AAPS PharmSciTech, Vol. 16, No. 5, October 2015 (© 2015)
DOI: 10.1208/s12249-015-0283-3

Research Article

Development of Novel Chitosan Microcapsules for Pulmonary Delivery of Dapsone: Characterization, Aerosol Performance, and *In Vivo* Toxicity Evaluation

Manoel Ortiz,[1] Denise Soledade Jornada,[1] Adriana Raffin Pohlmann,[1,2] and Sílvia Stanisçuaski Guterres[1,3]

Received 27 June 2014; accepted 5 January 2015; published online 5 February 2015

ABSTRACT. *Pneumocystis carinii* pneumonia (PCP) is a major opportunistic infection that affects patients with human immunodeficiency virus. Although orally administered dapsone leads to high hepatic metabolism, decreasing the therapeutic index and causing severe side effects, this drug is an effective alternative for the treatment of PCP. In this context, microencapsulation for pulmonary administration can offer an alternative to increase the bioavailability of dapsone, reducing its adverse effects. The aim of this work was to develop novel dapsone-loaded chitosan microcapsules intended for deep-lung aerosolized drug delivery. The geometric particle size ($D_{4,3}$) was approximately 7 μm, the calculated aerodynamic diameter (d_{aero}) was approximately 4.5 μm, and the mass median aerodynamic diameter from an Andersen cascade impactor was 4.7 μm. The *in vitro* dissolution profile showed an efficient dapsone encapsulation, demonstrating the sustained release of the drug. The *in vitro* deposition (measured by the Andersen cascade impactor) showed an adequate distribution and a high fine particles fraction (FPF= 50%). Scanning electron microscopy of the pulmonary tissues demonstrated an adequate deposition of these particles in the deepest part of the lung. An *in vivo* toxicity experiment showed the low toxicity of the drug-loaded microcapsules, indicating a protective effect of the microencapsulation process when the particles are microencapsulated. In conclusion, the pulmonary administration of the novel dapsone-loaded microcapsules could be a promising alternative for PCP treatment.

KEY WORDS: dapsone; dry powders inhalers; *in vivo* toxicity; microparticles; pulmonary drug delivery.

INTRODUCTION

Opportunistic diseases are frequent in patients with depressed immune systems. *Pneumocystis carinii* pneumonia (PCP) is a major opportunistic disease that affects patients with HIV, infecting between 80 and 85% of patients (1,2). This pneumonia, which has a mortality index of approximately 10 to 20%, is a local pulmonary infection. Its first-choice treatment is the combination of trimethoprim and sulfamethoxazole. This treatment is oral, has low bioavailability in the lungs, and can cause severe side effects, such as rash, nephrotoxicity, and fever (3,4).

Dapsone, 4-[(4-aminobenzene)sulfonyl]aniline, is used to treat several diseases and is effective for the treatment and prevention of PCP (5–7). This drug has a bacteriostatic activity, inhibiting dihydrofolic acid synthesis. When used orally, the drug undergoes a high hepatic metabolism, which decreases the therapeutic index and produces important metabolites that cause severe side effects, including hemolytic anemia, agranulocytosis, and methemoglobinemia (8–10). In this context, microencapsulation can offer an alternative to increase the bioavailability and reduce the adverse effects of dapsone because microencapsulation can protect the drug against extensive metabolism (10).

Microparticulate systems are described as effective drug delivery systems due to their characteristics, such as their high surface areas and their capacities to modulate the drug release profiles and increase the drug bioavailability (11,12). Many methods have been described to produce microcapsules. The main technique to produce dry-powder forms of microparticles is spray-drying because it is a simple method that is performed in one step and is easy to scale up (13). Microcapsules can encapsulate a higher amount of a drug than microspheres because of the lipophilic core of the microcapsules. Due to the different composition of microcapsules and microspheres, microcapsules have an internal phase with a higher viscosity. This feature reduces drug migration to the external phase during the formation and drying processes (14).

Drug administration by the pulmonary route, intended for local or systemic treatment, has been the focus of many studies over the past two decades (15–17). The lungs have a high-alveolar surface area for drug absorption without hepatic first-pass metabolism, also increasing the high amount of drug in the lungs and lowering the metabolism (15,18–20). Metered dose inhalers (MDI) are still the main type of device for

[1] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Avenida Ipiranga, 2752/Lab. 405, Porto Alegre, 90610-000RS, Brazil.
[2] Departamento de Química Orgânica, Instituto de Química, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9500, Porto Alegre, 91501-970RS, Brazil.
[3] To whom correspondence should be addressed. (e-mail: silvia.guterres@ufrgs.br)

pulmonary drug delivery used worldwide. However, dry powder inhalers (DPI) are gaining more visibility by presenting interesting advantages, such as a low cost and an ease of handling, increasing treatment compliance (15,20–22).

Microparticles have been produced using diverse organic polymers, such as polyesters and polysaccharides. Chitosan is a cationic polysaccharide obtained from the deacetylation of chitin, which is found in the exoskeleton of crustaceans. Chitosan is biocompatible and bioadhesive and it is approved by the FDA for pulmonary delivery. The cationic property of chitosan is important because it can open the tight junctions of the cell membrane in the lungs (23,24). In addition, it is able to form films, gels, and microparticles (25,26).

Considering the bioadhesive properties of chitosan, the advantages of microencapsulation, and the high loading capacity of microcapsules, the aim of this study was to develop a new microparticulate system containing dapsone intended for pulmonary delivery. To achieve this effect, dapsone was pre-emulsified with chitosan and raspberry oil in the presence of stabilizers, using a rotor-stator followed by high-pressure homogenization and spray-drying, to produce dry-powder microcapsules. *In vitro* aerosol performance and *in vivo* lung toxicity in rats were also evaluated.

