Isoniazid and Pyrazinamide pro-liposome dry powder aerosol

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Abstract

INH or PZA, the first line water soluble antituberculosis agent, was prepared as pro-liposome dry powder by spray dried methods. The spray dried mannitol and spray dried INH or PZA was blended in the concentration of 10% INH or PZA. The blended was dispersed in absolute ethanol containing the SPC and CH and then was spray dried. The obtained dry powder was examined for aerosol dispersion performance by using ACI apparatus. The MMAD of every formulation is higher than 5 μm (8.0 -9.0 μm). However the % FPF and % ED displayed satisfactory values for aerosol (30% FPF and 80% ED). From SEM images, the individual particles are in range of 1-5 μm which is able to reach deeply to the alveoli. All of pro-liposome formulations did not produce any toxicity to respiratory cell lines. In addition, after the powders were challenged with AM, no immunological action from AM to our products.

1. Introduction

Isoniazid (INH) and pyrazinamide (PZA) are hydrophilic first line antituberculosis drugs which cause rarely adverse drug events. The most occurred side effect of INH is hepatic injury, which may result in hepatitis in a small fraction of patients. The usual adult dose of INH is a single dose of 5 mg /kg/day up to a maximum of 300 mg daily. For PZA, the usual dose is 20–25 mg/kg daily. The most common (approximately 1%) side effect of PZA is joint pains and the most dangerous side effect of PZA is hepatitis, which is related to dose (Riedel, 2000).

TB is caused by the bacteria namely *Mycobacterium tuberculosis*. *M. tuberculosis* lodged to alveoli after direct inhalation of small aerosolized droplets that contain the tubercle bacilli and then is phagocyted by alveolar macrophage (AM) and initiate TB disease (Frieden, 2003). It is laborious to eradicate the tubercle bacilli which are secured from antibiotic under the phagosome. AM is likely to phagocyte the liposome vesicles (Ahsan, 2002). This is expected to facilitate intracellular delivery of antituberculosis encapsulated liposome to host cell of TB bacilli.

Liposomes, which has a size ranging from 10 nm to 20 μm, are composed of lipid bilayers surround the aqueous compartments. Hydrophobic drugs can be incorporated into the lipid bilayers, while

hydrophilic drugs are usually entrapped in the aqueous compartments. The preparation method is the one important influence to produce different types of liposome vesicle. For hydrophilic drug, it is quite difficult to obtain high % encapsulation (Ulrich, 2002). To overcome this problem, spray dryer was used to produce pro-liposome powder. Spray drying is able to produce the spherical particles in respirable ranges of 1-7μm which is suitable for the delivery of drugs as dry powder inhalers (Aulton, 2002). Pro-liposome is a dry free flowing particle which could form liposome vesicle after they are reconstituted in water. Additionally, pro-liposome also improve physical stability in aqueous suspension of liposome vesicle since it is in dry powder form.

Because of there is no natural barrier between AM and liposome delivered to the airway, thus aerosolized delivery is the most appropriate route of administration (Rooijen, 1998). Furthermore, localized delivery of drugs to the respiratory tract is the effective therapeutic method for treating of pulmonary disorders. Relatively small doses are required for effective therapy, reducing systemic exposure to drug and thus minimizing adverse effects (Suarez, 2000). The particle size plays an important role in indicating the amount of drug reaching the target site which related to the efficacy of therapeutic aerosols. Particles with aerodynamic diameter (d_{ae}) between 2

and 4 μm are able to deposit the peripheral (alveolar) airways (Mitchell, 2004).

DPIs create aerosols by drawing air through a dose of dry powder medication. DPIs rely on the the inspiratory flow rate to disperse the powder. A faster rate will generate an aerosol with finer particles, resulting in improved lung deposition. There is no need for coordination between actuation and inhalation (Shangle, 1999, James, 2000).

To target the pro-liposome powder to alveolar macrophage, the most important things to be concerned other than the fine particle size is the immunological responses of alveolar macrophage to the particles. The safety testing of particles reaching the airways will be determined in cell lines to ensure that product does not provoke any toxic chemicals in the airways.

