled inside a laminar flow hood (Bio48 Faster, Italy), respecting safety requirements to prevent contamination. The stocks of mycobacteria were preserved and stored in a -80ºC ultra-low temperature freezer (U725 Innova New Brunswick Scientific, USA).

Preparation of microparticles

Starch/CRG microparticles, either blank or containing an association of the antitubercular drugs INH and RFB, were prepared by spray-drying, according to a previously reported protocol [14]. The total concentration of the polymers in the dispersion was 2% (w/v), of which 80% corresponded to starch and 20% to CRG. The dispersion of the polymers was placed in a water bath at 85 ºC under slow stirring for 30 min to enable solubilization of both materials. Drug-loaded microparticles were produced at

starch/CRG/INH/RFB mass ratio of 8/2/1/0.5 and 10 mL of HCl at 0.01M was used to enable the solubilization of RFB. The total polymer concentration was kept at 2% (w/v). Due to the gelling ability of CRG, the spraying solutions were maintained at 85 ºC during the spray-drying process. This was performed using a laboratory mini spray dryer (Büchi B-290, Büchi Labortechnik AG, Switzerland) operating in open mode and equipped with a high-performance cyclone. Protection from light was ensured during the whole process. The operating parameters were: inlet temperature: 170 ± 2 ºC; aspirator setting: 90%; feed rate: 0.7 ± 0.1 mL/min; and spray flow rate: 473 L/h. These conditions resulted in outlet temperature of 110 ± 2 ºC. After spray-drying, microparticles were collected, placed in a dark flask and stored inside a desiccator until further use.

The spray-drying yield was calculated by gravimetry, comparing the total amount of solids initially added with the resultant weight of microspheres after spray-drying [15].

Fluorescent (unloaded) microparticles of starch/CRG were further prepared, to be used in the assay of macrophage capture. This encompassed the fluorescent labelling of CRG with fluorescein, by covalently binding it to the polymer in the presence of EDAC, an activator of the fluorescein carboxyl group. To do so, 1 g of CRG was dissolved at 1% (w/v) in 10^{-4} M HCl. Fluorescein sodium salt (23.5 mg dissolved in 2 mL of 96% (v/v) ethanol) and EDAC (16.3 mg dissolved in 30 mL of 10^{-4} M HCl) were previously mixed and added to the former solution. The reaction mixture was kept under stirring in the dark for 72 h and afterwards dialyzed (2000 Da Mw cut-off) against distilled water, protected from light. The dialysate was frozen and freeze-dried (FreeZone Benchtop Freeze Dry System, Labconco, Kansas City, MO, USA). The fluorescently-labelled polymer was stored in a desiccator until further use, under light protection. Fluorescent microparticles were produced by spray-drying, as described above.

Characterization of microparticles

The morphology of microparticles was characterized by field emission scanning electron microscopy (FESEM; FESEM Ultra Plus, Zeiss, Germany), after sputter-coating (model Q150T S/E/ES, Quorum Technologies, UK) the samples with iridium (5 nm thick).

The particle size distribution of starch/CRG microparticles was determined by laser light scattering (SprayTec, Malvern, UK). To do so, approximately 15 mg of dry powder were dispersed in 15 mL of 2-propanol and sonicated for 5 min. Various parameters were calculated automatically in the Spraytec software (Malvern instruments, UK) for volume-based size distribution and the particle sizes below which 10%, 50% and 90% of the spray lies, were determined, being expressed as Dv(10), Dv(50) and Dv(90). From these values, span was calculated as follows:

$$\text{Span} = \frac{Dv(90) - Dv(10)}{Dv(50)}$$

The analyses were carried out in triplicate with an obscuration threshold of 10%.

Determination of drug association and release

In order to determine the drug content, starch/CRG/INH/RFB microparticles were completely dissolved in HCl 0.1 M (30 min magnetic stirring). Samples were then centrifuged (16000 g, 10 min; Heraeus Fresco 17 centrifuge, Thermo scientific, USA) and filtered (0.45 µm). Drug content was determined by HPLC (Agilent 1200 series, Germany) using an adaptation of previously reported methods [16]. A gradient of

acetonitrile in 20 mM phosphate buffer pH = 4 (A) and acetonitrile (B) at a flow rate of 1 mL/min eluted isoniazid and rifabutin sequentially in a single injection. A LiChrospher® 100 RP-18 (5 µm) column of 4 mm i.d.×250 mm length with security guard cartridge was used. The elution conditions were: A:B starting at 95:5 and kept for 5 min, changed afterwards to 20:80 in 3 min and kept at this ratio for an additional 6 min. Detection was performed by a diode array detector at 275 nm. INH was eluted at 3.8 min and RFB at 12.6 min. Linear calibration plots for both drugs were obtained over the range 10-400 µg/mL (n = 3).

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

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

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 [17]. A determined amount of microparticles (30 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).

The release profiles of both drugs were compared using the similarity factor (f_2), according to the following equation:

$$f2 = 50 \times \log\left([1 + (1/n)\sum_{t=1}^{n}(R - T)^2]^{-0.5} \times 100\right)$$

where R is the mean of the dissolved drug from the reference batch at time t, T is the mean of the drug dissolved from the test batch at time t, n is the number of time points. f_2 values between 50 - 100 represent less than 10% difference between released drugs at each time point (i.e. similarity) and values <50 are considered dissimilar [18].