MATERIALS AND METHODS

Materials

Dapsone (Dap) was provided by Deg (São Paulo, Brazil), and chitosan (low molecular weight, deacetylation degree range from 75 to 85%) was purchased from Sigma-Aldrich (São Paulo, Brazil). Raspberry seed oil was obtained from Embapacs (Porto Alegre, Brazil). Sorbitan monooleate and polysorbate 80 were obtained from Sigma-Aldrich (São Paulo, Brazil). All of the other chemicals and solvents were of analytical or HPLC grade. All of the reagents and solvents were used as received.

Methods

Preparation and characterization of Chitosan Microcapsules

Pre-emulsification Step. Initially, an emulsion was prepared by mixing the aqueous phase (0.035 g of polysorbate 80 added to 100 mL of a 1% (*w*/*v*) chitosan solution in acetic acid) and a lipid phase (0.1 g of dapsone, 0.5 g raspberry seed oil, and 0.035 g of sorbitan monooleate solubilized in 20 mL of ethanol) using an Ultraturrax mixer for 5 min (T25 basic IKA®—Staufen, Germany). Afterwards, the emulsion was homogenized in a high-pressure homogenizer (Panda 2K Niro Soavi 1001L—Germany) over 3 cycles at 500 bar. Triplicate batches were produced.

Drying Step. The emulsion was spray-dried using a LabMaq MSD 1.0® (Ribeirão Preto, Brazil) equipped with a 0.7-mm nozzle, with an inlet temperature of 120°C, a feed rate of 0.3 L h^{-1}, an air pressure of 50 kPa, and an outlet temperature ranging from 75 to 85°C. The powder was termed Dap-MC.

Particle Size. The particle size, based on the median geometric diameter (d_{geo}), was measured by laser diffraction using a Mastersizer 2000 equipped with a Sirocco dry disperser (Malvern Instruments, UK) at a dispersing pressure of 2 bar and a feed rate of 70%.

The particle size distribution was measured (n=3), and the results were expressed as the mean diameters ($D_{4,3}$) as well as $d_{0.1}$, $d_{0.5}$, and $d_{0.9}$, which correspond to the respective diameters at 10, 50, and 90% under the cumulative size distribution profiles. The polydispersity was estimated using the span values, which were calculated as $d_{0.9}$ minus $d_{0.1}$, divided by $d_{0.5}$.

Tapped Density and Calculated Aerodynamic Diameter. The tap densities of the powders were determined using a Tapped Density Assessor (J. Engelshmann, Germany). The powder density (ρ) was measured after 1,250 taps. The measurements were performed in triplicate.

The theoretical aerodynamic diameter (d_{aero}) of the microcapsules was calculated from the geometric particle size (d_{geo}) and the tapped density (ρ) using the following Eq. 1 (27).

$$d_{aero} = d_{geo}\sqrt{\left(\frac{\rho}{\rho_0}\right)} \quad (1)$$

where ρ_0=1 g cm^{-3}.

Scanning Electron Microscopy

A morphological analysis (of the shape and surface) of the microcapsules was performed by scanning electron microscopy (SEM) (JEOL JSM-6060, Japan) at the *Centro de Microscopia Eletrônica—UFRGS* (Porto Alegre, Brazil). The powders were put on aluminum stubs with carbon conductive double-sided tape and were sputter-coated with a 15–20-nm layer of gold. The analyses were performed at an acceleration voltage of 10 kV at different magnifications.

HPLC Analysis

High-performance liquid chromatography analyses were performed using a PerkinElmer Series 200 UV/Vis (USA) chromatograph with a LiChrospher 100 RP18 (5 μm) (Merck) column, and the mobile phase used was acetonitrile/water/acetic acid (40:59:1, *v*/*v*/*v*) with a flow rate of 0.7 mL min^{-1} and an injection volume of 100 μL. The drug was detected at a wavelength of 293 nm. The linearity was evaluated with three different curves (0.1–10 μg mL^{-1}), and the correlation coefficient was R^2=0.9972. The limits of detection and quantification were 0.01 and 0.03 μg mL^{-1}, respectively. This method was validated in accordance with International Conference on Harmonisation (ICH).

In Vitro Aerosol Performance

The *in vitro* aerosol performance (n=3) of Dap-MC was evaluated by an 8-stage Andersen cascade impactor (ACI—Erweka, Germany). The ACI is composed of a USP

induction port throat and eight subsequent stages (0–7). Aiming to reduce particle bouncing, the ACI plates were coated with 0.1% (*w*/*v*) of Span 80® solution on *n*-hexane. An Aerolizer® (Novartis, Switzerland) was used as an inhalation device. A gelatin capsule (size no. 3) was filled with 20 mg of the Dap-MC powder. A pre-separator was not used in this experiment. The calibration of the equipment was adjusted using the following parameters: an inhalation rate of 60 L min^{-1} for 4 s and a pressure of 4 kPa. After inhalation, the particles retained in each stage were rinsed off with the mobile phase (acetonitrile/water/acetic acid—40:59:1, *v*/*v*/*v*). The quantity of dapsone in the collected solutions in each stage was analyzed by HPLC (a validated method).

In Vitro Drug Release

A dissolution assay was performed using a Vankel automatic sampling dissolution apparatus (VK 7010). Gelatin capsules (size no. 0) were filed with 150 mg of Dap-MC (n=3 for each). The USP I apparatus (i.e., the baskets) was used to carry out the release experiment. The dissolution medium was PBS at pH 7.4 and a temperature of 37±2°C (in a volume of 900 mL). The rotation rate was 100 rpm, and the *sink* conditions for the dapsone release were preserved. Samples (5 mL) were withdrawn for analysis at specific time points, and dapsone was quantified by UV spectroscopy (Shimadzu, UV-60A—Japan) using a validated method at 293 nm. The profiles were fit to a biexponential equation (Eq. 2) using Micromath Scientist 2.0 software.

$$\%R = 100\left[1\left(Ae^{-\alpha t} + Be^{-\beta t}\right)\right] \quad (2)$$

where $\%R$=cumulative release of dapsone; t=time; and α (0.0033) and β (0.045) are the apparent rate constants of the two lifetime components, into which the decay function is being decomposed. The exponentials α and β represent rapid and slow functions, which could be called the "burst phase" and the "sustained release," respectively (28). The parameters A (46%) and B (54%) correspond to the fractions of the drug release in the burst and the sustained phases, respectively.

