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Formulation and in vitro characterization of inhalable rifampicin-loaded PLGA microspheres for sustained lung delivery

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ABSTRACT

The solvent evaporation method with premix membrane homogenization was applied, with class-3 ethyl acetate as organic solvent, to produce narrowly size-distributed rifampicin (RIF)-loaded poly(lactide-co-glycolide) (PLGA) microspheres for sustained lung delivery as aerosol. Microsphere formulations (simple or multiple emulsions, different PLGA and RIF concentrations) and process parameters (transmembrane pressure, SPG membrane pore diameter) were investigated as their effects on RIF content, microsphere size, aerodynamic properties of the freeze-dried powder and in vitro release profiles. Narrowly size distributed microspheres with diameters from 2 to 8 μm, satisfactory RIF contents (from 4.9 to 16.5%), 80% RIF release from 12 h to 4 days, and adequate aerodynamic properties were prepared from a multiple emulsion and using SPG membrane pore diameter of 19.9 μm. The premix membrane homogenization appeared to be a rapid and efficient method to prepare monodisperse drug-loaded microspheres suitable for lung delivery as sustained-release microsphere aerosol.

1. Introduction

The search for more efficient therapeutic approaches for the treatments of patients with pulmonary infections has driven the research efforts to the development of inhaled antibiotics. Direct delivery into the lung is indeed expected to increase local drug concentrations for higher therapeutic efficiencies, and to lower systemic exposure for lower toxicity. Up to now the commercialized antibiotic aerosols are generated from aqueous solutions or suspensions using liquid nebulizers, and inhaled by patients for adequate periods several times a day (Traini and Young, 2009). No solid aerosols of pure drug particles that would be administered using dry-powder inhalers (DPI) and would reduce administration duration have been marketed so far, probably for two main reasons. First, administering a dose of antibiotics of several tens of milligrams as dry aerosol represent a technical challenge, since current commercial DPIs were mainly developed for the treatment of asthma, i.e. for delivering micrograms of drugs, and are not designed for high loads of drugs. Second, the deposition of high amounts of dry antibiotic powder on pulmonary epithelia may have irritant effects or acute toxicity (Le Brun et al., 2002) and may require less toxic prodrugs (Westerman et al., 2007). In addition, from a pharmaceutical point of view aerosols of pure drug powder are immediate-release dosage forms that may not be adequate for treatment efficiency and safety (due to high fluctuations of drug concentrations or to high concentrations in the vicinity of dissolving drug particles) or for patients' compliance with the regimen (due to the frequency of medications). Inhalable innovative drug delivery systems, such as microspheres, are expected to reduce direct contact of highly concentrated drug formulations with the lung tissue and therefore toxicity, and to minimize drug concentration fluctuation through sustained release. Rifampicin (RIF), an antibiotic used in the treatment of tuberculosis (TB), may benefit from innovative aerosol delivery. RIF-loaded poly(lactide-co-glycolide) (PLGA) microspheres administered into the lung by insufflation or nebulization reduced significantly the bacterial burden in a guinea pig model for tuberculosis (Suarez et al., 2001). They were shown to be phagocytosed by macrophages on a model of Mycobacterium bovis Bacillus Calmette–Guérin (BCG)-infected rat alveolar macrophage NR8383 cells, thus increasing intracellular RIF concentration and bactericide activity compared to the solution (Hirota et al., 2010). However, despite evidences of higher efficiency on in vivo TB models and a few, rather incomplete, in vivo pharmacokinetic studies (Sung et al., 2009; Coowanitwong et al., 2008), it is not known whether sustained-release microspheres will actually result in sustained high level of free drug in the lung area, a

Abbreviations: EA, ethyl acetate; HPβCD, 2-hydroxypropyl-β-cyclodextrin; RIF, rifampicin.

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requirement for eradicating free mycobacteria located in granulomas (Muttil et al., 2009). It is not known either which in vitro release profile would better suit the in vivo requirements for compensating RIF clearance from lungs and maintaining efficient RIF concentration in this area. The objective of the present work was therefore to prepare a series of RIF-loaded PLGA microspheres with different in vitro release rates, which will be screened for release profile selection in future pharmacokinetic studies in rats after lung administration. The microsphere preparation method was based on a dry-in-emulsion process with a premix membrane homogenization step (Doan and Olivier, 2009), a method recently proposed to produce monodisperse microspheres with a high productivity (Wei et al., 2008). Several formulation and process parameters were investigated, as their effect on microsphere diameter, aerodynamic properties and release profiles. For environmental and safety concerns, ethyl acetate (EA) was used as the dispersed solvent to dissolve PLGA in place of the more toxic chlorinated solvents (Sah, 1997).

