

# Inhalable microparticles containing large payload of anti-tuberculosis drugs

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## ABSTRACT

Microparticles containing large payloads of two anti-tuberculosis (TB) drugs were prepared and evaluated for suitability as a dry powder inhalation targeting alveolar macrophages. A solution containing one part each of isoniazid and rifabutin, plus two parts poly(lactic acid) (L-PLA) was spray-dried. Drug content and in vitro release were assayed by HPLC, and DSC was used to elucidate release behaviour. Particle size was measured by laser scattering and aerosol characteristics by cascade impaction using a Lovelace impactor. Microparticles were administered to mice using an in-house inhalation apparatus or by intra-tracheal instillation. Drugs in solution were administered orally and by intra-cardiac injection. Flow cytometry and HPLC were used to investigate the specificity and magnitude of targeting macrophages. Microparticles having drug content -50% (w/w), particle size -5  $\mu\text{m}$  and satisfactory aerosol characteristics (median mass aerodynamic diameter, MMAD = 3.57  $\mu\text{m}$ ; geometric standard deviation, GSD = 1.41; fine particle fraction, FPF <4.6  $\mu\text{m}$ , = 78.91:1: 8.4%) were obtained in yields of >60%. About 70% of the payload was released in vitro in 10 days. Microparticles targeted macrophages and not epithelial cells on inhalation. Drug concentrations in macrophages were -20 times higher when microparticles were inhaled rather than drug solutions administered. Microparticles were thus deemed suitable for enhanced targeted drug delivery to lung macrophages.

## Keywords:

Dry powder inhalation Respirable microspheres Targeting Macrophages Pulmonary delivery Tuberculosis

## 1. Introduction

TB bacilli reside and proliferate within lung macrophages, the very cells that have evolved to engulf and destroy microorganisms that reach the surface of the lungs along with inhaled air. Several researchers, including Hickey and colleagues (O'Hara and Hickey, 2000; Suarez et al., 2001a,b,c), Khuller's group (Pandey and Khuller, 2005a,b; Zahoor et al., 2005), Terada and colleagues (Hino et al., 2005; Hirota et al., 2007; Makino

et al., 2004; Yoshida et al., 2006) and the present authors (Sen et al., 2003; Sharma et al., 2007, 2001) have proposed the use of inhalable or respirable particulate delivery systems for chemotherapy of TB. This proposal is premised upon the uptake of inhaled particles by lung macrophages, where TB bacteria find sanctuary. It is well established that inhaled particulate or vesicular delivery systems enhance the efficacy of anti-TB drugs (Pandey and Khuller, 2005b; Sen et al., 2003; Suarez et al., 2001c), presumably by targeting

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macrophages and building up high intracellular drug concentrations (Makino et al., 2004; Sharma et al., 2001).

Microparticles reported for the purpose of inhalation therapy of TB usually contain a single drug such as rifampicin (Hino et al., 2005; Hirota et al., 2007; Makino et al., 2004; O'Hara and Hickey, 2000; Pandey and Khuller, 2005b; Suarez et al., 2001a,b,c; Yoshida et al., 2006) or capreomycin (Giovagnoli et al., 2007). Microparticles described in the reports cited above typically contain payloads ranging from 1 to 30% by weight. Larger proportions of the incorporated agent often show extremely rapid diffusion through the polymer matrix, such that, for instance, maximal *in vitro* drug release is observed within the first 24 h (Makino et al., 2004). These authors also reported that high molecular weight poly(lactic-co-glycolic acid) was more efficient in retarding release of rifampicin as compared to grades of lower molecular weight. We have been using L-PLA of high molecular weight to prepare inhalable microparticles since our first report (Sharma et al., 2001). Lower molecular weights of L-PLA, and/or copolymers of L-PLA and glycolic acid, and even blends of various grades of lactide-glycolide polymers were not able to achieve incorporation of equally high payloads in the polymer matrix, and attempts to increase drug content beyond 50% (w/w) in spray-drying processes resulted in particles of erratic morphology, as also reported by other investigators (Fu et al., 2001; O'Hara and Hickey, 2000). Apart from the choice of polymer, the presence of two drugs in microparticles reported here is another feature of interest. Since monotherapy presents a risk of development of drug resistant TB (Faustini et al., 2006), it was considered important to include at least two drugs in the microparticles as before (Sharma et al., 2001). Rifabutin was preferred over rifampicin primarily because the latter exhibited chemical incompatibility with isoniazid in organic solutions (Mariappan et al., 2004; Sharma et al., 2001). Rifabutin also has a longer half-life, lower effective concentration and equal propensity towards induction of drug resistance as compared to rifampicin (Weiner et al., 2005). Data generated by our industry collaborator, Lupin Laboratories Ltd. indicated that effective doses of isoniazid and rifabutin in humans were approximately equal. The ratio of rifabutin to isoniazid was therefore kept at 1:1, as compared to our previous reports on rifampicin-isoniazid microparticles (Sharma et al., 2001, 2007). Isoniazid and rifabutin also differ in solubility behaviour. As per the Merck Index, the aqueous solubility of rifabutin is 0.19 mg/ml, while that of isoniazid is 125 mg/ml. A solvent composition and order of mixing was therefore developed such that a true solution containing all three components could be spray-dried to produce microparticles of desired composition.

It was also considered important to establish whether a large drug payload was compatible with the ability of microparticles to deliver high drug levels to the cytosol of target cells. Drug release *in vitro*, uptake of large payload microparticles by alveolar macrophages, and intracellular drug concentrations resulting *in vivo* after inhalation or intratracheal instillation of microparticles, or injection or oral administration of free drugs to mice were therefore investigated. Thus, the primary objective of the present investigation was to establish whether high-payload, inhalable microparticles may be prepared by spray-drying, and whether such

microparticles would retain their payloads for sufficient time to allow phagocytosis by alveolar macrophages. Second, it was sought to be established whether inhalation of these microparticles would target alveolar macrophages and deliver large intracellular concentrations of the incorporated drugs to the cytosol.