In Vivo Lung Toxicity Evaluation

This protocol was approved by the Ethics Committee of the *Universidade Federal do Rio Grande do Sul* (# 21411). For the toxicity study, 16 male Wistar rats weighing between 250 and 350 g, purchased from *Fundação Estadual de Produção e Pesquisa em Saúde* (FEPPS, Porto Alegre, Brazil), were used.

The animals were kept in four separate cages with four animals per cage, and they were maintained on a diet of water and standard laboratory rat food until the beginning of the experiments.

Following the previously adapted reported protocol (17), the animals were intraperitoneally anesthetized with sodium pentobarbital (50 mg kg^{-1}), and anesthesia was maintained with ketamine/xylazine (50 and 13 mg kg^{-1}, respectively). For drug administration, the trachea was exposed, and an incision was performed between the fifth and sixth tracheal rings.

The animals were maintained at an inclination of 45°, and the corporal temperature was maintained at 37°C with the support of a 40-W incandescent heat lamp. The administered treatments were (1) the instillation of 100 μL of a 0.1% sodium dodecyl sulfate solution (for the positive control group), (2) the insufflation of 10 mL of air (for the negative control group), (3) the insufflation of free dapsone, and (4) the insufflation of spray-dried powders of Dap-MC. For the intratracheal administration of treatments 1 and 2, a calibrated syringe was used. Treatments 3 and 4, which are powders, were administered with assistance of a PennCentury (Model DP-4 Dry Powder Insufflator™—USA).

After 4 h, the animals were sacrificed, the lungs were removed, and bronchoalveolar lavage was performed by cannulating the trachea and infusing the lungs three times with 3 mL of saline. The bronchoalveolar lavage fluid (BALF) was centrifuged (4,578x*g* (RCF), 10 min, 4°C), and the supernatant was collected. The supernatant was analyzed, and biochemical parameters—the lactate dehydrogenase activity (29), the alkaline phosphatase activity (25) and the total protein (30,31)—were evaluated using commercial kits (Roche Diagnóstica®). Previous work demonstrated that 4 h is an adequate time interval to assess membrane toxicity (30). The statistical analyses were performed using one-way analysis of variance (ANOVA) following Tukey's posttest to compare the treatments (p<0.05).

Scanning Electron Microscopy of the Pulmonary Tissue

A piece of the deep lungs (near the termination of the bronchioles) was collected and maintained in 2.5% glutaraldehyde solution for fixation under low temperatures (2–4°C). After 1 week, the tissues were dehydrated with acetone and ethanol lavages. After this process, the tissue was dried by replacing acetone and ethanol by liquid carbonic gas (in a CO_2 chamber). Afterwards, the samples were placed on stubs, fixed with double-faced carbon tape, and sputter-coated with a 15–20-nm layer of gold. The samples were analyzed by SEM (JEOL JSM-6060, Japan) at the *Centro de Microscopia Eletrônica*—UFRGS (Porto Alegre, Brazil) using an acceleration voltage of 10 kV at different magnifications.

Statistical Analysis

A statistical analysis of the data was performed via one-way ANOVA with Tukey's posttest using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, USA); a value of p<0.05 was considered significant.

RESULTS AND DISCUSSION

The powders were successfully produced by spray-drying from a pre-formed emulsion. The Dap-MC powder showed a light yellow color and flowed freely. These characteristics are typical of chitosan powder formulations (32). The use of high-pressure homogenization before drying promoted a more homogenous formulation compared with the formulation prepared without this step, as can be observed by the span values (with homogenization, span=1.1; without homogenization, span=2.2).

The emulsion produced by the rotor-stator without using the high-pressure homogenization step showed a mean size of approximately 3 μm (data not shown) and, after spray-drying, the mean diameter of the dry-powder microcapsules was 31 μm. In comparison, the mean size of the emulsion after the rotor-stator followed by a high-pressure homogenization step was 430 nm (data not shown), generating microcapsules with a mean diameter ($D_{4,3}$) of 7 μm after spray-drying.

To estimate if these microcapsules are appropriate for pulmonary administration, we calculated the theoretical aerodynamic diameter. To this end, the median geometric diameter and tapped density of the powders were determined. The aerodynamic diameter is the most important parameter in the development of dry powders intended for lung administration (18,33).

The average particle size distribution (Fig. 1) was determined (n=3). The profile of the geometric particle size distribution was observed in the micrometer range, between 1 and 10 μm. These findings demonstrate that the formulation is uniform and reproducible, with a diameter $D_{4,3}$ of 7.7± 0.828 μm.

Table I shows the median geometric diameters, the span values, the tap density, the calculated theoretical aerodynamic diameters, and the mass median aerodynamic diameter (MMAD) for the mean of the batches of Dap-MC. The average tap density calculated was 0.2885±0.0183 g cm^{-3}. According to the literature, particles with a low density are targeted in powders for pulmonary administration because of their better dispersibility and aerosol performance (34). The average calculated aerodynamic diameter was 4.57±0.61 μm, a value appropriate for pulmonary administration (0.5–5 μm) (18,35). From the ACI, the MMAD obtained was 4.70 ±0.43 μm, a value similar to the calculated theoretical aerodynamic diameter. In the cumulative profile the of particle size (Fig. 2), 50% of the population have diameters <5 μm, corroborating the first results. Considering the findings, the chitosan microcapsules are suitable for pulmonary drug delivery in terms of their particle size.