2. Materials and Methods

2.1 Material

Cholesterol from lanolin, D-(+)-lactose monohydrate, L-α-phosphatidylcholine from soybean (SPC) and Dicetylphosphate (DCP) were purchased from Fluka, Switzerland. Isoniazid (INH), pyrazinamide (PZA), mannitol and lipopolysaccharide (LPS) from *E.coli* were obtained from Sigma Chemical company (St. Louis, MO, USA). Sodium dihydrogen

phosphate was purchased from Riedel-de Haën, Germany and Acetonitrile were obtained from J.T. Baker (NJ, USA). All these solvents were high performance liquid chromatography (HPLC) grades. All plastic wares of cell culture were obtained from Corning (NY, USA). All other used materials were of analytical grade and used as received. Milli-Q-water (Millipore, Watford, UK) was used in all preparations.

2.2 Spray dry mannitol, INH or PZA powder

Mannitol, INH and PZA were dissolve in deionized water in concentration of 10 mg/ml. The spray dryer (BÜCHI) was operated at inlet temperature 110° C, atomizer pressure at 800 KPa at a flow rate of 2.8 ml/min. This micronised particle were used as a core carrier of pro-liposome preparation.

2.3 Blended of micronised mannitol and INH or PZA and content uniformity determination

The micronised particle of either INH or PZA was blended with micronised mannitol in concentration of 10% w/w. The blended powder was mixed in the mixer for 20 minutes.

2.4 Pro-liposome containing INH or PZA

The pro-liposome powders were prepared by spray dry method. The ingredients of the formulations are shown in Table 1. SPC and CH was weighed and dissolved in mannitol saturated ethanol 100 ml. The powder blended, 1 g, obtained from step 2.3 was added and sonicated to deaggregate the fine particles. The spray dryer was operated at the conditions in Table 2. The powder was collected and separated from air flow by cyclone.

2.5 Scanning electron microscopy

The powder of pro-liposome was sprinkled in double-sided adhesive tape and coated with platinum of 50 nm thickness. The specimen was observed under JSM 6000F, high resolution field emission SEM, Jeol, Tokyo, Japan

2.6 Content uniformity of INH and PZA

10 mg of pro-liposome powder was weighed for 10 replicates. 0.025% Sodium dodecyl sulfate (SDS) in water was used to dissolve the pro-liposome and adjusted to desired volume of 50 ml volumetric flask. The clear solution was obtained and determined INH and PZA by HPLC technique. C18 was used with 0.05 M KH_2PO_4 : ACN 97: 3 as a mobile

phase system under the flow rate of 1.0 ml/min. The UV wavelength 254 nm was used to detect the absorbance of INH and PZA.

2.7 Encapsulation of reconsititue INH or PZA pro-liposome

5 mg of pro-liposome powder was dispersed in 2 ml phosphate buffer solution (PBS) pH 7.4 in microcentrifuge tube. The obtained suspension was centrifuged for 10 minutes at 13400 rpm. The supernatant was separated and analyzed the drug content as unencapsulated drug. The precipitate was dissolved with 0.025% SDS in water and determined the drug content as encapsulated drug. The percentage of encapsulation was calculated following this equation.

$$\text{Percent encapsulation} = \frac{\text{encapsulation drug amount} \times 100}{\text{unencapsulation drug amount} + \text{encapsulation drug amount}}$$

2.8 In vitro deposition of INH or PZA pro- liposome dry powder by Andersen Cascade Impactor (ACI)

Andersen Cascade Impactor (ACI, Atlanta, Georgia, USA) is the official apparatus using for determination aerosol particles size. ACI composed of 8 stages (stages 0-7), Each stage has multi-orifices which the orifices display the progressively smaller from top to bottom. The particles

should deposit on stage 5-7 which refer to alveoli *in vivo*. The ACI was applied with a vacuum pump under flow rate of 60 L/min for 10 sec. 0.025% SDS in water was used to rinse particles deposited on each stage. INH or PZA content deposited on each stage was determined by HPLC. The cumulative percentage of deposition was transformed to Z-value and plotted against log cut off diameter of each stage. The mass median aerodynamic diameter (MMAD) is the particle diameter at Z-value of zero. % Emitted Dose (%ED) and % Fine Particle Fraction (%FPF) were calculated from the percentage of drug propelled from delivery device, and the percentage of drug deposited on stage 1-7, respectively.