## 2. Materials and methods

### 2.1. Materials

Isoniazid and rifabutin of the Indian Pharmacopoeia were gifted by Lupin Research Park, Pune, India. Biodegradable poly(L-lactic acid) of apparent viscosity about 1 cP (L-PLA) purchased from Birmingham Polymers, USA was gifted by Dr. A.K. Panda, National Institute of Immunology, New Delhi, India. The number-average molecular weight of this polymer was about 100 kDa and the polydispersity index 0.91 as reported by the manufacturer. Fluorescein isothiocyanate and flow cytometry reagents were purchased from Sigma-Aldrich, Bangalore, India. Ascorbic acid, processing solvents of L.R. grade and HPLC solvents were obtained from Merck, India.

### 2.2. Spray drying

A solution containing 0.83% (w/v) of rifabutin and 1.67% (w/v) L-PLA was prepared in dichloromethane at room temperature ( $\geq 28^\circ\text{C}$ ) by mixing using a magnetic stirrer, for 15 min. A 5.35% (w/v) solution of isoniazid in methanol was prepared by warming to  $37^\circ\text{C}$ . Just before spraying, a true solution comprising the drugs and polymer in the methanol-dichloromethane solvent system was obtained by mixing not more than 16% (v/v) of the methanol solution with the dichloromethane solution to avoid polymer precipitation. This was done slowly with continuous stirring.

Spray drying was performed using a Buchi mini-spray dryer (Model 190 Buchi Laboratoriums Technik AG, Flawil, Germany). The homogeneous solution was atomized using a two-fluid pressure nozzle (0.7 mm diameter). Compressed air flow was maintained at  $>700$  but  $<800$  NL/h. Liquid feed was pumped to the nozzle by a syringe pump (model M362, Orion Sage) instead of using the built-in peristaltic pump. A 50 ml gastight syringe (Hamilton) was used to feed the solution to the spray dryer. Taking the volatile solution in the syringe rather than other glassware was helpful in avoiding evaporation of hazardous solvent and consequent changes in composition. The volume of feedstock in each batch was 25 ml, the inlet temperature  $54 \pm 1^\circ\text{C}$ , the feed rate 4.5 ml/min and the feed concentration was 3% total solids. Under these conditions, the outlet air temperature was  $33 \pm 1^\circ\text{C}$ . Free-flowing microparticles were collected from the product collection chamber. Material adhering to the chamber lid, as well as lower portion of the cyclone chamber taper was collected using a steel spatula.

### 2.3. Particle size analysis

Particle size of the microparticles in suspension was determined using a Malvern Mastersizer 2000 (Malvern, UK) apparatus with a manual accessory as previously described (Sharma et al., 2001). Briefly, about 5 mg of microparticles were

dry-mixed with an equal amount of sodium lauryl sulphate and suspended in 1 ml of distilled water by vortex-mixing. This slurry was added into the sampling beaker of the instrument till a laser obscuration factor of >10% was achieved.

#### 2.4. Cascade impaction

Aerodynamic particle size was determined using equipment supplied by In-Tox Products, Albuquerque, USA. Stainless steel collection disks of a Mercer (Lovelace) seven-stage cascade impactor were coated with a thin film of hydrocarbon grease as described by Beamer et al. (2000). The impactor was assembled and placed in one of the slots of a nose-only exposure chamber. About 250 mg of microparticles were accurately weighed and transferred to the fluid bed generator. Fluidization and collection conditions suggested by the manufacturer were employed. Thus, the powder was fluidized at a flow rate of about 20 l/min. Negative airflow at the rate of 1 l/min was applied to the exit port of the cascade impactor. Particles were collected over 5 min in four replicate experiments. The amount of microparticles collected on each stage was determined by HPLC estimation of isoniazid and rifabutin. MMAD was calculated from a best-fit plot of cumulative mass percentage undersize against the effective cut-off diameter (ECD) of the cascade impactor on a log-probability graph. The ECD at each individual stage was determined as  $ECD = D_1 \times \sqrt{1/Q}$  where  $D_1$  is the cut-off diameter at the negative flow rate of 1 l/min (i.e., 4.595, 3.0, 2.090, 1.624, 1.061, 0.719, 0.328  $\mu\text{m}$  for stages 1–7, respectively), and  $Q$  is the flow rate employed in the test (i.e., 1). The experimental MMAD of the particles was calculated using the intercept at 50%. The ratio between 84% undersize and 50% size (MMAD) represented the GSD. The relative fine particle fraction (FPF<sub><4.6 $\mu\text{m}$</sub> ) was also determined from the size distribution obtained using the cascade impactor.

#### 2.5. Analytical methods

A Shimadzu (Japan) Class VP HPLC system with a Luna C18 column (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm, Phenomenex, Torrance, USA) was used. Analytical methods were developed based on literature reports (Khuhawar and Rind, 2002; Lewis et al., 1991). Briefly, a gradient of ACN in 0.05 M potassium dihydrogen phosphate eluted isoniazid and rifabutin sequentially after a single injection (Muttil et al., unpublished results).

Drug amounts collected at various stages of the cascade impactor and in samples of serum and alveolar macrophage lysate were extracted by adding fourfold excess of MeOH, vortexing for 2 min and centrifugation at 3600  $\times g$  for 15 min. The supernatant was vacuum-evaporated (Maxi-dry Lyo, Heto-Holten, Denmark), the residue reconstituted in 50  $\mu\text{l}$  mobile phase, centrifuged again and 20  $\mu\text{l}$  of the supernatant injected on to the column. The mobile phase for bioanalytical samples was phosphate buffer at pH 7.0, modified by acetonitrile in a gradient program. Isoniazid eluted at  $\sim$ 6 min and rifabutin at  $\sim$ 17 min.

Standard curves were generated in the concentration range of 1.0–25  $\mu\text{g/ml}$  in MeOH for assay in formulation and 0.1–2.5  $\mu\text{g/ml}$  for analysis of biological samples. Standards for bio-analysis were prepared by spiking known amounts of drugs in separate tubes containing 100  $\mu\text{l}$  normal mouse serum or lysate of  $2 \times 10^6$  macrophages.

The linearity of the standard curve, range, specificity, sensitivity, accuracy, precision and efficiency of extraction of drugs from samples were determined by standard procedures described in guidelines issued by the International Commission on Harmonization of Laboratory Data (Anonymous, 1996).