The shape, topography, and size of the primary particles and the presence or absence of pores were evaluated by scanning electron microscopy (Fig. 3) because this technique is able to characterize these properties (34,36). The samples showed a spherical shape, corroborating the results of the previous works carried out with solid chitosan particles (32,37). The images showed microcapsules with smooth surfaces and without macropores, and the size of the primary particles was below 5 μm, lower than that measured using laser diffraction. This difference can be attributed to a part of the population of particles that remain agglomerated during the laser diffraction analysis.

Table I. Characterization of Three Batches of Dap-MC

Formulation	d_{geo} (μm)	Span	ρ (g cm^{-3})	d_{aero} (μm)	MMAD (μm)
Mean	7.71	1.21	0.29	4.57	4.70
S.D. (±)	0.82	0.07	0.01	0.61	0.43

d_{geo} median geometric diameter (dispersing pressure of 2 bar), P tap density, d_{aero} theoretical aerodynamic diameter calculated, *MMAD* median mass aerodynamic diameter

To verify the *in vitro* aerosol performance, the powders were tested using an ACI. The *in vitro* aerosol evaluation of Dap-MC showed an adequate distribution (Fig. 4). The amounts of aerosol in the inhalation device and in the capsule were insignificant. Approximately 40% of the total amount of microcapsules, in terms of the drug content, was in stage 0, which represents the total amount from stage 0 to the USP throat. This stage represents the oropharyngeal region (i.e., the mouth and the throat) of the respiratory tract. This result most likely occurred due to a fraction of particles that do not deagglomerate in the moment of inhalation. However, approximately 60% of the microcapsules were distributed along the other stages. The deposition of Dap-MC in stages 2 to 7 represents the fraction of particles with particle sizes lower than 5.8 μm. The fine particle fraction (FPF) represents the fraction that can arrive in the deepest portion of the lungs and the therapeutic fraction of the dry-powder inhalers. The aerodynamic characteristics of Dap-MC revealed high FPF values, which reached approximately 50%. Elevated FPF values are a requirement for the treatment of pulmonary diseases, aiming for deposition in the deepest parts of the lungs (38). These results indicate the high aerosol performance of Dap-MC. An important aspect is that the powder was prepared without needing drying adjuvants, and the powder showed significant aerodynamic characteristics for its use for pulmonary administration.

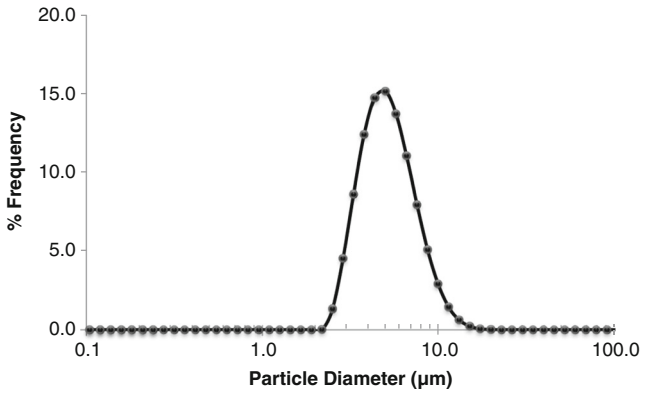

Fig. 1. Laser diffraction of geometric size distribution of Dap-MC. Average analysis of three different batches using Scirocco dry disperser

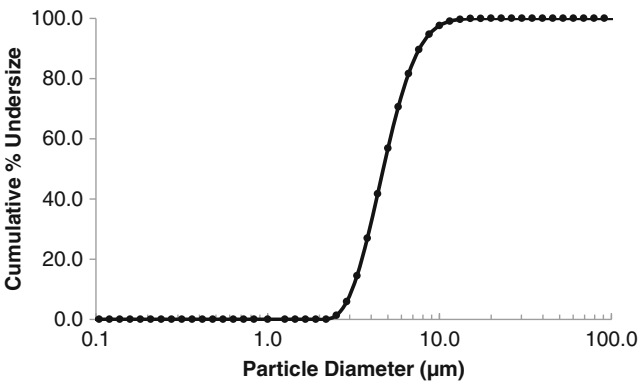

Fig. 2. Cumulative size distribution of Dap-MC, measured by laser diffraction

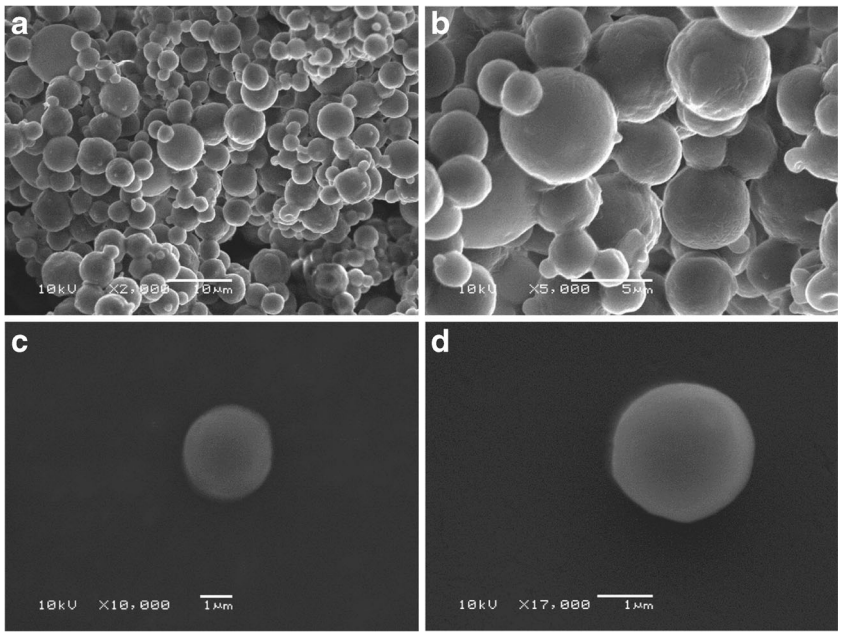

Fig. 3. SEM images of Dap-MC at different magnifications: **a** (×2,000); **b** (×5,000); **c** (×10,000); and **d** (×17,000)

The *in vitro* dapsone dissolution/release profiles were developed using a dissolution apparatus (Fig. 5). The aim of this study was to compare the dissolution of the microcapsules with the dissolution of free dapsone and to evaluate the influence of the microcapsules on the dissolution profile of the drug. For the free drug, it can be noted that approximately 80% was dissolved within 30 min of experiment, and after 200 min, all of the drug was dissolved in the PBS medium. In the case of Dap-MC, after 200 min, 60% of the drug was released, and after 1,440 min, almost all of the drug was released. The result showed that the dissolution of the free drug was significantly different of that of Dap-MC.