2.9 Cytotoxicity of RIF encapsulated liposome to alveolar macrophage.

2.9.1 Cell culture

Rat alveolar macrophage, AM (NR8383) obtained from ATCC were cultured in modified Ham F 12 K supplemented with 15% FBS. Cell culture was incubated at 37 $^{\circ}$C in 5% CO_2 incubator. The cell density of $5x10^5$ cells/ml (100 µl) was seeded into each well and incubated overnight. After incubation, the supernatant in the well was discarded and warmed fresh media (100 µl) was added. 100 µl of samples was added into each well.

Cultured media (100 µl) was used as a negative control while LPS from *E.coli* was used as a positive control in all experiments.

2.9.2 MTT assay

MTT was used to examine the toxicity of INH or PZA pro-liposome. After incubation the pro-liposome powder with cell line 24 h, the supernatant was taken from the well to examine the level of toxic cytokine. Each well was carefully washed each wells with 200 µl fresh media for 3 times. 150 µl of warmed fresh media and 50 µl of MTT solution (5 mg/ml in PBS) were added into each well and further incubated for 4 h. The supernatant was carefully removed and the remaining precipitated formazan salt was dissolved by 200 µl DMSO. The absorbance of formazan salt solution was measured by the microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 540 nm. Percent viable cell was calculated as compared to negative control of untreated well.

2.10 Immunological responses of alveolar macrophage to RIF encapsulated liposome

2.10.1 Nitric oxide assay by the Griess reaction

Nitric oxide was determined by the Griess reaction as a following description. 100 µl of Griess reagent (0.1% NED (N-(1-naphthyl)-

ehtylenediamine dihydrochloride) and 1% sulfanilamide in 2.5% phosphoric acid) was mixed with an equal volume of experimental cell supernatant. This causes a pink solution for a positive and yellow solution for negative result. The reaction was recorded quantitatively at 540 nm based on a standard curve of $NaNO_2$.

2.10.2 IL-1β examination by ELISA technique

ELISA kits (Quantikine®, R&D Systems, MN, USA) were used to determine IL-1β in cell supernatants following the procedure described in manufacture manual. The minimum detectable dose of both IL-1β is typically less than 5 pg/ml.

Result and Discussion

INH and PZA are water soluble antituberculosis agents. Generally, it is very difficult to obtain high percent encapsulation of water-soluble drug in liposome. To Coat the SPC and CH on the very fine particles of INH or PZA produce the pro-liposome type vesicle. Directly deliver of this pro-liposome powder to alveolar macrophage may produce intracellular INH and PZA concentration after phagocytosis this particles of AM.

The spray dryer is the one of important instrument used for produce the very fine particles suitable for aerosol delivery. As showed in SEM image

(Figure 1), we can produce 3 μm mannitol and 3 μm PZA by spray dried method. For INH, spray dry process under the above condition gave irregular shaped particles as showed in the Figure 1 which displayed the blended of mannitol and INH. In addition, we try to spray dry INH by several conditions; we cannot get the high yield of INH spray dry powder. For this reason, we can not complete INH experiment. However, we did the spray dry to produce the pro-liposome powder for 2 conditions as shown in Table 2. The significantly different of powder yield was shown as the condition 2 gave higher yield (> 500 mg) while condition 1 gave very low yield (< 100 mg). After preparation of pro-liposome powder by spray dry powder technique, the content uniformity result was determined and the results are shown in Table 3. The PZA content in the formulation has uniformity in every formulation. However, by the spray dry condition 2, we obtained the higher yield of with the lower drug content (1200-1300 μg with condition 1 and 500-600 μg with condition 2). This may due to the more content of mannitol was sucked to the cyclone. The drug content obtained from both conditions are very low as compared to the theoretical content (~1800 μg). This may due to our PZA individual particle is very small, thus the particle was sucked out to the exhaust air and loss some amount of drug. The percentage of encapsulation of PZA or INH in the liposome after