#### 2.6. In vitro drug release

A USP Type II tablet dissolution test apparatus (Labindia, DISSO 2000) was used at a stirring speed of 150 rpm for these studies. A dialysis membrane (Sigma, molecular weight cut-off >900 kDa, 25 mm  $\times$  16 mm) was cut into equal pieces of about 6 cm  $\times$  2.5 cm and pre-treated as suggested by the manufacturer. Microparticles (5 mg) were accurately weighed out on the pre-treated dialysis membrane and sealed with clips. The pouch thus formed was attached to the paddles of the apparatus using rubber bands wound over the clips. Two kinds of release media were employed, so that release properties at the cytosolic pH (7.4) could be compared with release at late phagosomal pH of 5.2 (Llopis et al., 1998). Nine-hundred millilitres of phosphate-buffered saline containing 1% ascorbic acid and 0.05% sodium azide, at a pH of either 5.4 or 7.2 was used for this purpose to ensure sink conditions given the aqueous solubilities of the two drugs.

Samples of 5 ml were drawn (and fresh medium replaced) hourly for the first 6 h, then at 12 and 24 h, followed by daily sampling over the next 15 days. Release from microparticles was compared with that from a physical mixture of the two drugs, by themselves as well as in combination with an equal weight of L-PLA. For preparing the mixture, the drugs alone or drugs and L-PLA were weighed in the same proportion as present in the microparticle composition. All the samples were tested in duplicates. Samples were filtered through Millipore 0.22  $\mu\text{m}$  disposable filter and filtrate was stored at  $-20^\circ\text{C}$  till analysis by HPLC as described above.

Various models of matrix-controlled drug release (Fickian diffusion as a zero- or first-order process, Higuchi equation and Peppas equation) were fit to the drug release profiles from 0 time to 10 days using least-squares regression (Costa and Sousa Lobo, 2001).

#### 2.7. Differential scanning calorimetry (DSC)

A Pyris Diamond DSC system (Perkin-Elmer) was used to investigate the effects of drug incorporation on L-PLA matrix properties, in an attempt to explain the nature of drug release observed. Microparticles containing either of the two drugs alone were prepared for these studies. Samples (2 mg) were weighed in aluminum pans, sealed and subjected to temperature ramping at 10  $^\circ\text{C/min}$  from 10 to 300  $^\circ\text{C}$  under nitrogen. Thermograms were integrated using software supplied by the manufacturer.

#### 2.8. Targeting to macrophages in vivo

Animals were bred and housed ethically at the Laboratory Animals Division of the Institute. Experiments were conducted with the permission/monitoring of the Institutional Animal Ethics Committee, under conditions equivalent to European

Community guidelines on accepted principles for the use of experimental animals. Four female Swiss mice weighing 22–25 g each were administered fluorescent microparticles, while another four received non-fluorescent microparticles as controls.

About 10 mg microparticles were placed in the in-house inhalation apparatus. This consists of a 15 ml centrifuge tube with one hole in the wall to accommodate the muzzle of a mouse, and another in the apex of the taper to admit an aeration tube. Microparticles placed in the cap were fluidized by admitting turbulent air through the tube by means of actuating a rubber pipette bulb. Mice were restrained with their nares inserted in the apparatus, and the rubber bulb actuated for 30 s. The animals were then euthanized, their thoracic cavity exposed, and bronchioalveolar lavage (BAL) was conducted. The trachea was cannulated with a 24-gauge butterfly canula and secured by a silk thread to ensure patency, and five sequential aliquots of ice-cold saline were used to lavage the lungs and airways.

### 2.9. Flow cytometry

The lavage fluid recovered was pooled, centrifuged at  $200 \times g$  for 10 min, and a cell count performed using a hemocytometer. The cell pellet was resuspended in saline to obtain approximately  $10^5$  cells per  $100 \mu\text{l}$ , and stored on ice. Cells were then fixed with ethanol and stained for flow cytometry using phycoerythrin-conjugated ED1 antibody (Sigma–Aldrich, Bangalore, India) for the surface marker CD68. ED1 is reported to stain alveolar and airway-luminal macrophages (Lehnert et al., 1990). Cultured NIH 3T3 fibroblasts were added in different numbers to the fixed BAL cell suspension before staining to control for the reported overlap of fibroblast surface markers (Inoue et al., 2005). To block the Fc receptor,  $25 \mu\text{l}$  of mouse serum was added to the cell suspension and incubated at room temperature for 30 min. ED1-phycoerythrin was then added at a dilution of 1:300 and incubated for 4 h at  $4^\circ\text{C}$ . Samples were then subjected to flow cytometry using a FACS Calibur (Becton-Dickinson, USA) flow cytometer equipped with an Ar-ion laser. Histograms of cell counts versus fluorescence intensity due to phycoerythrin were recorded for preparations containing different proportions of macrophages and fibroblasts.

In two-color experiments, BAL cells recovered from mice that had inhaled fluorescent microparticles containing fluorescein isothiocyanate and stained with phycoerythrin as above were compared with BAL cells from mice receiving non-fluorescent microparticles. Scatter graphs of fluorescence intensity versus counts were recorded for 10,000 events at two photomultiplier tubes specific for fluorescein isothiocyanate and phycoerythrin, respectively. Instrument settings were used to exclude free fluorescent microparticles from recorded fluorescence events, based on their distinct forward and side scatter. These settings did not exclude either unstained bronchioalveolar lavage cells or 3T3 cells.

### 2.10. Drug amounts in serum and lung macrophages

Five groups of six mice each were taken for this study. Group I received microparticles by inhalation alone for 30 s. For dosing Group II, a solution was prepared by dissolving rifabutin

in a minimal volume of dimethylsulfoxide, isoniazid in normal saline, and mixing the two to obtain final concentrations of 1 mg/ml. Mice in Group II received oral gavages of this solution at a dose level of 5 mg/kg, and were subjected to blood and BAL sampling. Animals in Groups III, IV and V were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital, one by one, and their thoracic cavity exposed. Animals in Group III received an intra-tracheal instillation of  $100 \mu\text{l}$  of a suspension of microparticles containing  $500 \mu\text{g}$  microparticles. Animals in Group IV were administered an injection of  $100 \mu\text{l}$  sterile-filtered drug solution prepared above in the left ventricle. The last group was administered an intra-tracheal instillation of the same solution used in case of Group IV. All animals were subjected to cardiac puncture and BAL within 5–10 min of dose administration. Serum was separated by retracting the clot and centrifugation at  $3000 \times g$  for 10 min at  $4^\circ\text{C}$ .