Aerodynamic characterization of microparticles using an Andersen cascade impactor

HPMC size 3 capsules (Quali-V-I, Qualicaps, Spain) were filled with 30 mg of starch/CRG/INH/RFB microparticles. The content of three capsules was discharged in each aerodynamic test using the high 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) which operates 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 in USP 38 and Ph.Eur.8 [19, 20].

The 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. 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 as described above. The quantification of drug deposited inside the impactor allows calculating different aerodynamic parameters. Emitted dose (ED), mass median aerodynamic diameter (MMAD), fine particle dose (FPD) and fine particle fraction (FPF) were calculated as described elsewhere [21].

The recovery (%) is the percentage of MD versus the labelled dose. The recovery ranged between 77-91% in all the experiments, generally complying with the pharmacopeial requirements.

***In vitro* biocompatibility study**

The biocompatibility of microparticles and raw materials was evaluated in the context of a lung delivery application. A metabolic test (MTT assay) and the determination of released LDH were performed after exposure of two cell lines of high relevance within the scope of tuberculosis infection, A549 and macrophage-differentiated THP-1 (dTHP-1) cells, to the samples. 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 acquire the macrophage-phenotype. To do so, 3.5 x 10^4 THP-1 cells/well were seeded on 96-well plates 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.

For the MTT assay, starch/CRG and starch/CRG/INH/RFB microparticles were exposed in the form of a suspension prepared in pre-warmed CCM without FBS, as well as their individual components (starch, CRG, INH and RFB) and evaluated at the concentrations of 0.1, 0.5 and 1.0 mg/mL, for 24 h. 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. Formazan crystals were then solubilized 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.

The integrity of cell membrane after exposure to raw materials and microparticles was determined by the quantification of LDH released by the cells. This was performed in A549 and dTHP-1 cells upon 24 h exposure to a concentration of 1 mg/mL of starch/CRG and starch/CRG/INH/RFB microparticles and their individual components (starch, CRG, INH and RFB). The chosen concentration corresponds to the maximum concentration tested in the MTT assay.

In simultaneous assays with the MTT, cell supernatants were collected upon exposure to the samples, centrifuged (800 g, 5 min, 4 ºC, Heraeus Fresco 17 centrifuge, Thermo scientific, USA) 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 a positive control corresponded to the lysis solution (1% triton-X in cell CCM). Released LDH (%) upon incubation with each sample was determined by comparison with the 100% of the positive control. All measurements were performed in triplicate.

***In vitro* determination of antitubercular activity: Minimum inhibitory concentration**

The antitubercular activity of the developed drug-loaded microparticles was evaluated *in vitro* on strains of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). The Minimum Inhibitory Concentration (MIC) value of free and microencapsulated INH and RFB was determined by the microdilution method [22]. After

sterilization (UV light, 10 min) of starch/CRG/INH/RFB microparticles and free drugs, a solution of the dry powder was prepared at 1 mg/mL and appropriately diluted to meet the desired drug concentrations. In parallel, 1 mg/mL stock solution of INH was prepared by dissolving the drug in the M7H9 supplemented medium. RFB was previously solubilized in DMSO (1 mg/mL) and then diluted with M7H9 broth. Stock solutions were mixed at concentrations proportional to the drug mass ratio contained in the microparticles, ensuring that free and microencapsulated drugs were tested at the same concentrations. Two-fold dilutions of the antibiotics/formulations were performed to obtain final concentrations of RFB from 0.001 to 0.125 µg/mL and of INH from 0.008 to 1 µg/mL. A 1/10 dilution of a McFarland 1.0 turbidity standard suspension of *M. bovis* BCG was inoculated (20 µL of inoculum in 180 µL of medium or test solution) with a multichannel pipette, delivering approximately 10^4 CFU per well. The outside lane of wells (a frame-like) was filled with sterile distilled water to avoid the evaporation of microplate content. The plates were covered with the lid, sealed with parafilm, and incubated at 37 °C (Binder, USA) for 7 days. Afterwards, 30 µL of MTT sterile solution were added to each well, followed by 4 h of incubation at 37 °C. Then, 50 µL of DMSO were added into the wells to dissolve the tetrazolium blue crystals that was produced, which are proportional to the growth rate of mycobacteria. The absorbance was measured by spectrophotometry (Infinite M200, Tecan, Austria) at 540 nm. Assays were performed in triplicate. The MIC value was considered the lowest that inhibited mycobacteria growth by 95 – 100% [23].

Internalization of microparticles by macrophages

The determination of macrophage ability to capture microparticles was determined *in vitro* in dTHP-1 cells. THP-1 cells (3.5 x 10^5 cells/mL) were suspended in RPMI supplemented with 50 nM PMA to allow differentiation and seeded at 3 mL/well in an individual cell culture dish for cells in suspension (growth area of 8 cm^2).