reconstitute the pro-liposome powder in PBS pH 7.4 is shown in Table 4. The encapsulation capacity of PZA or INH is very low and independent to the lipid content. The drug amount encapsulated in the liposome vesicles was not significantly different in each formulation. The SEM images of the pro-liposome dry powers are shown in Figure 2 for PZA and Figure 3 for INH. The individual particles are smaller than 5 μm. However, these particles become to aggregate to be a bigger particles since the SPC displayed a sticky material and act as a binder of each particles. These are able to observe the linkage between particles in the high magnification SEM images (B). The more SPC, Rx3 and Rx4, produced the larger particle aggregations. The aerosol dispersions of these pro-liposome powders are shown in Table 5. Even if, the MMAD is larger than 5 μm, the obtained % FPF and % ED is suitable for aerosol formulation. However, from PZA data, the spray dry condition 2 tends to give the smaller MMAD. To obtain the MMAD less than 5 μm is to break the aggregate between individual particles caused by the SPC. Surprisingly, from SEM images, the aggregated particles were a larger than 10 μm but the MMAD displayed only 8 μm and not significantly different among different formulations. This may indicate that the aggregated particles may break up after exposed to the inhaled air.

These pro-liposome formulations are likely to be safe for respiratory tract. The pro-liposome did not cause any cell death when the cell lines were exposed with the products for 48 h. MTT was used to determine the viability of the AM cell line. As shown in Figure 5, the % viability of cell line compared to untreated cell lined is almost 100%. For the immunological examination, IL-1β, TNF-α and NO, the AM did not produce any of these toxic chemicals as compared to lipopolysaccharide (LPS) from *E. coli* as shown in Figure 4 (data not shown for TNF-α).

When the lipid amount increased, percent encapsulation and aerosolised performance were not changed. The suitable spray dry conditions may improve the aerosol dispersion of the pro-liposome powder. Furthermore, the different core carrier like the co-spray dry of mannitol and INH/PZA may improve the percent encapsulation. However, all of our formulations are likely to be safe to deliver to the alveoli as they did not cause immunological responses.