BAL fluid was centrifuged as above to obtain a cell pellet. Despite exsanguination, the pellet was red in color, indicating the presence of erythrocytes. These, as well as microparticles that had not been internalized by macrophages were removed by magnetic separation of cells bearing the CD11b surface marker. The MiniMACS System (Miltenyi Biotec, Germany) was used as per manufacturer's instructions. Briefly, the cell pellet was dispersed in  $500 \mu\text{l}$  of MACS separation buffer and  $100 \mu\text{l}$  of magnetic beads with the ligand (CD11b) conjugated to them were mixed with the cell suspension and incubated for 15 min at  $4\text{--}8^\circ\text{C}$ . The cells were then washed with 10–20 vol. of separation buffer and redispersed in 1 ml of buffer. A magnetic separation column was placed in the magnetic field of the MACS separation unit and conditioned by two passes of  $500 \mu\text{l}$  of buffer. The cell suspension was applied to the column and free microparticles, erythrocytes as well as small amounts of lymphocytes and neutrophils were collected in the eluent as the negative fraction. The column was washed with  $3 \times 500 \mu\text{l}$  portions of buffer. The column was removed from the magnet and placed on a collection tube. One millilitre of buffer was applied on to the column and the positive fraction firmly flushed out using the plunger.

Since BAL typically yields  $0.5\text{--}0.75 \times 10^6$  cells from one mouse, and the analytical method was standardized for  $2 \times 10^6$  cells, BAL cells from three mice were pooled to obtain the required number. Two samples of purified macrophages were thus obtained from six mice in a group. Purified macrophages were lysed by removal of the buffer by centrifugation and addition of  $250 \mu\text{l}$  of triple-distilled water. Samples were stored at  $-80^\circ\text{C}$  till analysis, within a week.

## 3. Results

### 3.1. Microparticle size and drug content

Microparticles were recovered in yields approaching 60% of starting material. Ten representative batches, each of size 750 mg showed product recovery ranging from 449 to 456 mg. The mean yield from these batches was 59.88% of starting material. Microparticles typically contained about one part isoniazid and one part rifabutin for every two parts of L-PLA as estimated by HPLC (Table 1). Residual solvent was analysed by gas chromatography (Perkin-Elmer) in a method wherein

**Table 1 – Content and uniformity of microparticles assayed by HPLC**

Amount sampled (mg)	Isoniazid (%)	S.D. (n = 3)	Rifabutin (%)	S.D. (n = 3)
1	24.93	1.89	24.01	3.19
3	23.85	0.83	21.69	0.67
10	23.20	0.28	22.04	1.03

methanol eluted at 2.5 min and dichloromethane at 4.75 min. The residual solvents were below 0.05 ppm (data not shown).

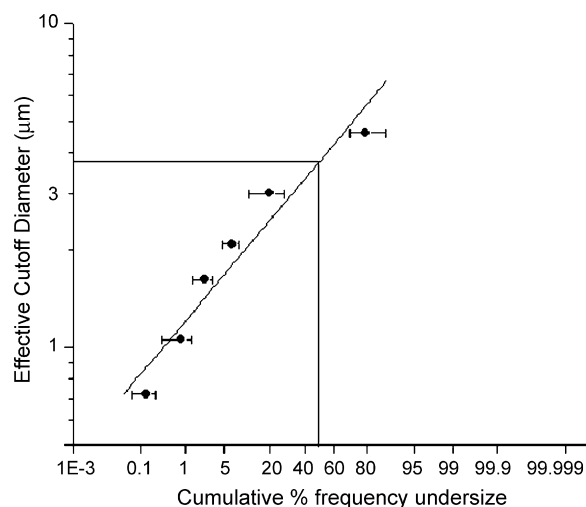
The particles displayed a log-normal distribution of volume-average sizes between 1 and 10  $\mu\text{m}$ , with a median particle size of about 5  $\mu\text{m}$ . The size distribution had a single mode, and >92% of the microparticles were within the range of 1–10  $\mu\text{m}$  (Fig. 1). We have found such a size range adequate for targeting to lung macrophages in experiments with both mice (Sen et al., 2003; Sharma et al., 2007) and rats (Sharma et al., 2001).

### 3.2. Microparticle respirability

The log-probability plot of the cumulative mass distribution of microparticles collected at various stages in a Mercer or Lovelace cascade impactor is illustrated in Fig. 2. The amounts collected on the seven stages of the cascade impactor were rather small, raising concerns about accurate estimation by gravimetry as suggested by the manufacturer. HPLC was therefore employed to estimate amounts collected at various cut-off diameters. The MMAD and GSD calculated from this plot were 3.57 and 1.41  $\mu\text{m}$ , respectively. The powder exhibited a uni-modal, log-normal distribution appropriate for inhalation delivery. The FPF<sub><4.6 $\mu\text{m}$</sub>  of the powder was 78.91  $\pm$  8.4%. The line fitting the data showed a regression coefficient of about 0.93, indicating a satisfactory linearity on the log-probability graph.