The evaluation of microparticle uptake by macrophages was performed by flow cytometry (FacScalibur cell analyzer, BD Biosciences, Belgium) upon exposure to fluorescently-labelled starch/CRG microparticles (50 µg/cm^2 and 250 µg/cm^2). dTHP-1 cells were exposed to microparticles in the presence of a residual amount of CCM that was kept to ensure the hydration of cell surface. Microparticles were aerosolized using the Dry Powder Insufflator™ (Model DP-4, Penn-Century™, USA) and 2 h incubation at 37 ºC was allowed. The phagocytic process was stopped by the addition of a cold solution of PBS with 3% FBS (5 mL, two applications). 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 with 3% FBS and centrifugation was repeated thrice. As some microparticles may remain in the cell pellet, APC-CD11b antihuman antibody (BioLegend, USA) was incubated with the cells to ensure a double stain, eliminating the interference of non-phagocytosed microparticles in the final reading. The cell pellet (50 µL) was incubated with an antibody dilution of 1:100 in PBS.3%FBS for 1 h on ice. At the end, cells were washed 3 more times to remove the unbound antibody, 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 the flow cytometre, 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 number of cells exhibiting

a double fluorescent signal (APC from the antibody and FITC from fluorescent microparticles) was considered that phagocytosing microparticles. The assay was replicated at least three times for each dose.

Macrophage activation by microparticles

To evaluate the capacity of microparticles to activate macrophage-like cells, dTHP-1 cells (3.5 x 10^5 cells/well) were incubated with starch/CRG/INH/RFB microparticles, as well as with starch and CRG polymer, at 0.5 mg/mL in CCM. After 24 h of incubation, cell-free supernatants were collected and IL-8 and TNF-α quantified with ELISA kits according to the manufacturer protocol. The amount of each cytokine was expressed in pg/mL based on reference standard curves. Cytokines released from cells treated with LPS (100 μg/mL) and untreated cells were used as control. The absorbance of samples was determined at 450 nm and corrected for background absorbance at 540 nm.

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. All analysis was run using the GraphPad Prism (version 6.07) and differences were considered to be significant at a level of $p < 0.05$.

Results/Discussion

Preparation and characterization of starch/CRG microparticles

Assuming tuberculosis as model disease and considering that the lung is the primary infected organ, the developed drug carriers (starch/CRG microparticles) were designed as an inhalable platform aimed at targeting antitubercular drugs to the infection site. Unloaded starch/CRG microparticles were successfully produced by spray-drying with a yield of 58%, which increased significantly to 71% ($p < 0.05$) when the model drugs were associated to the formulation (Table 1). This difference in the yield may be explained by the addition of HCl in the drug-loaded formulation, which was visually observed to decrease the viscosity of the solution, as reported elsewhere [24], improving the spraying characteristics. A small amount of HCl was in fact required to dissolve RFB. A possible explanation for the decreased viscosity may be the occurrence of some depolymerization of CRG [25], although the amount of HCl involved in the experiment is possibly too low for that. On the other hand, CRG used in this work is a mixture of potassium and sodium salt forms. Potassium has been reported to lead to double-helix conformation associated with increased viscosity. It is possible that the addition of HCl in the drug-loaded formulation interfered in the total ionic concentration, consequently altering hydrogen bonds in the double-helix and decreasing the viscosity of the solution [26]. The influence of HCl in the viscosity of polymeric solutions was reported in a previous work of our group regarding locust bean gum, a very viscous polymer [14].

As depicted in Fig. 1, the morphological analysis of starch/CRG microparticles suggested an increase in the number of small particles upon the inclusion of INH and RFB in the formulation. In fact, this was demonstrated in the characterization of particle volume diameter, evidencing a decrease from 11.3 μm in the unloaded particles to 7.0 μm ($p < 0.05$) in drug-loaded microparticles (Table 1). The microparticles corresponding to both dry powders exhibited dimpled surface morphology.