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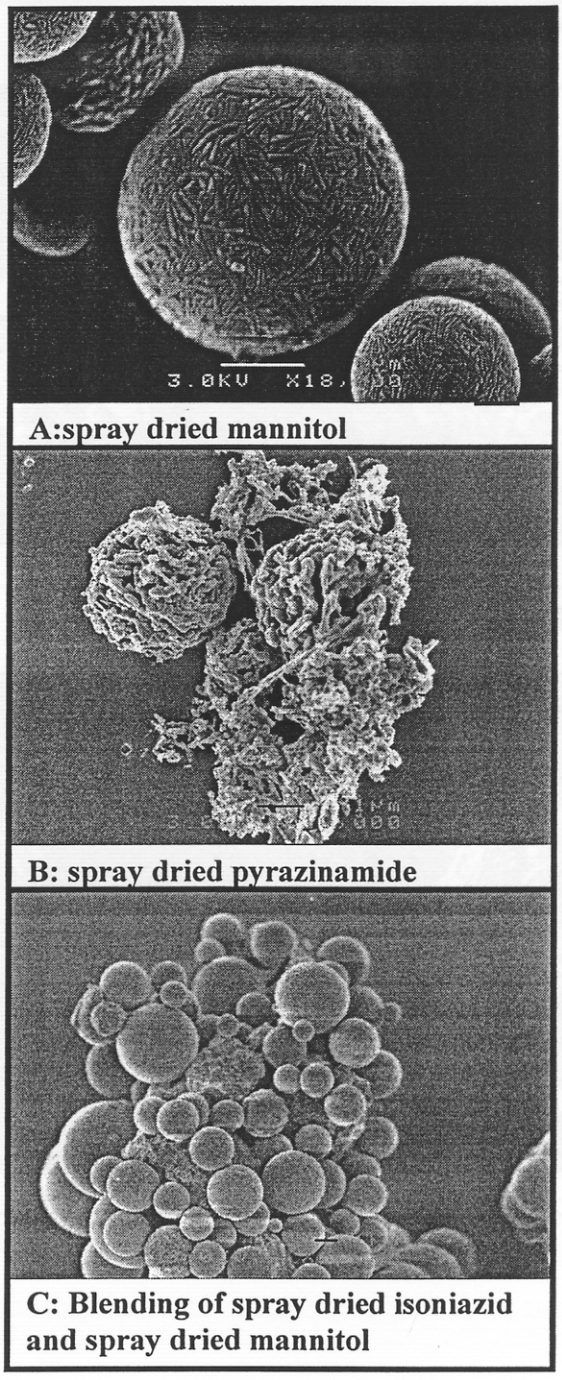
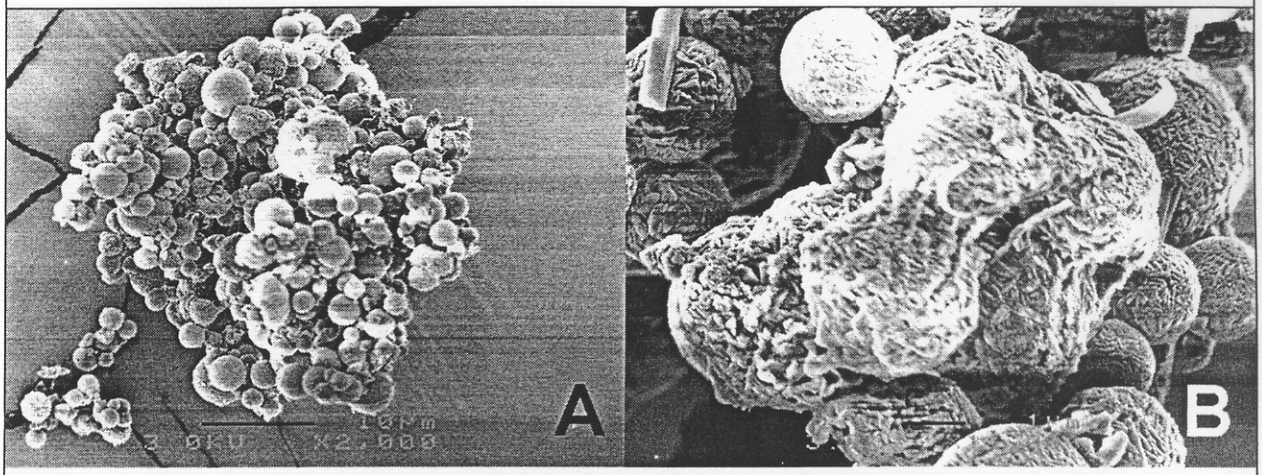
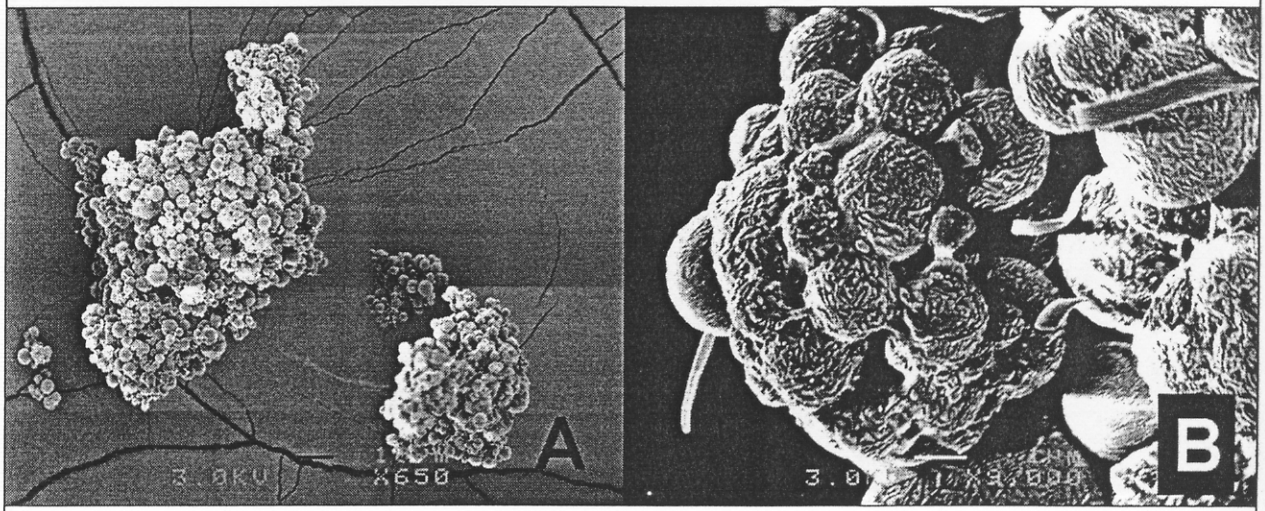


Figure 1. SEM photographs of the spray dried particles used as a core carrier of pro-liposome powder (bar = 1 μm)