### 3.3. Drug release

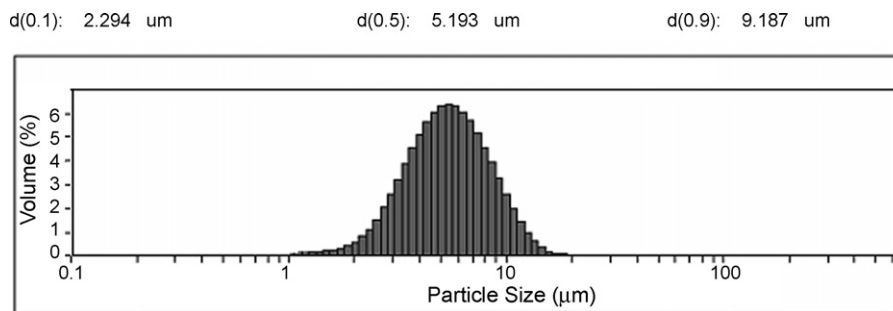
Microparticles or a physical mixture of drugs with and without L-PLA were placed in dialysis bags of porosity  $\sim$ 900 kDa and subjected to dissolution testing at pH 7.4 and 5.2. Almost the entire amount of isoniazid was released in 6 h, while >90% of rifabutin was detected in the sample withdrawn at 12 h when no polymer was present in the dialysis bag to



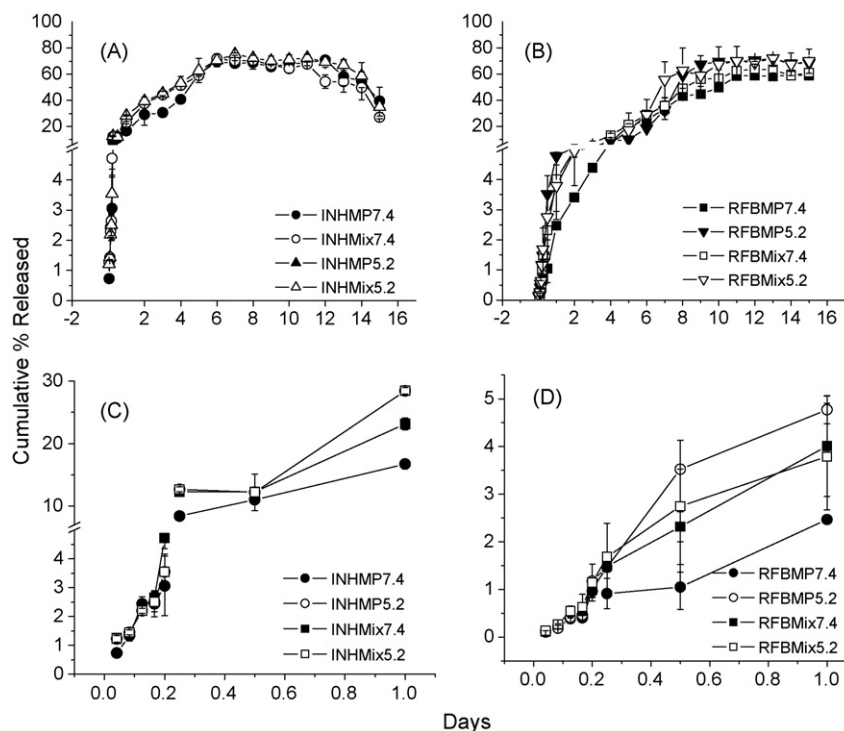
**Fig. 2 – The amount of microparticles deposited on each collection disc of the cascade impactor plotted against the effective cut-off diameter (ECD) on a log-probability chart. Error bars represent S.D. of four experiments. The MMAD (size corresponding to 50% cumulative mass oversize) calculated from this data was 3.57  $\pm$  1.41  $\mu\text{m}$  (GSD, ratio of 84% undersize to 50% size). The FPF<sub><4.6 $\mu\text{m}$</sub>  was 79  $\pm$  8.4%.**

establish a Donnan membrane equilibrium (data not shown). Isoniazid (Fig. 3A) was released more rapidly than rifabutin (Fig. 3B), regardless of pH. At a confidence level of  $P < 0.05$  in ANOVA using Bonferroni's post-test to compare group means, no significant differences were observed between the release profiles of rifabutin and isoniazid, whether tested as the microparticle formulation or physical mixtures of drugs and L-PLA over the entire 15-day period.

Drug release during the first day alone is plotted in Fig. 3C and D. ANOVA as above revealed differences in the burst release patterns of isoniazid from microparticles and from the physical mixture of drugs and L-PLA (Fig. 3C). About 15% of the isoniazid content was released from microparticles in the first day at pH 7.4, while at pH 5.2, the extent of release was nearly 22%. In case of the physical mixture, too, larger amounts were released at pH 7.4. Fig. 3D shows rifabutin release over the first day. In contrast to isoniazid, more drug was released at pH 5.2 than at 7.4 from the microparticle formulation. Amounts released from the physical mixture were interme-



**Fig. 1 – Log-normal, volume-average particle size distribution of a typical batch of microparticles prepared by the standardized process as assessed by laser scattering. The median diameter was 5.19  $\mu\text{m}$  and >90% of particles were below 9.19  $\mu\text{m}$  in size.**



**Fig. 3 – In vitro drug release of (A) isoniazid and (B) rifabutin, at cytosolic (pH 7.4) or phagolysosomal (pH 5.2) conditions. There was no significant difference between any of the release profiles over the 15-day period, but isoniazid appeared to degrade during the course of the study. Drug release in the USP type II apparatus over 1 day indicated a much higher quantum of release of isoniazid (C) than rifabutin (D). Larger amounts of isoniazid were released at pH 7.4 than 5.2, while the inverse was observed with rifabutin over 1 day.**

diate and comparable, indicating lack of pH-dependence. The total amount of rifabutin release from microparticles was less than 5%, as compared to 22% in the case of isoniazid.

Various models of drug release were employed in attempts to describe drug release from the microparticles (Table 2). The best fit was obtained with the Peppas model, which incorporates components of non-Fickian diffusion in the drug release profile (Costa and Sousa Lobo, 2001).

To further elucidate the reason for differences in drug release profiles of isoniazid and rifabutin, DSC was undertaken to investigate plasticising effects of the two drugs on the L-PLA matrix (Nishino et al., 2007). As shown in Fig. 4, the glass-transition temperature of L-PLA microparticle matrix

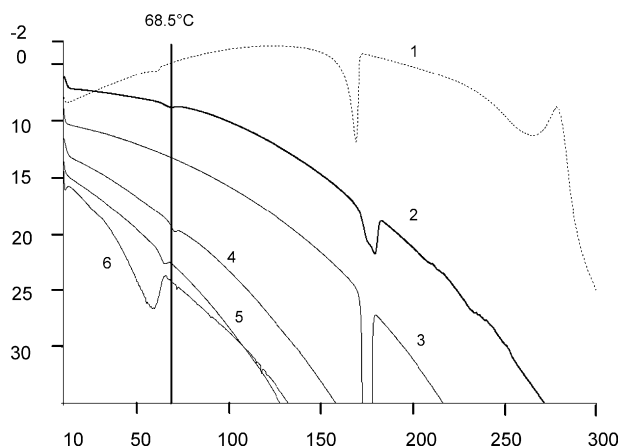
was about 68.5 °C. This was reduced drastically as a result of incorporation of rifabutin alone, less so when isoniazid alone was present in the matrix, and to an intermediate value when both drugs were present in the matrix.