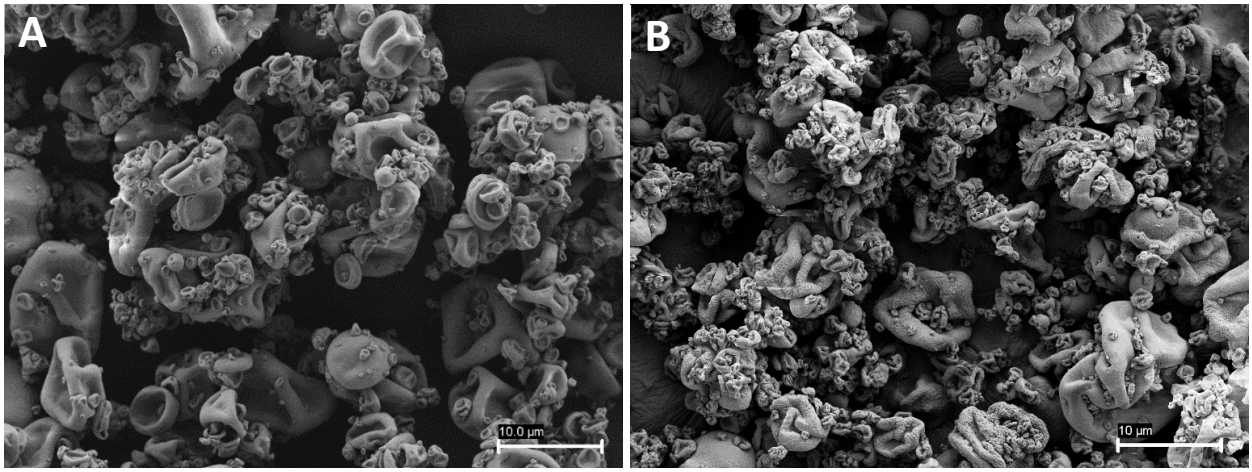

Fig. 1 - Microphotographs of unloaded starch/CRG microparticles (A) and starch/CRG/INH/RFB microparticles (B) as obtained by scanning electron microscopy. Scale bar is 10 µm.

Drugs were successfully associated to microparticles, with efficiencies of 96% for INH and 74% for RFB. The significantly lower value obtained for RFB ($p < 0.05$) is possibly a consequence of its low water solubility (125 mg/mL for INH and 0.19 mg/mL for RFB). Although a solution was previously prepared for spray-drying, when the spray is formed, water starts to evaporate and a phase separation may occur, depending on drug solubility [27]. In fact, after the atomization process, the walls of the drying chamber assumed a reddish hue, which indicates a certain deposition of RFB. The reported association efficiency corresponded to loading capacities of 8.4% for INH and 3.2% for RFB, which compare with the theoretical values of 8.6% for INH and 4.3% for RFB.

Table 1 – Spray-drying production yield, volume diameter - Dv50 and span, drug association efficiency and loading capacity of starch/CRG microparticles (mean ± SD, n = 3). Different letters represent significant differences in each parameter ($p < 0.05$).

Microparticles (Mass ratio)	Production Yield (%)	Dv50 (µm)	Span	Association Efficiency (%)	Loading Capacity (%)
Starch/CRG (80/20)	58.2 ± 5.0^{a}	11.3 ± 0.3^{c}	2.3 ± 0.5^{e}	n. a.	n. a.
Starch/CRG/INH/RFB (80/20/10/5)	71.0 ± 1.7^{b}	7.0 ± 0.7^{d}	2.5 ± 0.2^{e}	INH: 96.3 ± 3.4^{f}	INH: 8.4 ± 0.3^{h}
				RFB: 74.4 ± 11.9^{g}	RFB: 3.2 ± 0.5^{i}

CRG: carrageenan; INH: isoniazid; n.a.: not applicable; RFB: rifabutin

To evaluate the potential of starch/CRG microparticles for inhalation purposes, their aerodynamic properties were characterized using an Andersen cascade impactor (Fig. 2). Despite the differences observed in drug loading, the deposition profiles show an even distribution of the two drugs through the

stages, which indicates that the drugs were homogeneously distributed into the particles, without a size-dependent preferential allocation.

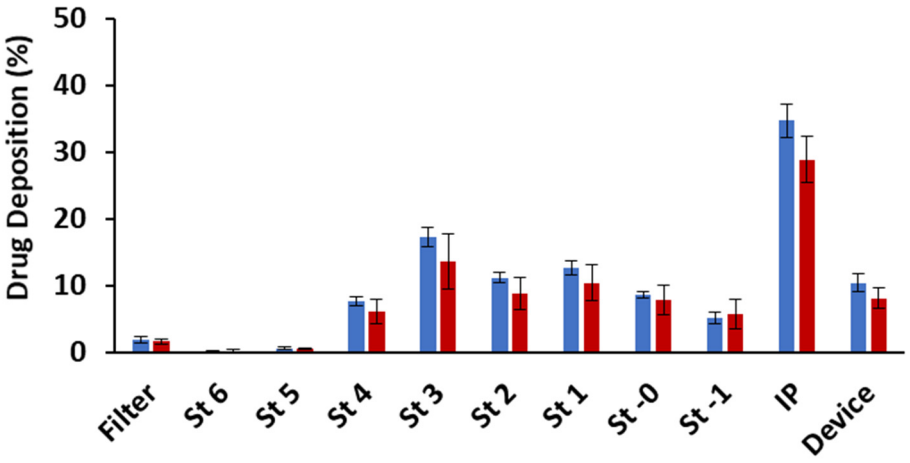

Fig. 2 – Stage-by-stage deposition profiles of isoniazid (INH, blue) and rifabutin (RFB, red) inside the Andersen cascade impactor after aerosolization with RS01 inhaler at 60 L/min, inhalation volume of 4L (values are mean ± SD, n = 3). IP: induction port; St: stage.

The mass deposition profiles allowed the calculation of other aerodynamic parameters, detailed in Table 2. As a consequence of the homogeneous distribution of both drugs, already referred above, no significant differences were found in the aerodynamic parameters when using INH or RFB as the reference. The calculated MMAD is within 3.3 – 3.9 μm and the determination of FPF indicated that 32% - 38% of microparticles have less than 5 μm, having suitable properties to reach the respiratory zone. The results certainly had a positive contribution from particle morphological features, while the values are in agreement with those usually determined for high dose antibiotic powders formulated without lactose as carrier [21, 28]. The emitted dose is also satisfactory, 91%. It should be taken in consideration that antibiotic therapy requires a consistent amount of delivered drug. In this respect, patient convenience and adherence to therapy could be improved by increasing the amount of powder to administer in a bigger reservoir device capable to control and release the powder in successive inhalation acts [29]. Nevertheless, an optimization of the formulation is envisaged to mitigate the high deposition in the induction port (almost 50%). This area represents the throat, which is difficult to overcome for particles of large size [30]. The high deposition observed in this zone is certainly a consequence of the exhibited Dv50 of 7.0 μm.