2. Materials and methods

2.1. Materials

Resomer® RG 502 H (PLGA 50:50, 0.19 dL/g inherent viscosity (25 °C, 0.1% in chloroform), M_w 10 500, M_n 6100 (M_w/M_n = 1.71)) was supplied by Boehringer Ingelheim (Ingelheim, Germany). Rifampicin (purity ≥97% (HPLC)), ethyl acetate (EA) and dimethyl sulfoxide (DMSO) were obtained from Sigma (France). Rhodoviol 4/125 (polyvinylalcohol (PVA), degree of hydrolysis of 88%) was provided by Prolabo (France). Kleptose® HP (2-hydroxypropyl-β-cyclodextrin (HPβCD), degree of substitution per glucose unit: 0.6–0.9 and M_w 1510 g/mol) was obtained from Roquette (France). Purified water was produced using a MilliQ Gradient® Plus Millipore system.

2.2. Methods

2.2.1. SPG porous membrane cleaning and conditioning

Shirasu porous glass (SPG) membranes were dipped in a detergent solution and water-bath sonicated for 30 min. They were then dipped in EA for 2 h, and finally in 2 M hydrochloric acid for 2 h. After calcination at 500 °C for 4 h, they were regenerated in HCl 2 M at 70 °C for 2 h and finally rinsed with purified water. For conditioning before the emulsion homogenization process SPG membranes were water-bath sonicated for 30 min in the emulsion continuous phase.

2.2.2. Preparation of RIF-loaded PLGA microspheres from O/W emulsions

A series of microspheres was prepared, based on the method previously described (Doan and Olivier, 2009). A coarse emulsion or premix was first prepared by mixing under magnetic stirring (at 200 rpm for 5 min) 3 mL of a solution of PLGA and of RIF in EA (dispersed phase O) with 7 mL of a solution of PVA (3%, m/v) in water acidified to pH 4 with 2 M hydrochloric acid and saturated with 8.5 vol.% EA (continuous phase W). Then the premix was subjected to three homogenization cycles in an open system under positive transmembrane pressure ΔP_{tm}, using an external pressure-type micro kit emulsification device (SPG Technology, Sadowara, Japan) equipped with an SPG membrane (5.9–19.9 μm pore size, SPG Technology). The obtained emulsion was poured quickly into 100 mL of a 0.4% (w/v) PVA solution at pH 4 and left under magnetic stirring (300 rpm) for 4 h to allow EA evaporation. Microspheres were collected and washed three times with water at pH 4 by centrifugation at 100–980 × g (depending on the size of the microspheres) for 10 min, and finally freeze-dried using a FreeZone Triad freeze-drier from Labconco Corp. (Kansas City, MO), with initial freezing temperature of −30 °C and freeze-drying under 4 Pa pressure at shelf temperatures set at −10 °C for 4 h, then at +10 °C for 16 h. The studied parameters for formulation optimization were the concentrations of PLGA (3–30 wt.%) and of RIF (1–3 wt.%) in the dispersed phase, the membrane pore size (5.9, 10.0, and 19.9 μm) and the applied transmembrane pressure ΔP_{tm} (5–100 kPa).

2.2.3. Preparation of RIF-loaded PLGA microspheres from W/O/W emulsions

A W_1/O primary emulsion was prepared by emulsifying 0.6 mL of a HPβCD–RIF complex aqueous solution (18 mg/mL RIF and 0.066 M HPβCD in 50 mM borate buffer, pH 9, prepared as previously (Tewes et al., 2008)) into 3 mL of a solution of PLGA (3–30 wt.%) and RIF (2 wt.%) in EA using a Ultraturrax® homogenizer (13 500 rpm for 15 s). It was then coarsely emulsified under magnetic stirring (200 rpm for 5 min) in 7 mL of a 3% (m/v) PVA aqueous solution (W_2) at pH 4 saturated with EA (8.5 vol.%). Then, the W_1/O/W_2 premix was subjected to three homogenization cycles through an SPG membrane (19.9 μm porosity) under ΔP_{tm} of 25 kPa. After dilution in 100 mL 0.4% (w/v) PVA solution at pH 4 and EA evaporation, microspheres were collected, washed and freeze-dried as described in Section 2.2.2.

2.2.4. Microsphere RIF content

RIF contents were determined by spectrophotometry as described previously (Doan and Olivier, 2009). RIF contents were expressed as the amount of RIF (mg) per mg of microspheres (including entrapped RIF). Entrapment efficiencies (%) were calculated as the percent ratios of the determined contents to the theoretical contents calculated considering a 100% entrapment efficiency.