### 3.4. Uptake by mouse lung macrophages

The airways and lungs of mice administered fluorescent and control microparticles using the in-house apparatus were lavaged within 5 min of inhalation. Cells recovered by BAL were stained for surface markers specific for lung and airway macrophages and subjected to flow cytometry analysis. Panel A in Fig. 5 shows histograms indicating that the proportion of

**Table 2 – Model fitting to release data over 15 days**

Model	Parameter	Isoniazid				Rifabutin			
		Microparticles		Mixture		Microparticles		Mixture	
		7.2	5.4	7.2	5.4	7.2	5.4	7.2	5.4
1st order	R <sup>2</sup>	0.66	0.69	0.63	0.65	0.87	0.80	0.88	0.81
	K	0.35	0.37	0.34	0.36	0.54	0.49	0.55	0.51
Higuchi	R <sup>2</sup>	0.97	0.96	0.96	0.97	0.84	0.80	0.77	0.86
	K	13.27	13.24	13.52	14.51	7.80	6.46	10.78	9.23
Peppas	R <sup>2</sup>	0.97	0.97	0.95	0.96	0.96	0.96	0.96	0.97
	K	6.57	7.28	6.03	6.18	31.80	29.87	25.35	13.64
	n	0.80	0.83	0.79	0.82	1.07	1.00	1.08	1.04



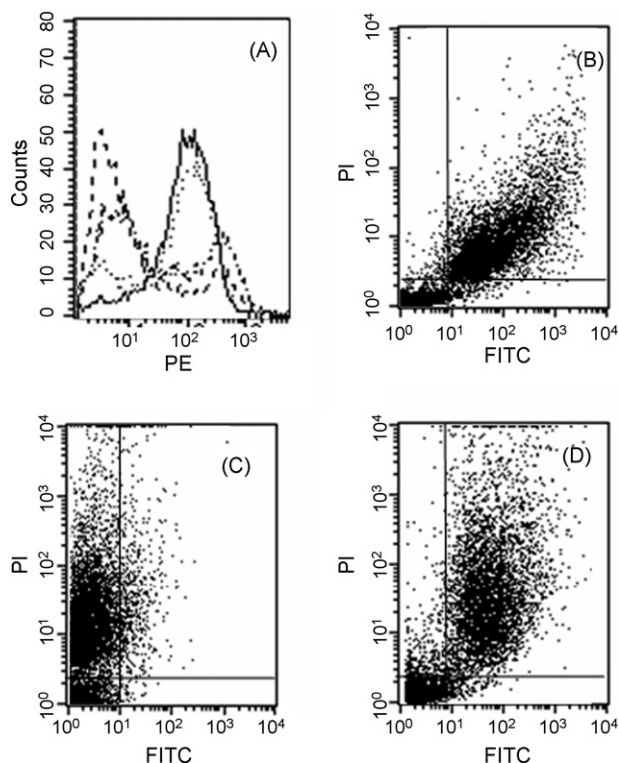
**Fig. 4** – Thermograms showing modification of glass transition and melting temperature of L-PLA microparticle matrix (curve 2) containing isoniazid alone (curve 1), rifabutin alone (curve 6) and the combination of both (curve 5). Isoniazid by itself (curve 3) showed a melting endotherm overlapping with that of L-PLA at 180 °C, while rifabutin (curve 4) showed a transition near the  $T_g$  of L-PLA.

cells strongly stained with anti-CD68-phycoerythrin was the same as the proportion of BAL cells in the sample. The population represented by the area under the curve peaking at high fluorescence intensity reduced as the proportion of 3T3 cells was increased. As expected, the fluorescence intensity of stained cells was higher when there were more BAL cells in comparison to 3T3 cells. The scatter graphs of double staining in the next two panels show that BAL cells recovered from mice administered fluorescent particles, but not stained for CD68 do stain strongly with fluorescein isothiocyanate (Fig. 5B), while those from mice receiving non-fluorescent particles, but stained for surface CD68 show high fluorescence corresponding only to phycoerythrin (Fig. 5C). These two samples containing either of the fluorescent dyes alone demonstrated that bleed-through of fluorescence resulting from one dye did not significantly impact on the results recorded on the non-cognate detector.

Fig. 5D indicates that >70% of cells that stain for CD68 also exhibit fluorescence due to fluorescein isothiocyanate contained in microparticles. It is expected that surface-bound microparticles would fluoresce more strongly than internalized microparticles, and efforts are underway to resolve internalized microparticles from those that are just attached to the macrophages surface after inhalation delivery. Also, when double-staining was undertaken, fluorescence due to fluorescein isothiocyanate fell in terms of total intensity, an effect that needs to be compensated for by incorporating anti-quench agents and equalizing steps of washing and incubation in the two cases.