Table 2 - Aerodynamic properties of starch/CRG/INH/RFB (8/2/1/0.5, w/w) microparticles (mean ± SD, n = 3). Different letters represent significant differences in each parameter ($p < 0.05$).

Emitted dose (%)	Drug	MMAD (µm)	GSD (µm)	FPD (mg)	FPF < 5 µm (%)
91.0 ± 3.8	INH	3.9 ± 0.3[a]	2.2 ± 0.1[b]	2.3 ± 0.6[c]	32.1 ± 9.4[d]
	RFB	3.3 ± 0.4[a]	2.2 ± 0.1[b]	1.4 ± 0.3[c]	37.9 ± 8.0[d]

Release of INH and RFB from starch/CRG microparticles

The *in vitro* release of INH and RFB encapsulated in starch/CRG/INH/RFB microparticles was assessed in PBS pH 7.4 added of 1% (v/v) Tween 80®. The pH resembles that of the lung lining fluid [31] and the addition of Tween 80® intends to mimic the surfactant content [32]. The registered release profiles are depicted in Fig. 3. INH release was faster than that of RFB, reaching 100% in 60 min, while RFB took 240 min to reach the same value. Differences in the release profiles started to be statistically significant at 15 min, remaining as such until 180 min. This difference was confirmed by a calculation of the similarity factor, which reached 33 and, being lower than 50, indicates the existence of differences in the release patterns. These observations were expected in some way, because of the lower water solubility and higher molecular weight of RFB. Additionally, similar behaviors were reported for these drugs when other polymers with gelling ability, as CRG, were used as microparticle matrix [21, 33].

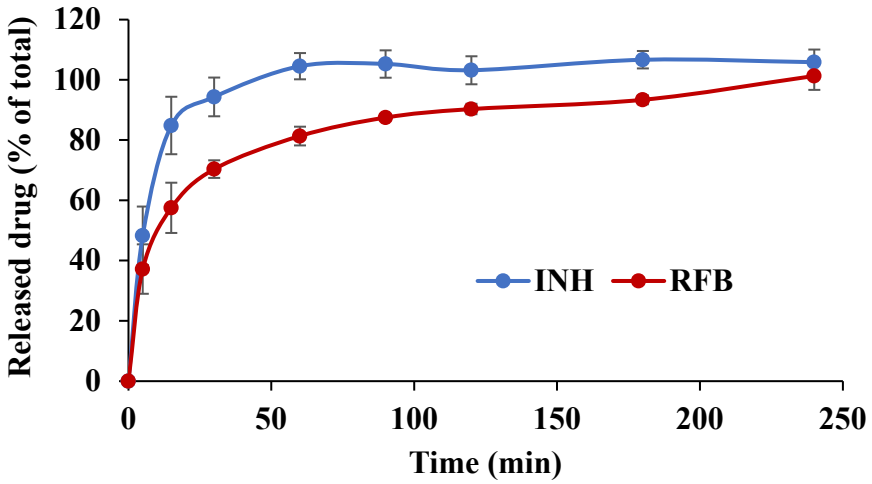

Fig. 3 - *In vitro* release profile of isoniazid (INH) and rifabutin (RFB) from starch/CRG/INH/RFB (8/2/1/0.5, w/w) microparticles in PBS pH 7.4-Tween 80®, at 37 °C (mean ± SD, n = 3). * $p < 0.05$ comparing INH and RFB at a given time point.

Overall, the release rate of RFB was slower than that of INH, although sustained release is not considered to occur, as 80% of the drug releases within 60 min. The solubility difference between both drugs may explain the faster release of INH. Nevertheless, a fitting to the Korsmeyer–Peppas equation [34] results, for RFB, in r^2 of 0.991 and a n value of 0.245, which may result from a polydisperse particle size distribution evident in Fig. 1. Different particle size distributions, geometries and aspect ratios may affect the diffusional exponent [35, 36]. The present study suggests that the rate of RFB diffusion (Fickian diffusion or quasi-Fickian diffusion) through the microparticle matrix is mostly governing the release.