2.2.5. Microsphere size

Microspheres were dispersed in purified water and analyzed using laser light diffraction (Microtrac® X100 particle size analyzer) as described previously (Doan and Olivier, 2009). Particle size was expressed as the mean diameter of the volume distribution (D_v) calculated using the Microtrac Particle Size Analyzer application program (version 9.0g).

The width of microsphere size distribution, or span, was calculated according to Eq. (1) (Vladisavljević and Schubert, 2003)

$$\text{Span} = \frac{d_{90} - d_{10}}{d_{50}} \quad (1)$$

where d_{10}, d_{50}, d_{90} are the diameters corresponding to 10, 50 and 90 vol.% on a relative cumulative microsphere size distribution curve. Microspheres were considered as satisfactorily narrowly size-distributed when span values were below or equal to 1.

2.2.6. Aerodynamic diameter

The aerodynamic properties of freeze-dried microsphere powder aerosolized with a Model DP-4 dry powder insufflator™ for rat (Penn-Century Inc., USA) were evaluated using an NGI cascade impactor (Copley Scientific) as previously described (Doan and Olivier, 2009), using RIF as a marker. Calculations were made on the recovered mass of RIF, using the Microsoft® Excel Office software (version 2003). The percent cumulative mass fractions were plotted versus log aerodynamic diameters. The mass median aerodynamic diameter (MMAD) was estimated by linear interpolation using the equation of the linear interpolant that links the curve points immediately below and above 50% deposition. For respirable fractions (i.e. 1–5 μm aerodynamic diameter cumulated fractions in percentile), the same interpolation method was applied to estimate the fractions corresponding to 1 μm and 5 μm cut-offs, because the NGI cut-offs did not correspond to these values.

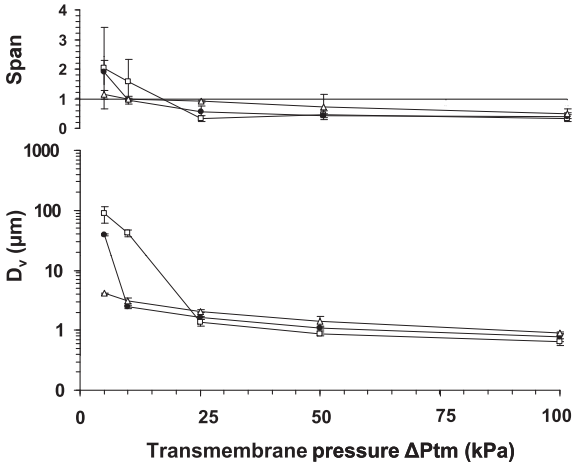

Fig. 1. Effect of transmembrane pressure ΔP_{tm} and of membrane pore size d_p on microsphere volume diameter (D_v) and span. d_p: 5.9 (□), 10.0 (●), 19.9 (△) µm (mean ± SD, $n = 3$). O/W emulsion formulae before dilution: O (3 mL): 3 wt.% PLGA and 2 wt.% RIF, W (7 mL): 3% (m/v) PVA.

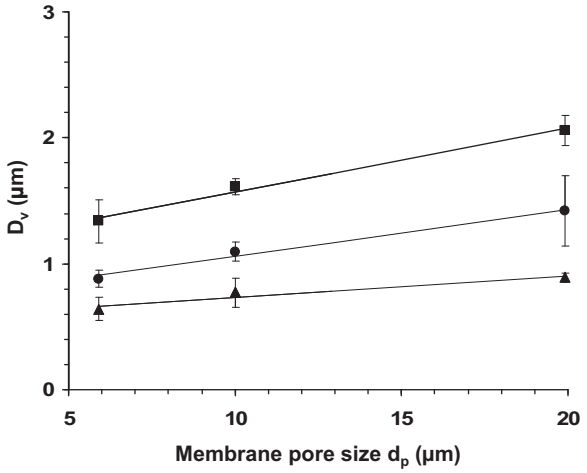

Fig. 2. D_v versus d_p for $\Delta P_{tm} = 25$ (■), 50 (●) and 100 (▲) kPa (mean ± SD, $n = 3$). Data and experimental conditions as in Fig. 1.

2.2.7. Microscopy

The microspheres were observed by scanning electron microscopy (SEM) using a JSM-840A JEOL electron microscope (JEOL Ltd., Tokyo, Japan) at 15 kV, after gold-sputtering the microspheres in an argon atmosphere.