### 3.5. Intracellular and serum concentrations

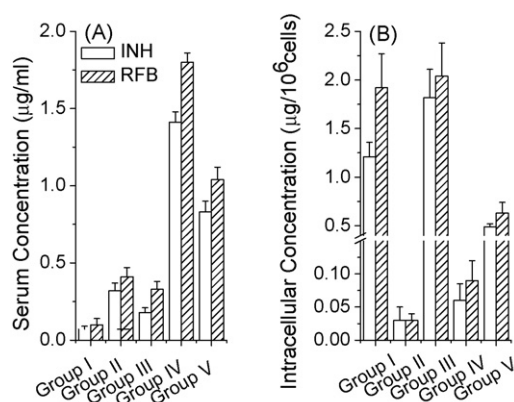
Robust methods of HPLC analysis of the two drugs, isoniazid and rifabutin were developed and used to determine drug concentrations in the lysate of BAL cells as well as in the serum of animals administered microparticles or pure drugs



**Fig. 5** – (A) Histograms of cell counts vs. fluorescence intensity due to phycoerythrin staining of CD68 surface marker in preparations containing different proportion of NIH 3T3 fibroblasts and primary cells recovered from mice by BAL. The reduction in the high-intensity peak due to macrophages corresponds to amplification of low-intensity peak due to unstained fibroblasts. Instrument settings employed recorded autofluorescence of unstained cells at an intensity of 10 arbitrary units, while stained cells gave signals ranging from ~100 to 1000 units. (B) Scatter plots of BAL cells recovered from mice administered fluorescein isothiocyanate-microparticles, but not stained for CD68. (C) BAL cells from mice administered non-fluorescent microparticles and stained for CD68. (D) Scatter plot obtained when BAL cells were recovered from mice administered fluorescein isothiocyanate-microparticles and stained with anti-CD68-phycoerythrin.

by different routes. The standard curves prepared by adding drugs to serum or cell lysate were linear in the concentration range of 0.1–2.5  $\mu\text{g/ml}$ . Quality control samples at concentrations of 0.25 and 1.25  $\mu\text{g/ml}$  of blank serum or lysate of  $2 \times 10^6$  macrophages showed recoveries between 77.6 and 80.2%.

Fig. 5 shows the concentrations of the two drugs in serum and lysate of alveolar macrophages, approximately 5–10 min after administration. As shown in Fig. 6A, the concentrations of both isoniazid and rifabutin were more than fourfold higher in serum after intra-cardiac injection as compared to routes and modalities other than intra-tracheal instillation of free drugs. When free drugs were instilled into the trachea, about half the concentration achieved by intra-cardiac injection was observed.



**Fig. 6 – (A) Serum concentrations of isoniazid (open bars) and rifabutin (hatched bars) resulting from administration of microparticles inhalation for 30 s (Group I), 5 mg/kg dissolved drugs by oral gavage (Group II), 500 µg microparticles by intra-tracheal instillation (Group III), 5 mg/kg drug solution by intra-cardiac injection (Group IV) and 5 mg/kg dissolved drug by intra-tracheal instillation to mice. Error bars show S.D.,  $n = 6$ . (B) Intracellular concentrations of isoniazid and rifabutin in lung macrophages. Cells from different animals were pooled to obtain  $>2 \times 10^6$  cells per sample and concentrations normalized accordingly. Group nomenclature was the same. Error bars show S.D.,  $n = 2$ .**

Intracellular concentrations were 17–19 times higher than serum concentrations when microparticles were introduced into the airways of the animals, whether by inhalation or intra-tracheal instillation (Fig. 6B). Intra-cardiac administration of free drugs lead to intracellular concentrations that were about a fifth of those achieved by instillation into the trachea. However, microparticles inhalation or instillation resulted in approximately four times greater intracellular concentrations as compared to instillation of free drugs.

#### 4. Discussion

Microparticles of acceptable size (Fig. 1) and aerosol characteristics (Fig. 2) could be prepared by spray drying a true solution of drugs and L-PLA. The drug content of the microparticles reported here (Table 1) is unusual. Much lower drug content is usually aimed at, since temporal control of drug release is by far the major preoccupation of investigations on biodegradable microparticles, especially as delivery systems for therapeutic peptides and vaccines. The primary objective of the work reported here was, however, to target airway and lung macrophages with large amounts of anti-TB drugs rather than achieve temporal control of drug release.

Despite the differences in the solubilities of isoniazid and rifabutin (125 mg/ml versus 0.19 mg/ml), the two drugs could be incorporated within the L-PLA matrix with approximately equal efficiency. Fu et al. (2001) have investigated the preparation of L-PLA microparticles by spray drying in detail. These authors encountered low product yields, compromised incorporation efficiency, irregular morphology and poor control of

burst release when drug:polymer ratios  $\geq 1:1$  were employed. The microparticles prepared during this study were recovered in yields approaching 60% and the incorporation efficiency of the process was  $\geq 98\%$ .

It is known that biodegradable microparticles such as those used in this study exhibit an initial burst. For instance, spray-dried L-PLA microparticles containing 1:5 ratio of drug to polymer released above 55% of their content of carboplatin over the first day when tested *in vitro* (Gavini et al., 2005). Similar results were obtained for carboplatin by Fu et al. (2001) and by Makino et al. (2004) for rifampicin. Despite the higher drug content of the microparticles of the present investigation, the burst release observed was not excessive. This observation may be attributed to the lower diffusivity of the solutes through the high-molecular-weight polymer matrix. As indicated in Table 2, the release data showed best fit to the Peppas model of drug release. Unlike swellable polymers, release from L-PLA matrices cannot be explained by considering hydration of the bulk and consequent changes in dimensions and diffusivity of the matrix. The good concordance observed with the Peppas model might be due to progressive hydration of the void spaces left behind after drug-rich domains of the matrix are depleted as drug diffuses into the release medium. Such a situation would be analogous to matrix swelling, but experiments are required before this speculation can be confirmed.

Fig. 3 also compares drug release at cytosolic and lysosomal pH (Llopis et al., 1998). The choice of two different pH conditions was prompted by an understanding that on administration to infected animals, microparticles may be localized either in “normal” phagosomes and experience progressive reduction of pH; or may co-localize with bacteria in maturation-arrested phagosomes (Hestvik et al., 2005). Results depicted in Fig. 3 indicate that microparticles released up to 60% of their content within the first 4–8 days, even at cytosolic pH. Total amounts of rifabutin released were apparently larger, but since these results were not corrected for degradation rates *in vitro*, this observation is likely to be an artifact. Further, in the absence of correction for degradation, the extent of release depicted herein is likely to be an underestimate. Nevertheless, the microparticles lost about 25% of their mass, in the form of drug content, within the first 4 days *in vitro*. Leaching out of the drug may be expected to create void spaces permitting greater hydration of the matrix. Blasi et al. (2005) have demonstrated that hydration of lactide-glycolide polymer matrices tends to lower the glass transition temperature. This phenomenon has recently been employed to modulate micropore formation in microparticle matrices and thereby engineer drug release profiles (Matsumoto et al., 2006, 2005). It is therefore expected that high-payload microspheres would be hydrated more rapidly and extensively *in vivo*, and may exhibit more rapid degradation than has been estimated for L-PLA microparticles containing smaller payloads. In addition, differential scanning calorimetry indicates that both rifabutin and isoniazid decrease the glass transition temperature of L-PLA (Fig. 4). Nishino et al. (2007) have recently demonstrated that surfactant incorporation into polylactide matrix results in reduction of initial burst release of incorporated irinotecan and provides smoother surface morphology to the microparticle. Rifamycins such as rifabutin have long been known to possess surfactant properties (Pelizza et al., 1976), and can