To identify the most appropriate mathematical model to describe the drug release profile, different kinetic models were studied. The release of dapsone from Dap-MC showed two different phases. These experimental data fit a biexponential model. The determined coefficient of determination was 0.9858. A burst phase, represented by 54.5% of the dapsone being released from the microcapsules within the first 30 min, and a sustained phase, showing a controlled release profile until the end of the experiment (i.e., 45.5% of the dapsone), were observed. The burst phase can occur due to three main reasons: (1) the presence of an amount of non-encapsulated drug solubilized by the surfactant during the preparation, (2) the superficial location by the sorption of the drug on the microcapsules, and (3) the high polydispersity of the formulation, with the simultaneous occurrence of small and large particles in the sample. Drug crystals were not found in the sample according to the SEM analysis. The particle surfaces did not present macropores where the drug could be superficially retained. Therefore, the dapsone release profile from the microcapsules is most likely due to the polydispersity of the powder. Theories suggest that the release rate of a spherical particle shape with radius r is directly proportional to $1/r^2$. This relation justifies why smaller particles can show a rapid initial release (28,39).

The analysis of the BALF is a useful method for sampling the biochemical components from the lung, and it can indicate if local damage has occurred. This protocol was performed aiming to investigate whether Dap-MC causes any damage in the lungs compared with the administration of the free drug. Generally, the activity of lactate dehydrogenase (LDH) is an important biochemical parameter to evaluate pulmonary cell damage because LDH is a cytosolic enzyme, and the presence of LDH in the BALF is evidence of cell membrane injury (30). The LDH levels were evaluated (Fig. 6). The group treated with the positive control (SDS) showed a level of LDH activity twofold higher (398.00±28.87 U/L) compared with that of the insufflated air group (186.75±7.17 U/L). The negative control (air), free dapsone, and Dap-MC showed significantly ($p<0.05$) lower levels (186.75±7.17, 297.00±32.63, and 220.75 ±1.93 U/L, respectively) than the positive control group (SDS, 398.00±28.87 U/L). Comparing the dapsone group and the microcapsules group, no significant difference was observed. However, the Dap-MC group was statistically equal to the

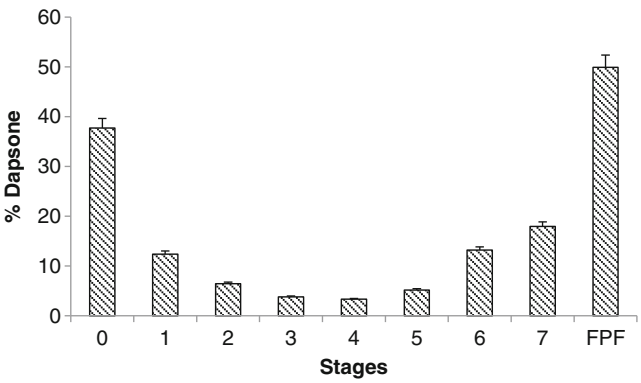

Fig. 4. Aerosol performance of Dap-MC using Andersen cascade impactor simulating an *in vitro* inhalation. FPF represented the sum of stages 2–7. Each point represents the mean±S.D. (n=3)

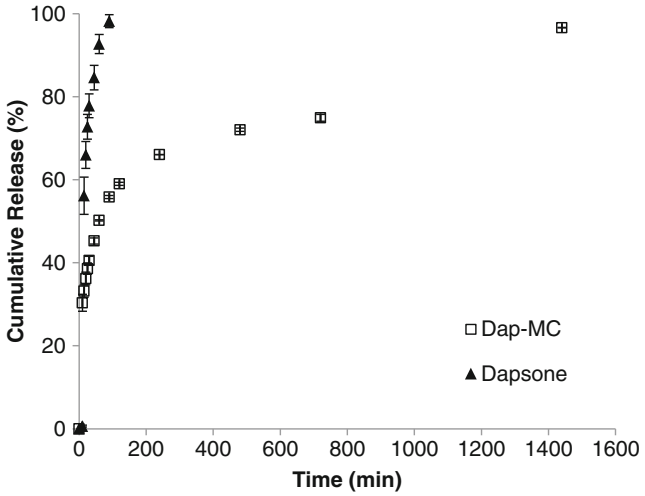

Fig. 5. The *in vitro* cumulative release profiles of free drug and Dap-MC in PBS, pH 7,4 at 37°C

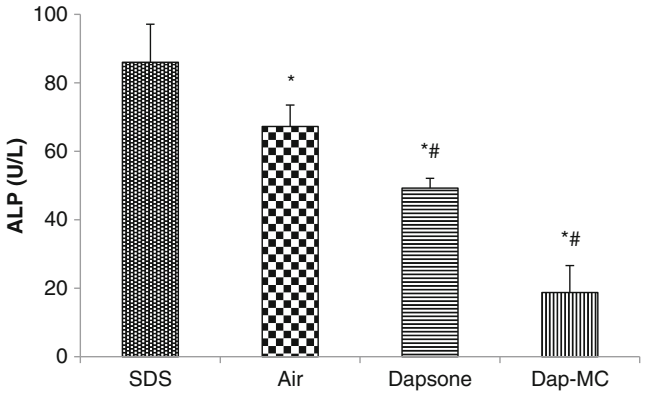

Fig. 7. Alkaline phosphatase levels in the bronchoalveolar lavage fluid (BALF) after intratracheal administration of the following: sodium dodecyl sulfate (SDS), insufflated air, dapsone, and Dap-MC. The *asterisk* denotes significant differences when compared with SDS. The *hashtag* denotes significant differences when compared with dapsone

negative control group (i.e., the air group). These results indicate that the chitosan microcapsules presented low lung toxicity, considering the analysis of the bronchoalveolar lavage fluid.