2.2.8. In vitro RIF release study

RIF-loaded microspheres (amount corresponding to a 500 µg/mL RIF final concentration, i.e. sink conditions) were incubated at 37 °C, under magnetic stirring (990 rpm), in 5 mL phosphate-buffered saline (PBS), pH 7.4, containing ascorbic acid (200 µg/mL) as an antioxidant. At pre-defined time points, microspheres were sedimented by centrifugation at 630 × g for 5 min, and supernatants were collected for RIF determination and replaced with fresh PBS. RIF concentrations were determined spectrophotometrically at 475 nm using a Varian Cary 50 UV-Visible spectrophotometer, with a RIF calibration curve (0.010–0.050 mg/mL concentration range in PBS supplemented with ascorbic acid).

2.2.9. Release profile modeling

RIF release kinetics were analyzed using Eq. (2) that describes the drug release from a spherical matrix (so-called Baker–Lonsdale model), in order to clarify the RIF release mechanism from microspheres (Costa and Sousa Lobo, 2001):

$$ft = \frac{3}{2}\left[1 - \left(1 - \frac{Q_t}{Q_\infty}\right)^{2/3}\right] - \frac{Q_t}{Q_\infty} = k \cdot t \qquad (2)$$

where Q_t/Q_∞ is the fraction of drug released at release time t, k a release constant.

The correlation between experimental data and the Baker–Lonsdale model was evaluated using the correlation coefficient r.

3. Results

3.1. Effect of ΔP_{tm} and SPG membrane pore diameter

The effect of transmembrane pressures ΔP_{tm} and SPG membrane pore diameter d_p on the size of the microspheres prepared from a O/W emulsion was first studied (Fig. 1). With d_p of 19.9 µm, D_v decreased gradually from 4.09 ± 0.03 to 0.89 ± 0.04 µm when ΔP_{tm} was increased from 5 to 100 kPa. Span values were satisfactory for $\Delta P_{tm} > 10$ kPa. With d_p of 10.0 or 5.9 µm and below ΔP_{tm} threshold values of 10 and 25 kPa respectively, microspheres were polydisperse and larger than the membrane pore diameters ($D_v > 38$ µm and span > 1.5). Above these threshold values, D_v were less than 2 µm and gradually decreased with increasing ΔP_{tm}, and span values were satisfactorily below 1. It is worth noting that above the ΔP_{tm} threshold value, the porosity d_p had moderate impact on D_v (Fig. 2). RIF contents and entrapment efficiencies were close in all the conditions (0.10–0.12 mg RIF per mg microsphere and 25–31%, respectively), except for d_p of 5.9 µm with ΔP_{tm} of 50 or 100 kPa (0.07 mg RIF per mg, around 18%, respectively).

As they permitted to prepare monodisperse microspheres, ΔP_{tm} of 25 kPa and membranes of 19.9 µm pore size were used for further investigations.

3.2. Effect of RIF concentration

The effect of RIF concentration in the dispersed phase at constant PLGA concentration (3 wt.%) is presented in Table 1. Increasing RIF concentration from 1 to 3 wt.% had no impact on D_v, but resulted in higher RIF contents, with a maximum entrapment efficiency observed with a 2 wt.% RIF concentration. The higher RIF concentration led to a RIF precipitation at the membrane surface at the 3rd homogenization cycle and to unsatisfactory span values.

3.3. Effect of PLGA concentration

As a rule, an increase in PLGA concentration resulted in microspheres with larger diameters, lower RIF contents and higher entrapment efficiencies, and in higher MMAD of the powder (Tables 2 and 3).

3.4. Effect of the emulsion type

Compared to the O/W preparation method (Table 2), the $W_1/O/W_2$ preparation method (Table 3) which permitted to provide an additional input of RIF in the form of a RIF–HPβCD complex solubilized in the inner aqueous phase increased the RIF content at PLGA concentrations up to 6 wt.%, but had no impact at higher PLGA concentrations. It also led to microspheres with lower MMAD:D_m ratio, except with 3 wt.% PLGA concentration. Microspheres deriving from simple or multiple emulsions had close respirable fraction values, except at the 30% PLGA concentration. SEM observation showed spherical narrowly size-distributed microspheres (Fig. 3).

Table 1
Effect of RIF concentration in ethyl acetate on the size, RIF content and entrapment efficiency of microspheres prepared using the O/W process (mean ± SD, n = 3 different batches analyzed).

RIF (wt.%)	D_v (μm)	Span	RIF content (mg/mg)	Entrapment efficiency (%)
0	2.16 ± 0.14	0.80 ± 0.05	–	–
1	2.23 ± 0.10	0.85 ± 0.08	0.048 ± 0.004	19.1 ± 1.5
2	2.06 ± 0.12	0.92 ± 0.03	0.126 ± 0.005	31.5 ± 1.2
3	2.12 ± 0.30	1.14 ± 0.24	0.136 ± 0.005	27.2 ± 1.1

Conditions for O/W emulsion preparation: d_p = 19.9 μm, ΔP_{tm} = 25 kPa, O: 3 wt.% PLGA and 0–3 wt.% RIF (3 mL), W: 3% (m/v) PVA (7 mL).