Biocompatibility assessment

Considering the purpose of inhalation and the fact that the proposed materials have not been frequently explored for such an application, the biocompatibility of the developed carriers is a relevant matter. The evaluation of cell metabolic activity upon 24 h exposure to microparticles and their individual components was performed in two different cell lines using the MTT test. The chosen cells are relevant for the application, representing the alveolar epithelium (A549 cells) and macrophages (dTHP-1). Different concentrations were evaluated, between 0.1 and 1.0 mg/mL. For the effects of the discussion, a toxic effect was considered to exist when cell viability was below 70%, the threshold indicated by ISO 10993-5 [37]. The results obtained upon exposure to the highest tested concentration (1 mg/mL for microparticles and concentrations of free drugs corresponding to theoretical drug loadings) are depicted in Fig. 4A. Free RFB was the only tested sample reducing cell viability clearly beyond 70% in both cell lines, reaching values of 49%. Despite this observation, the toxic effect was overcome by drug microencapsulation, as starch/CRG/INH/RFB microparticles induced cell viability of 67% and 78%, in A549 and dTHP-1 cells, respectively ($p < 0.05$). The same trend of toxicity reversal by microencapsulation was observed in other works [21, 33, 38]. Lower concentrations showed absence of toxicity, with cell viabilities between 83% and 107% (data not shown).

The metabolic activity assay was complemented with the determination of the release of cytoplasmic enzyme LDH, upon 24 h exposure to the samples at a concentration corresponding to the highest tested in the MTT assay (Fig. 4B). Surprisingly, exposure to CRG led to the release of a significantly lower amount of LDH, when compared to CCM ($p < 0.05$). Free drugs (INH and RFB) affected LDH release from dTHP-1 cells, inducing a significant increase ($p < 0.05$) that was not perceived in A549 cells. Although this correlates with the MTT results in the case of RFB, the same was not observed for INH. Starch and starch/CRG microparticles did not induce alterations in LDH release, while drug-loaded microparticles led to increased release only in A549 cells. Nevertheless, the latter was a very slight alteration of less than half of that presented for the positive control.

Although the observed responses are not exactly coincident, the general indication is of low toxicity of the system. Nevertheless, the need to deepen the analysis with complementary assays is identified, in order to reach more robust conclusions.

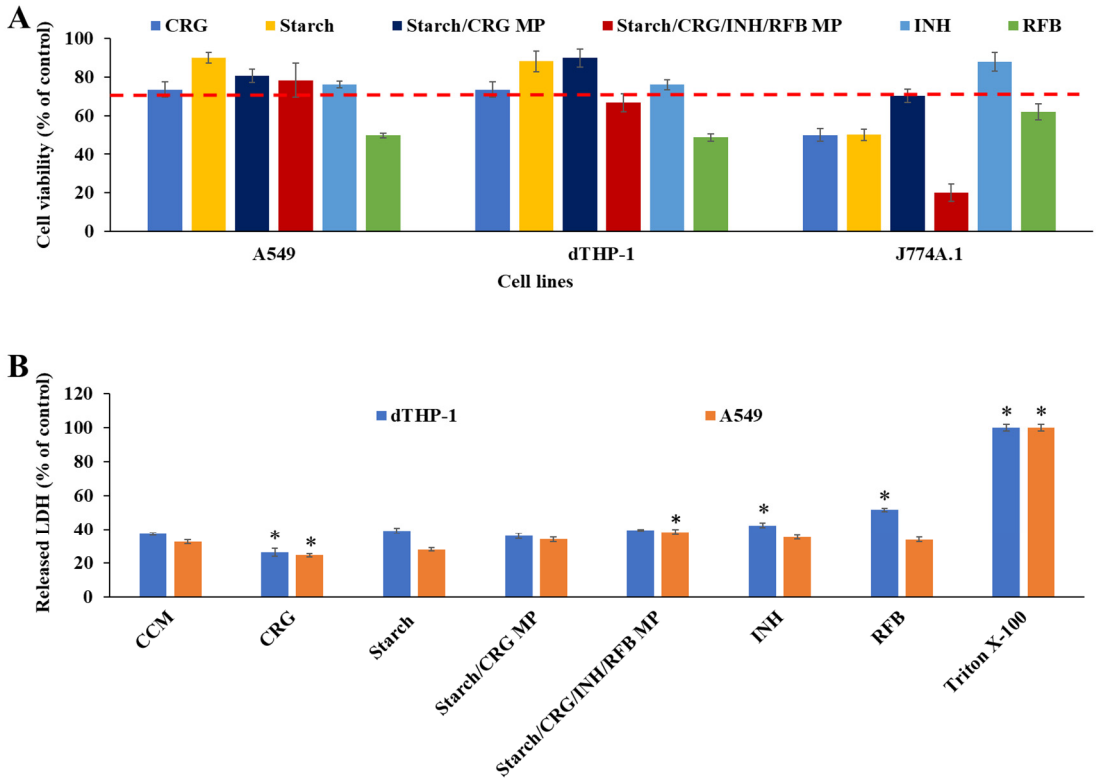

Fig. 4 A - Cell viability after 24 h incubation with 1 mg/mL of starch, CRG, starch/CRG unloaded and drug-loaded microparticles, 0.01 mg/mL of free INH and 0.05 mg/mL of free RFB. Dashed line indicates 70% cell viability. Data represent mean ± SEM (n = 3); **B** - LDH released by A549 and macrophage-differentiated THP-1 (dTHP-1) cells after 24 h exposure to CRG and starch as polymers, starch/CRG unloaded and drug-loaded microparticles, and free drugs. Triton X-100 was the positive control and cell culture medium (CCM) the negative control. Data represent mean ± SEM (n = 3). * $p < 0.05$ compared to respective CCM.