Likewise, alkaline phosphatase (ALP), which is a membrane-bound enzyme, has been considered as a marker to verify pulmonary cell damage (40). The determination of ALP levels in the BALF demonstrated high ALP values for the SDS-treated group (86.00±6.42 U/L). However, the negative control (air), the free dapsone group, and the Dap-MC group showed significantly lower ALP values ($p<0.05$) (67.25 ±3.61, 49.25±1.65, and 18.75±4.55 U/L, respectively) (Fig. 7). In addition, comparing the free dapsone group (49.25±1.65 U/L) with the Dap-MC group (18.75±4.55 U/L), a significant decrease can be observed, demonstrating that the microencapsulation of dapsone reduces the cytotoxic effect of the drug.

Another marker to evaluate pulmonary cell damage is the determination of total protein in the BALF, which indicates membrane lysis, because proteins are released if the cell membrane is damaged (40). The total protein contents in the BALF (Fig. 8) were higher for the SDS-treated group (91.33 ±0.67 U/L) and significantly lower for the free dapsone group (89.00±1.73 U/L) and for the Dap-MC group (75.33±1.67 U/L). Taking the results of all of the biochemical parameters in BALF into account, we can conclude that the microencapsulation of dapsone reduces pulmonary cytotoxicity.

The SEM images of the lungs were observed after the BALF experiment for the different treated groups (Fig. 9). Segmented and solid arrows indicate erythrocytes and microcapsules, respectively. Tissue damage can be observed due to the presence of erythrocytes in the SDS group (Fig. 9c). Similar damage of the pulmonary tissue, represented by a more pronounced presence of blood cells, was observed in the dapsone group (Fig. 9b). This finding indicates that vascular injury and an overflow of erythrocytes to the pulmonary tissue occurred, which corroborates the biochemical parameters in the BALF experiment, indicating the cytotoxicity of dapsone. The Dap-

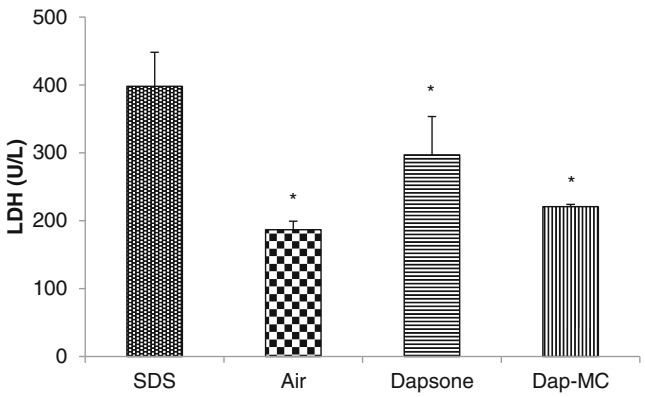

Fig. 6. Lactate dehydrogenase activity (LDH) in the bronchoalveolar lavage fluid (BALF) after intratracheal administration of the following: sodium dodecyl sulfate (SDS), insufflated air, dapsone, and Dap-MC. The *asterisk* denotes significant differences when compared with SDS

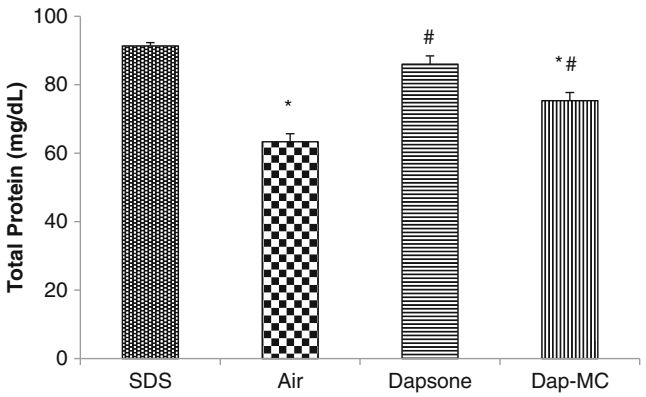

Fig. 8. Total protein content in the bronchoalveolar lavage fluid (BALF) after intratracheal administration of the following: sodium dodecyl sulfate (SDS), insufflated air, dapsone, and Dap-MC. The *asterisk* denotes significant differences when compared with SDS. The *hashtag* denotes significant differences when compared with dapsone