Table 2
Effect of PLGA concentration in ethyl acetate on the size, RIF content, entrapment efficiency and aerodynamic properties of microspheres prepared using the O/W process (mean ± SD, n = 3 different batches analyzed, except *: n = 1 batch analyzed).

PLGA (wt.%)	D_v (μm)	Span	RIF content (mg/mg)	Entrapment efficiency (%)	MMAD (μm)	MMAD:D_v ratio	Respirable fraction (%)
3	2.06 ± 0.12[a]	0.92 ± 0.03[a]	0.126 ± 0.005[a]	31.5 ± 1.2[a]	3.36 ± 0.09	1.63	70.2 ± 2.2
6	2.61 ± 0.12	0.81 ± 0.17	0.088 ± 0.012	35.2 ± 4.9	3.78*	1.45*	54.1*
10	3.01 ± 0.3	0.76 ± 0.21	0.075 ± 0.006	45.0 ± 3.7	4.41*	1.47*	51.3*
20	4.02 ± 0.25	0.86 ± 0.28	0.054 ± 0.004	51.8 ± 11.0	4.89*	1.22*	49.8*
30	5.51 ± 0.84	0.99 ± 0.19	0.046 ± 0.002	73.4 ± 3.7	6.26 ± 0.90	1.14	33.4 ± 7.92

Conditions for O/W emulsion preparation: d_p = 19.9 μm, ΔP_{tm} = 25 kPa, O: 3–30 wt.% PLGA and 2 wt.% RIF (3 mL), W: 3% (m/v) PVA (7 mL).
[a] Data reported from Table 1.

Table 3
Effect of PLGA concentration in ethyl acetate on the size, RIF content, entrapment efficiency and aerodynamic properties of microspheres prepared using the $W_1/O/W_2$ process (mean ± SD, n = 3 different batches analyzed, except *: n = 1 batch analyzed).

PLGA (wt.%)	D_v (μm)	Span	Content (mg/mg)	Entrapment efficiency (%)	MMAD (μm)	MMAD:D_v ratio	Respirable fraction (%)
3	2.08 ± 0.10	0.90 ± 0.04	0.165 ± 0.009	35.0 ± 2.0	3.43 ± 0.05	1.65	69.9 ± 1.3
6	3.48 ± 0.25	1.85 ± 0.22	0.102 ± 0.008	34.6 ± 2.6	3.92*	1.13*	60.7*
10	4.51 ± 0.29	1.34 ± 0.14	0.069 ± 0.003	35.2 ± 10.3	4.41*	0.98*	57.9*
20	6.71 ± 0.52	0.74 ± 0.14	0.051 ± 0.007	47.8 ± 6.2	4.72*	0.70*	46.7*
30	8.43 ± 0.60	0.56 ± 019	0.049 ± 0.002	66.9 ± 2.1	4.93 ± 0.32	0.58	51.3 ± 10.5

Conditions for $W_1/O/W_2$ emulsion preparation as in Fig. 4.

At a 3 wt.% PLGA, both O/W and $W_1/O/W_2$ processes provided microspheres with similar smooth surface (Fig. 3a and c). At a 30 wt.% PLGA concentration, O/W process produced microspheres with smooth surface and internal void spaces (Fig. 3b, insert), while with the $W_1/O/W_2$ process microspheres presented pores on their surface (Fig. 3d).

3.5. *In vitro release profiles*

The in vitro RIF release profiles from the microspheres obtained from $W_1/O/W_2$ emulsions (Fig. 4) are characterized by an initial fast release ("burst" release of 45–74% of the RIF content within 6 h, Table 4). Generally, increasing PLGA concentrations increased