Effect of spray-drying on antitubercular activity of drugs

M. bovis BCG constitutes a live attenuated vaccine against tuberculosis, which is one of the most commonly administered vaccines worldwide, being also commonly used in experiments as a surrogate for virulent *M. tuberculosis* [39]. For this reason and because BCG is a slow growing strain with similarities to *M. tuberculosis*, it was selected to test the effect of spray-drying on the antibacterial activity of the drugs.

The MIC value of free and encapsulated drugs was determined by exposing *M. bovis* BCG to different concentrations of the drugs for 7 days. The MIC values obtained for the free drugs were 0.125 µg/mL for INH and 0.004 µg/mL for RFB, indicating higher sensitivity of *M. bovis* BCG to RFB than to INH. The literature reports variable values, depending on the bacterial strains and determination methods that are used, but the values obtained herein were similar to those reported in other studies [39, 40]. A 2/1 ratio of INH/RFB was tested in combination in the form of free drugs, respecting the ratio present in the microparticles. In that case, RFB ruled the inhibition effect and the same 0.004 µg/mL concentration (0.004 µg/mL RFB and 0.008 µg/mL INH) led to inhibition of the growth of *M. bovis* BCG. Drug-loaded

starch/CRG microparticles evidenced the same MIC value observed for the corresponding free RFB concentration and to the concentration of RFB in the mixture of INH/RFB. This indicates the spray-drying process did not affect the antimicrobial effect of the drugs, as reported in other similar works [38, 41, 42]. In summary, *in vitro* susceptibility of *M. bovis* BCG was observed towards drug-loaded starch/CRG microparticles with considerable growth inhibition.

Study of microparticle-macrophage interaction

Bearing in mind the application proposed in this work, where macrophages are the target cells of the prepared microparticulate systems, the evaluation of the potential interaction occurring between the carriers and the cells is of utmost importance. Different concentrations of fluorescently-labelled starch/CRG unloaded microparticles were aerosolized onto a monolayer of dTHP-1 cells and the capacity of microparticles to undergo macrophage uptake established. Unexposed cells (incubated with CCM only) were taken as negative control (0% of phagocytosis) and the increase in fluorescence was considered to correspond to cells that phagocytose microparticles.

Fig. 5 displays the obtained results, showing that there is an affinity of starch/CRG microparticles for macrophages, with the level of phagocytosis varying between 36% and 65%, in a dose-dependent process. In comparison, a similar dose of fucoidan microparticles was reported to reach a lower level of phagocytosis [38], while locust bean gum, a polymer with mannose moieties, reached practically 100% [14]. Nevertheless, the experimental conditions in these cases present some differences, making a direct comparison difficult. Moreover, it should be stressed that while in the formulations mentioned above, fucoidan and locust bean gum were the only excipients present in the tested microparticles, in the present study CRG composes only 20% of the carriers' matrix material. Galactose sulphate and anhydrogalactose units are believed to drive macrophage recognition, leading to phagocytosis. However, previous studies also reported the uptake of starch-based materials by macrophages [43], indicating the need to deepen this uptake study, perhaps using individual polymers as controls, in order to establish more rigorously the existing affinities. Additionally, using as control a material devoid of units potentially recognized by macrophages and complementing cytometry data with confocal microscopy images, would be beneficial in corroborating the data obtained so far. Finally, perhaps testing different amounts of CRG in the microparticles could provide a hint on the best option towards effective macrophage targeting.

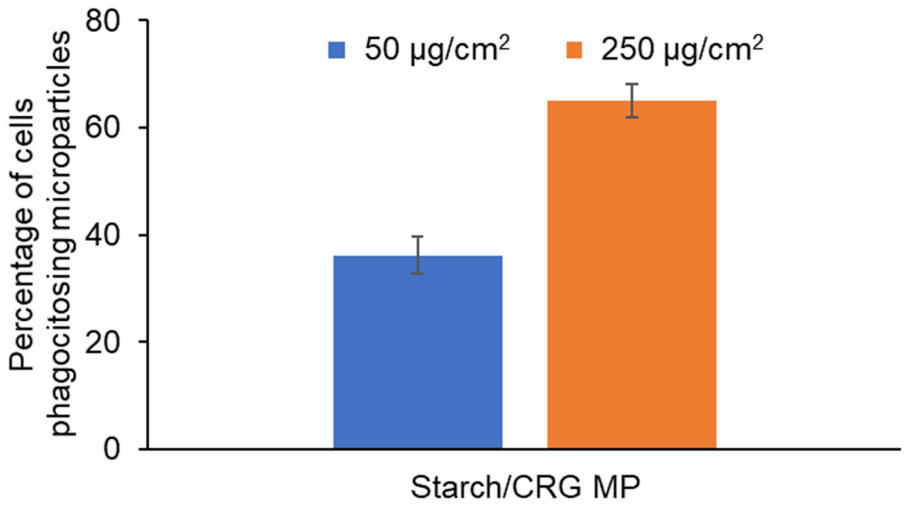

Fig. 5 – Percentage of dTHP-1 cells phagocytosing fluorescently-labelled unloaded starch/CRG microparticles after 2 h exposure, as obtained by flow cytometry. Data represent mean ± SEM (n = 3).