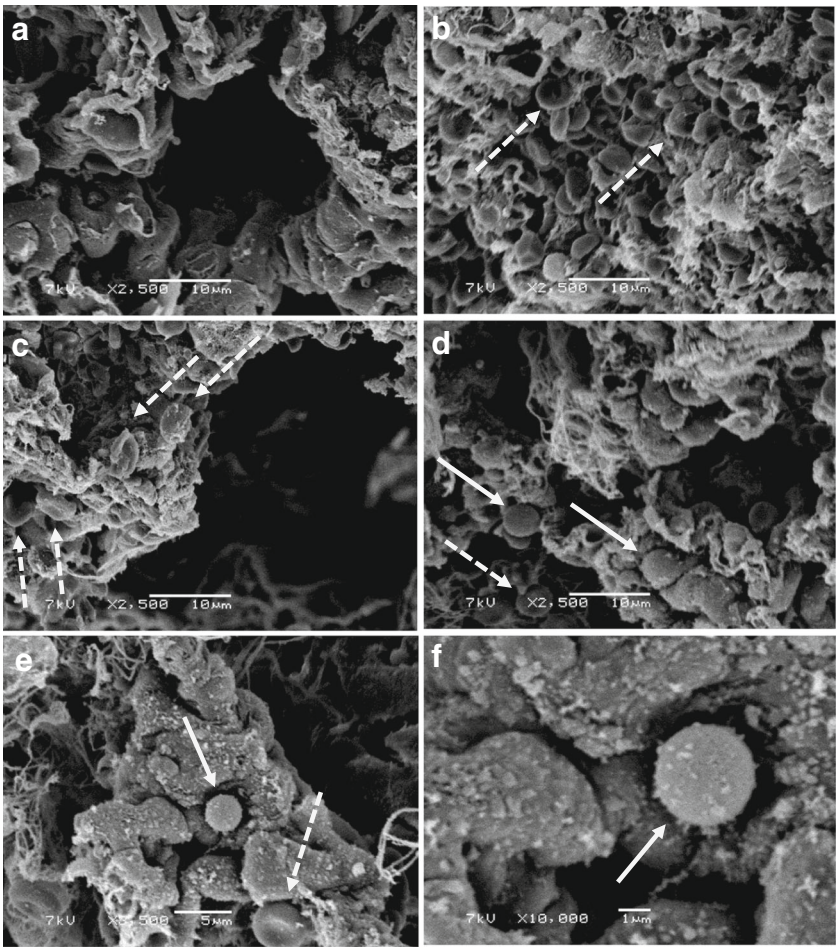

Fig. 9. Lungs scanning electron microscopy (SEM) images of each treated group. **a** air group; **b** dapsone group; **c** SDS group; **d–f** Dap-MC. (*segmented arrows* indicate erythrocytes, the *solid arrows* indicate microcapsules)

MC group (Fig. 9d–f) showed few erythrocytes in the tissue, and the incidence was much less intense than that observed for the dapsone group and for the SDS group. Interestingly, the presence of one isolated Dap-MC was visualized with a size of approximately 2.5 μm in the deep pulmonary tissue (Fig. 9f). The microcapsule was inserted into a bronchiole tube with a diameter of 3 μm, indicating that Dap-MC could arrive in the deepest parts of the lungs.

CONCLUSION

The dapsone-loaded chitosan microcapsules prepared by spray-drying demonstrated promising results for pulmonary drug delivery. The aerosol performance of Dap-MC showed an elevated FPF, indicating that 50% of the particles are within the size range adequate for therapeutic action. The BALF experiment showed the low cytotoxicity of the microencapsulated drug, when compared to the control groups, shown by the biochemical parameters and by the photomicrography of the pulmonary tissues. SEM analysis demonstrated an adequate deposition of Dap-MC in the deepest part of the lung. All of the results suggest that the pulmonary administration of the novel dapsone-loaded microcapsules could be a promising alternative for the treatment of PCP.

ACKNOWLEDGMENTS

The authors wish to thank CAPES, Rede Nanotecnologia Farmacêutica CAPES, CNPq/Brasilia/Brazil, INCT-IF CNPq/MCT, Pronem and Pronex FAPERGS-CNPq, and FAPERGS.

Conflict of Interest The authors report no conflicts of interest.

REFERENCES

1. Gutierrez S, Morilla R, Leon JA, Martin-Garrido I, Rivero L, Friaza V, *et al*. High prevalence of *Pneumocystis jiroveci* colonization among young HIV-infected patients. J Adolesc Health. 2011;48(1):103–5.
2. Bennett NJGSAR, Burton F, McLean, J C, Murray C, Schreibman T S, Rigsby M. *Pneumocystis* (*carinii*) *jiroveci* pneumonia. 2010. http://www.patient.co.uk/doctor/pneumocystis-jirovecii-pneumonia