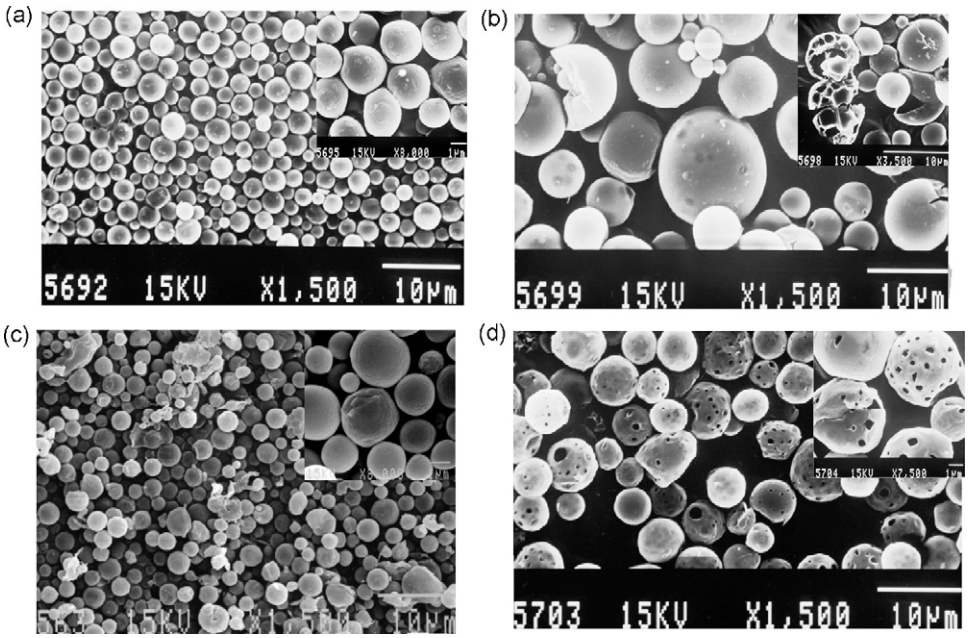

Fig. 3. Scanning electron micrograph of RIF-loaded PLGA microspheres prepared from O/W (a and b) or $W_1/O/W_2$ (c and d) emulsions. Preparation conditions and emulsion formulae before dilution: d_p = 19.9 μM, ΔP_{tm} = 25 kPa, W_1 (0.6 mL): 18 mg/mL RIF as HPβCD complex in water, O (3 mL): 3 wt.% PLGA (a and c) or 30 wt.% PLGA (b and d) and 2 wt.% RIF, W or W_2 (7 mL): 3% (m/v) PVA.

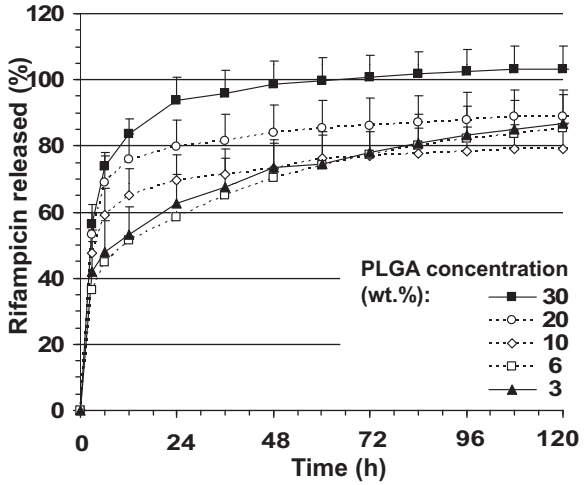

Fig. 4. In vitro release profiles of RIF-loaded microspheres prepared from $W_1/O/W_2$ emulsion with different PLGA concentrations in phase O (mean ± SD, $n = 3$). Conditions: 500 μg/mL RIF, PBS, pH 7.4, with 200 μg/mL ascorbic acid. Preparation conditions and emulsion formulae before dilution: d_p = 19.9 μM, $\triangle P_{tm}$ = 25 kPa, W_1: 18 mg/mL RIF as HPβCD complex in water (0.6 mL), O: 3–30 wt.% PLGA and 2 wt.% RIF (3 mL), W_2: 3% (m/v) PVA (7 mL).

release rates, with around 80% RIF released over 12 h (30 wt.% PLGA concentration) to 4 days (3–10 wt.% PLGA concentration). With 3 or 6 wt.% PLGA concentration, the release profiles were found to correlate with the Baker–Lonsdale model (Table 4, $r > 0.99$). With PLGA concentrations from 10% and above, release profiles over the effective release period (i.e. 0–108 h) did not fit the Baker–Lonsdale model ($r < 0.96$), except the late minor fractions released after 6–24 h (Table 4).