Alveolar macrophages are cells programed to process and remove inhaled particulate matter from the airways. After internalization of bacteria, such as *M. tuberculosis*, activation of macrophages normally occurs and the phagocytosis of other particulate matter has been reported to result in similar activation states [44-46]. It has been argued that this leads to increased ability for bacteria elimination, for instance, as the bactericidal activity of macrophages is reported to increase upon activation [47]. One of the hallmarks of this activation process is the release of pro-inflammatory cytokines. Two of the cytokines involved in the elimination of pathogens in airway diseases are TNF-α and IL-8 [48, 49]. These cytokines were also reported to be the most predominant in the supernatant of THP-1 macrophages [50]. Therefore, the secretion of TNF-α and IL-8 to cell supernatant was analyzed after 24 h exposure of dTHP-1 cells to the samples. Negative control consisted of cells incubated only with CCM and positive control comprised an exposure to LPS, which is known to induce activation of macrophage cells, thus resulting in cytokine expression. The obtained results are displayed in Fig. 6, showing different outcomes for the exposure of dTHP-1 cells to the samples and controls. Despite the concern about CRG inflammatory capacity, the polymer did not increase cytokine secretion in comparison with the negative control. On the contrary, the exposure to starch led to increased production of both cytokines ($p < 0.05$). This could be a consequence of the incomplete solubilization of starch in the incubation medium, which led to the presence of some insoluble granules in the micro size range that possibly underwent phagocytosis by dTHP-1 cells, inducing activation, despite the reported absence of inflammatory properties of starch [51]. When comparing starch/CRG/INH/RFB microparticles with starch, the level of released TNF-α was similar, but a statistically significant increase was observed for IL-8 ($p < 0.05$). Most of this induction is certainly caused by the presence of starch, but a possible effect from the drugs should also be ascertained in future tests. In this context, however, a study has reported that the particulate forms of drugs rather than the solutions, are those contributing to higher induction of cytokine secretion [52].

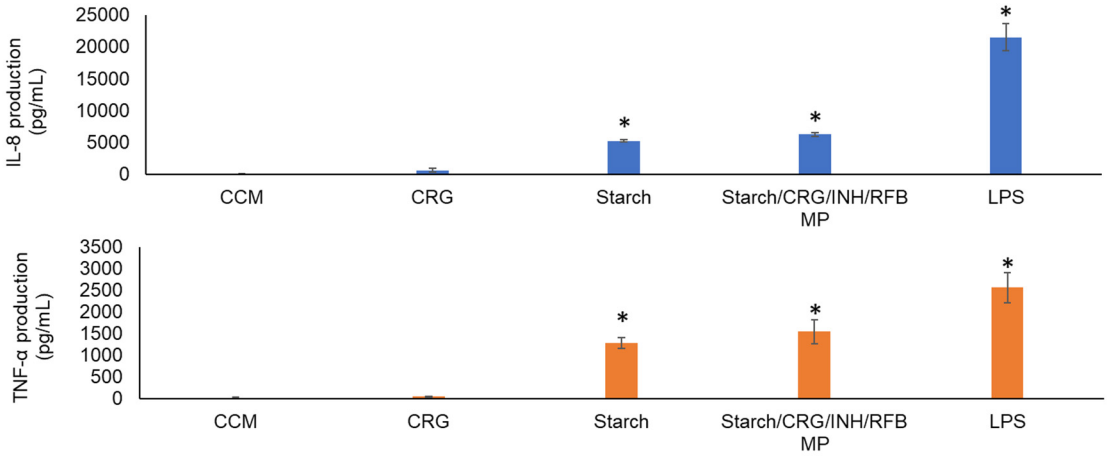

Fig. 6 – IL-8 and TNF-α secretion after 24 h incubation with CRG, starch and starch/CRG/INH/RFB (8/2/1/0.5, w/w) microparticles. Cell culture medium (CCM) was used as negative control and lipopolysaccharide (LPS) as positive control. Data represent mean ± SEM (n = 3). * $p < 0.05$ compared to CCM.

The performed assays demonstrate favorable interaction of starch/CRG microparticles with macrophages, indicating the potential of the microparticulate system in the context of macrophage intracellular disease therapy.

Conclusions

Starch/CRG/INH/RFB microparticles were successfully produced, efficiently associating the model drugs, which antibacterial effect was not affected by the microencapsulation process. The powder aerosolization performance was efficient, with emitted doses of 91% and MMAD of 3.3 – 3.9 μm, for RFB and INH indicating a co-deposition of drugs in the deep lung region. Low toxic effect was observed in alveolar epithelial cells and macrophages, but further testing is needed to reinforce the potential of the system, particularly if more prolonged treatment schedules are envisaged. Starch/CRG microparticles demonstrated some ability to interact with macrophages and induced moderate activation of these cells. As a whole, this work provides indications on the potential of the carriers to interact with macrophages, thus providing a platform for drug delivery in the context of macrophage intracellular diseases. Additionally, if tuberculosis is focused, these microparticles can be used as inhalable drug carriers.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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