3. Fishman JA. Treatment of infection due to *Pneumocystis carinii*. Antimicrob Agents Chemother. 1998;42(6):1309–14.
4. Kovacs JA, Gill VJ, Meshnick S, Masur H. New insights into transmission, diagnosis, and drug treatment of *Pneumocystis carinii* pneumonia. JAMA-J Am Med Assoc [Article]. 2001;286(19):2450–60.
5. Ramesh M, Chandrasekar PH. Effective alternates to trimethoprim-sulfamethoxazole as antimicrobial prophylaxis in stem cell recipients: are there any? Pediatr Transplant. 2008;12(8):823–6.
6. Wolf R, Tuzun B, Tuzun Y. Dapsone: unapproved uses or indications. Clin Dermatol. 2000;18(1):37–53.
7. Blum RN, Miller LA, Gaggini LC, Cohn DL. Comparative trial of dapsone versus trimethoprim/sulfamethoxazole for primary prophylaxis of *Pneumocystis carinii* pneumonia. J Acquir Immune Defic Syndr. 1992;5(4):341–7.
8. Coleman MD. Dapsone-mediated agranulocytosis: risks, possible mechanisms and prevention. Toxicology. 2001;162(1):53–60.
9. Reddy C, Kannan G, Vasantha J, Kousalya K, Rani N, Thennarasu P, *et al*. Drug usage evaluation of dapsone. Indian J Pharm Sci. 2009;71(4):456–60.
10. Chougule M, Padhi B, Misra A. Development of spray dried liposomal dry powder inhaler of dapsone. AAPS PharmSciTech. 2008;9(1):47–53.
11. Ravi Kumar MN. Nano and microparticles as controlled drug delivery devices. J Pharm Pharm Sci. 2000;3(2):234–58.
12. Jyothi NV, Prasanna PM, Sakarkar SN, Prabha KS, Ramaiah PS, Srawan GY. Microencapsulation techniques, factors influencing encapsulation efficiency. J Microencapsul. 2010;27(3):187–97.
13. Oneda F, Re MI. The effect of formulation variables on the dissolution and physical properties of spray-dried microspheres containing organic salts. Powder Technol. 2003;130(1):377–84.
14. El-Gibaly I. Development and in vitro evaluation of novel floating chitosan microcapsules for oral use: comparison with non-floating chitosan microspheres. Int J Pharm. 2002;249(1–2):7–21.
15. Frijlink HW, De Boer AH. Dry powder inhalers for pulmonary drug delivery. Expert Opin Drug Deliv. 2004;1(1):67–86.
16. Grenha A, Seijo B, Remuñán-López C. Microencapsulated chitosan nanoparticles for lung protein delivery. Eur J Pharm Sci. 2005;25(4–5):427–37.
17. Cruz L, Fattal E, Tasso L, Freitas GC, Carregaro AB, Guterres SS, *et al*. Formulation and in vivo evaluation of sodium alendronate spray-dried microparticles intended for lung delivery. J Control Release [Article]. 2011;152(3):370–5.
18. Pilcer G, Amighi K. Formulation strategy and use of excipients in pulmonary drug delivery. Int J Pharm. 2010;392(1–2):1–19.
19. D'Addio SM, Chan JG, Kwok PC, Prud'homme RK, Chan HK. Constant size, variable density aerosol particles by ultrasonic spray freeze drying. Int J Pharm. 2012;427(2):185–91.
20. Zhang X, Ma Y, Zhang L, Zhu J, Jin F. The development of a novel dry powder inhaler. Int J Pharm. 2012;431(1–2):45–52.
21. de Boer AH, Wissink J, Hagedoorn P, Heskamp I, de Kruijf W, Bunder R, *et al*. In vitro performance testing of the novel Medspray wet aerosol inhaler based on the principle of Rayleigh break-up. Pharm Res. 2008;25(5):1186–92.
22. Xu L, Dong XW, Shen LL, Li FF, Jiang JX, Cao R, *et al*. Simvastatin delivery via inhalation attenuates airway inflammation in a murine model of asthma. Int Immunopharmacol. 2012;12(4):556–64.
23. Mukhopadhyay P, Mishra R, Rana D, Kundu PP. Strategies for effective oral insulin delivery with modified chitosan nanoparticles: a review. Prog Polym Sci. 2012;37(11):1457–75.
24. Zhang J, Zhu X, Jin Y, Shan W, Huang Y. Mechanism study of cellular uptake and tight junction opening mediated by goblet cell-specific trimethyl chitosan nanoparticles. Mol Pharm. 2014;11(5):1520–32.
25. Price N, Newman B. Demonstration of the principles of enzyme-catalysed reactions using alkaline phosphatase. Biochem Mol Biol Educ. 2000;28(4):207–2010.
26. Vargas M, Albors A, Chiralt A, González-Martínez C. Characterization of chitosan–oleic acid composite films. Food Hydrocolloids. 2009;23(2):536–47.
27. Wang H-C, John W. Particle density correction for the aerodynamic particle sizer. Aerosol Sci Technol. 1987;6(2):191–8.
28. Washington C. Drug release from microdisperse systems: a critical review. Int J Pharm. 1990;58(1):1–12.
29. Babson A, Philips GE. A rapid colorimetric assay for serum lactic dehydrogenase. Clin Chemica Acta. 1965;12:265–75.
30. Hussain A, Majumder QH, Ahsan F. Inhaled insulin is better absorbed when administered as a dry powder compared to solution in the presence or absence of alkylglycosides. Pharm Res. 2006;23(1):138–47.
31. Layne E. Total protein-modified according to Layne, E., spectropho-tometric and turbidimetric methods for measuring proteins. II. Protein estimation with the Folin-Ciocalteu reagent. Methods Enzimol. 1957;3:447–54.
32. Learoyd TP, Burrows JL, French E, Seville PC. Sustained delivery by leucine-modified chitosan spray-dried respirable powders. Int J Pharm. 2009;372(1–2):97–104.
33. Okamoto H, Shiraki K, Yasuda R, Danjo K, Watanabe Y. Chitosan-interferon-beta gene complex powder for inhalation treatment of lung metastasis in mice. J Control Release. 2011;150(2):187–95.
34. Vehring R, Foss WR, Lechuga-Ballesteros D. Particle formation in spray drying. J Aerosol Sci. 2007;38(7):728–46.
35. Pilcer G, Wauthoz N, Amighi K. Lactose characteristics and the generation of the aerosol. Adv Drug Deliv Rev. 2012;64(3):233–56.
36. Li FQ, Yan C, Bi J, Lv WL, Ji RR, Chen X, *et al*. A novel spray-dried nanoparticles-in-microparticles system for formulating scopolamine hydrobromide into orally disintegrating tablets. Int J Nanomedicine. 2011;6:897–904.
37. Alhalaweh A, Andersson S, Velaga SP. Preparation of zolmitriptan-chitosan microparticles by spray drying for nasal delivery. Eur J Pharm Sci. 2009;38(3):206–14.
38. Aquino RP, Prota L, Auriemma G, Santoro A, Mencherini T, Colombo G, *et al*. Dry powder inhalers of gentamicin and leucine: formulation parameters, aerosol performance and in vitro toxicity on CuFi1 cells. Int J Pharm. 2012;426(1–2):100–7.
39. Wu P-C, Huang Y-B, Chang J-S, Tsai M-J, Tsai Y-H. Design and evaluation of sustained release microspheres of potassium chloride prepared by Eudragit®. Eur J Pharm Sci. 2003;19(2–3):115–22.
40. Henderson RF. Use of bronchoalveolar lavage to detect respiratory tract toxicity of inhaled material. Exp Toxicol Pathol. 2005;57 Suppl 1:155–9.