4. Discussion

The premix membrane emulsification is a membrane emulsification method that permits the preparation of monodisperse emulsions with productivities of several orders of magnitude higher than the direct membrane emulsification (Vladisavljević and Williams, 2005). In the present work, it was applied to the preparation of RIF-loaded PLGA microspheres by the dry-in-emulsion process. Formulation and process parameters were investigated with the objectives of an in vitro sustained RIF release over 1–4 days combined with adequate aerodynamic properties for intra-tracheal delivery of respirable microspheres in rats using a Penncentury dry powder insufflator™. Targets for aerodynamic properties were based on requirements for lung administration in humans (Sung et al., 2007) and MMAD between 1 and 5 μm and respirable fraction above around 50% were considered as satisfactory. The class-3 ethyl acetate (EA) solvent was used as organic solvent in place of the more toxic class-2 dichloromethane for toxicity and environmental concerns (Sah, 1997). PLGA was chosen for its well-known biocompatibility and biodegradability (Anderson and Shive, 1997). The low molecular weight (M_w 10 500) and a 50:50 PGA:PLA ratio of the selected Resomer® RG 502 H grade, which was shown to be extensively degraded within one week in vitro when formulated in 1.6-μm microspheres (Díez and Tros de Ilarduya, 2006), should avoid deleterious accumulation in lung in case of long-term aerosol treatments.

Table 4
Early RIF released fractions, and correlation of release profiles to the Baker–Lonsdale model.[a]

PLGA (wt.%)	Mean RIF release fractions within 6 h	Correlation coefficient r (release time range) and corresponding mean RIF released fraction ($n = 3$)
3	48%	0.9917 (0–180 h), 88.9%
6	45%	0.9948 (0–180 h), 88.1%
10	59%	0.9534 (0–108 h), 79.1%
		0.9767 (3–108 h), 31.6%
		0.9904 (6–108 h), 20.0%
20	69%	0.9292 (0–108 h), 88.8%
		0.9566 (3–108 h), 35.6%
		0.9837 (6–108 h), 20.0%
		0.9916 (12–108 h), 12.9%
30	74%	0.9378 (0–108 h), 103.4%
		0.9520 (3–108 h), 46.9%
		0.9671 (6–108 h), 29.4%
		0.9783 (12–108 h), 19.6%
		0.9938 (24–108 h), 9.7%

[a] Same experimental data as in Fig. 4.

The microsphere preparation from an O/W emulsion was first investigated, applying three homogenization cycles, which was previously shown to result in monodisperse microspheres (Doan and Olivier, 2009). The transmembrane pressure ΔP_{tm} was set above the critical transmembrane pressure P_c defined by Vladisavljević et al. (2004) as the minimal pressure that makes the emulsion to flow through the porous membrane. In order to obtain monodisperse emulsions, and consequently monodisperse microspheres, ΔP_{tm} should be above a minimal level, i.e. $P_{disruption}$, that generates shear stress sufficient to break up and homogenize the coarse emulsion droplets (Vladisavljević et al., 2004). Beyond $P_{disruption}$, mean emulsion droplets are monodisperse and below the membrane pore size d_p. For d_p = 5.9 or 10.0 μm, $P_{disruption}$ was estimated to be 25 or 10 kPa respectively (Fig. 1). With d_p = 19.9 μm, $P_{disruption}$ was below the lowest ΔP_{tm} (i.e. 5 kPa), since narrowly size-distributed microspheres with size below d_p were obtained at this pressure. For a given d_p and above $P_{disruption}$ increasing ΔP_{tm} resulted in smaller microspheres, therefore smaller emulsion droplets, in agreement with the observations of Vladisavljević et al. (2004). At a given ΔP_{tm} above $P_{disruption}$, varying the pore size from 5.9 to 19.9 μm had remarkably little impact on D_v and an apparently linear relationship was found between D_v and d_p. Microsphere diameters ranged from 4.1 ± 0.1 μm (d_p = 19.9 μm, ΔP_{tm} = 5 kPa) to 0.64 ± 0.1 μm (d_p = 5.9 μm, ΔP_{tm} = 100 kPa) (Fig. 2).

Based on these studies, d_p of 19.9 μm and ΔP_{tm} of 25 kPa were selected for investigating the effect of RIF and PLGA concentrations in the dispersed phase on RIF loading, microsphere diameter and aerodynamic properties, since these two conditions should give robust results in terms of microsphere diameters, i.e. small variations of ΔP_{tm} and d_p would have virtually no impact on microsphere size and span. A RIF:PLGA ratio not higher than 2:3 (i.e. 2 wt.% RIF and 3 wt.% PLGA in the dispersed phase) resulted in satisfactory diameter and span (Table 1) and aerodynamic properties (Table 2). Increasing PLGA concentrations increased diameters and entrapment efficiencies, since at a constant ΔP_{tm}, i.e. a constant shear stress, higher viscosity of the dispersed phase results in larger emulsion droplets (Bahl and Sah, 2000), which solidify into larger microspheres. On SEM, freeze-dried microspheres appeared as narrowly size-distributed spherical spheres with smooth surface (Fig. 3a and b). Microspheres prepared from dispersed phase with 30 wt.% PLGA concentration apparently had internal void space (Fig. 3b, insert), which was attributed to the migration of the polymer towards the surface of the emulsion droplets and its early precipitation close to the oil–water interface as shell-like structures (Li et al., 2008). MMAD increased with D_v according to a non proportional relationship, as shown by the decrease of the MMAD:D_v ratio, and was beyond target with 30 wt.% PLGA concentration (Table 2).