be expected to play a similar role in the present formulation. Changes in crystallinity brought about as a result of admixture of the two drugs in large proportions would obviously modulate the release profiles. Results shown in Fig. 3 indicate that release is retarded if the matrix is rendered more crystalline. Detailed investigations in this regard are currently in progress. The choice of high molecular weight L-PLA as the preferred excipient could thus provide the benefit of achieving high drug incorporation efficiency without imposing the cost of prolonged residence *in vivo*.

Drug release up to day 10 appeared to follow the Pepas model (Costa and Sousa Lobo, 2001) most faithfully, as shown in Table 2. Release-controlling effects of the microparticles formulation are discernible in the differences between the K-values of functions fitting release from the microparticles and the mixture of drugs and polymer. This parameter also highlights the differences in rates of release of isoniazid and rifabutin, as well as minor dependence on pH of the medium.

Taking the question of drug payload to its extreme, the need for any polymer matrix at all appears moot if the objective is to target as much drug as possible to macrophages. Indeed, standardized formulations of drug agglomerates are reported for pulmonary delivery (Ikegami et al., 2003). However, the alveolar surface is rich in lung surfactant that may be expected to almost instantaneously dissolve typical drug carriers such as lactose as well as pure drug particles formulated as agglomerates (Kendall et al., 2004). Dissolved drug would then enter surrounding cells by diffusion and permeation, curtailing bioavailability to lung macrophages that make up just 2–10% of lung tissue (Pinkerton et al., 1992). When the efficacy of rifampicin-containing microparticles was compared with drug alone administered to the respiratory tract of infected guinea pigs, a clear advantage in the microparticle formulation was observed (Suarez et al., 2001b). The polymeric component of the microparticles matrix may therefore be expected to maintain structural integrity, while the high drug content would render the surface more hydrophilic than would have been the case if the microparticles matrix was mostly polymer. The extent of microparticles phagocytosis by lung macrophages is related to the surface hydrophobicity (Evora et al., 1998). It is therefore speculated that the high-payload L-PLA microparticles reported here would be more amenable to phagocytosis by lung macrophages than those containing a smaller proportion of drugs.

To assess microparticles targeting to macrophages on administration of inhalations, a flow cytometry experiment was conducted. It was first established that the surgical procedure used to recover macrophages from the lungs and airways yielded reasonably pure populations. The antibody to CD68 that is reported to be specific to lung and airway macrophages (Lehnert et al., 1990) was found capable of distinguishing between primary macrophages recovered by BAL and exogenously added fibroblasts (Inoue et al., 2005). Moreover, the estimates of cell numbers arrived at by flow cytometry tallied with the values used to prepare different admixtures of macrophages and fibroblasts. It was concluded that the staining was specific to macrophages. Flow cytometry also confirmed that microparticles delivered using the apparatus

were specifically targeted to lung macrophages. Our previous report (Sharma et al., 2001) had employed single-color flow cytometry to assess the association of fluorescent microparticles with cells recovered by BAL, and fluorescence microscopy to demonstrate that these cells possessed macrophage morphology. The present data not only specifies that macrophages and not epithelial cells represent the cell type interacting with inhaled microparticles (Fig. 5A), but also that almost all macrophages recovered by BAL take up microparticles (Fig. 5C and D). In view of extensive targeting of the microparticles, it was also of interest to establish the actual drug concentrations that were achieved within the cells as a consequence of microparticles uptake, and to compare these with the concentrations reaching the serum.

Determination of drug concentrations in alveolar macrophages and serum after administration of 'nose-only' dry powder inhalation (Fig. 6) reconfirmed our previous findings in rats (Sharma et al., 2001). Both isoniazid and rifabutin were present in appreciable amounts in serum almost immediately after inhalation of microparticles. This indicates that there is a component of burst release *in vivo* that is rapidly available for biodistribution through the bloodstream entering the lungs for oxygenation. Drug released in the lungs also resulted in the development of higher serum concentrations as compared to oral administration. Concentrations of isoniazid detected were consistently lower than those of rifabutin, despite a marginal excess of isoniazid content in the microparticles (Table 1). This was expected, since rifabutin has a much longer serum half-life, although it does not interact pharmacokinetically with isoniazid (Breda et al., 1992; Weiner et al., 2005). It is likely that the concentration difference arises due to loss of isoniazid to metabolism. The analytical method used here did not address metabolites, so further studies are required in this direction.

Instillation of free drugs in the trachea resulted in intracellular drug concentrations that were about one-fourth of those observed when microparticles were administered. This observation supports the view that free drugs or appropriately sized agglomerates might not be as efficient at targeting drugs to macrophages.

Thus, high-payload microparticles may offer the following advantages. They possess the obvious ability to deliver more drug per targeting event. Their powder characteristics are compatible with dry powder inhalation, as evident from the MMAD of 3.57  $\mu\text{m}$ . They retard drug release, so that burst effects do not deplete the content drastically. Finally, the presence of a large proportion of diffusible drugs in the matrix suggests that drug release would result in the creation of voids that may be readily hydrated. This is expected to facilitate rapid biodegradation.

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## 5. Conclusions

Inhalable microparticles containing high drug payload can be prepared as a dry powder inhalation targeting alveolar macrophages rather than lung epithelium. These may be expected to control drug release inside classical or maturation-arrested phagosomes over a few days. Microparticle residence and biodegradation *in vivo* need to be established.

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