In order to keep MMAD within targets, i.e. between 1 and 5 μm aerodynamic diameter, the preparation of microspheres from $W_1/O/W_2$ emulsions was investigated, since the inner aqueous phase W_1 was shown to leave void spaces within the polymer matrix after drying, thus decreasing the microsphere apparent den-

sity (Edwards et al., 1997). For increasing the RIF microsphere content, an additional RIF fraction was dissolved in the W_1, as a cyclodextrin complex for solubility reasons (Tewes et al., 2008). At a 3 wt.% PLGA concentration, microspheres deriving from $W_1/O/W_2$ emulsions possessed D_v, MMAD and MMAD:D_v ratios similar to microspheres deriving from O/W emulsion (Tables 2 and 3) and similar non-porous surface under SEM observation (Fig. 3a and c), suggesting a similar non porous structure. At low PLGA concentration, the viscosity of the intermediate O phase of the $W_1/O/W_2$ emulsion may not have been high enough to preserve the W_1/O inner emulsion before polymer solidification. Above a 3 wt.% PLGA concentration, microspheres deriving from $W_1/O/W_2$ emulsions (Table 3) were larger than those deriving from O/W emulsions (Table 2). The presence of an inner aqueous phase W_1 may have reduced microsphere shrinkage during solvent removal (Crotte and Park, 1995), reducing the MMAD:D_v ratios compared to microspheres prepared from W/O emulsions and leading to satisfactory respirable fractions (Table 3). The respirable fraction of the microspheres with a D_v of 8.43 μm was in agreement with that of the large porous microspheres reported by Edwards et al. (1997) (diameter of 8.5 μm and a density below 0.1 g cm^{-3} with respirable fraction of 50 ± 10%). It is worth noting that for a given PLGA concentration microspheres prepared from simple or multiple emulsions had similar entrapment efficiencies (Tables 2 and 3). When PLGA concentrations were 3 or 6 wt.%, the additional input of RIF provided by the W_1 phase (i.e. 9 mg) increased RIF contents of the microspheres deriving from the $W_1/O/W_2$ emulsions compared to microspheres deriving from the simple emulsion. At higher PLGA concentrations, the entrapment efficiency of the RIF contained in the oil phase being higher, the advantage of this additional input of RIF provided by W_1 became negligible. RIF contents of microspheres deriving from $W_1/O/W_2$ emulsions were suitable for lung administration as aerosol. Indeed, based on a dosing of 1–2 mg RIF per kg shown to give therapeutic activity in rats (Suarez et al., 2001), the amount of microspheres to be administered into the lungs of a 250-g rat should be from 1.5–10.2 mg.

Due to their appropriate RIF contents and aerodynamic diameters, microspheres deriving from $W_1/O/W_2$ emulsions were selected for in vitro RIF release studies (Fig. 4). Release rates depended on the PLGA concentrations used in the organic phase, with 80% release time ranging from 12 h (30% PLGA concentration) to 4 days (10% PLGA concentration). Microspheres prepared with 3 or 6 wt.% PLGA concentrations gave virtually superimposed release profiles with similar initial burst release (45 and 48% of RIF contents released within 6 h respectively). A good correlation ($r > 0.99$) was obtained with the Baker–Lonsdale model (Table 4), indicating that release was mainly governed by Fickian diffusion (Costa and Sousa Lobo, 2001). Higher PLGA concentrations (10–30%) resulted in higher burst release (59–74% of RIF contents released within 6 h) and to a bad correlation with the Baker–Lonsdale model (Table 4). This observation was attributed to the higher porosity of the microspheres (Fig. 3d) which resulted in larger surface exchange with the release medium and in higher burst release probably mainly governed by a dissolution-controlled mechanism (Gurny et al., 1982). Only a minor RIF fraction, i.e. 9.7–20.0% of the RIF content, was released lately by a diffusion-controlled mechanism (Table 4).

5. Conclusion

Narrowly size-distributed RIF-loaded PLGA microspheres with 80% release of RIF content within from 12 h to 4 days and satisfactory aerodynamic diameters were prepared from $W_1/O/W_2$ emulsions using the premix membrane homogenization method combined with the solvent evaporation process. This work provides a set of microspheres that will be used for further pharmacokinetic studies in rats to define adequate release profiles for RIF sustained release within lungs after pulmonary delivery as aerosols.

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