Rx 1: 0.06 mmole lecithin /1 g powder



Rx 2: 0.12 mmole lecithin /1 g powder



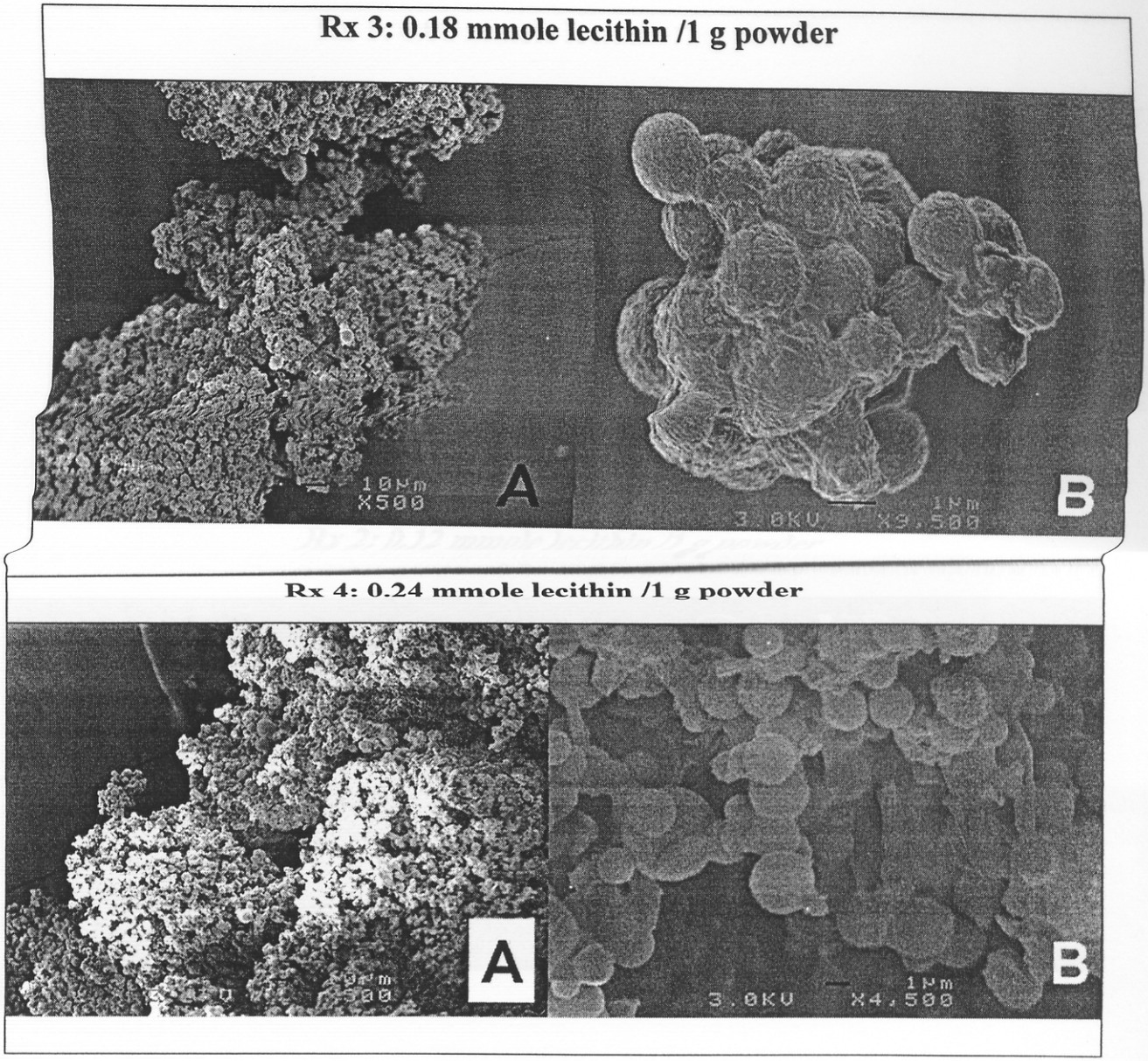


Figure 2. SEM photographs of the spray dried PZA pro-liposomes powder

(A: low magnification, bar = 10 μm B: high magnification, bar = 1 μm)

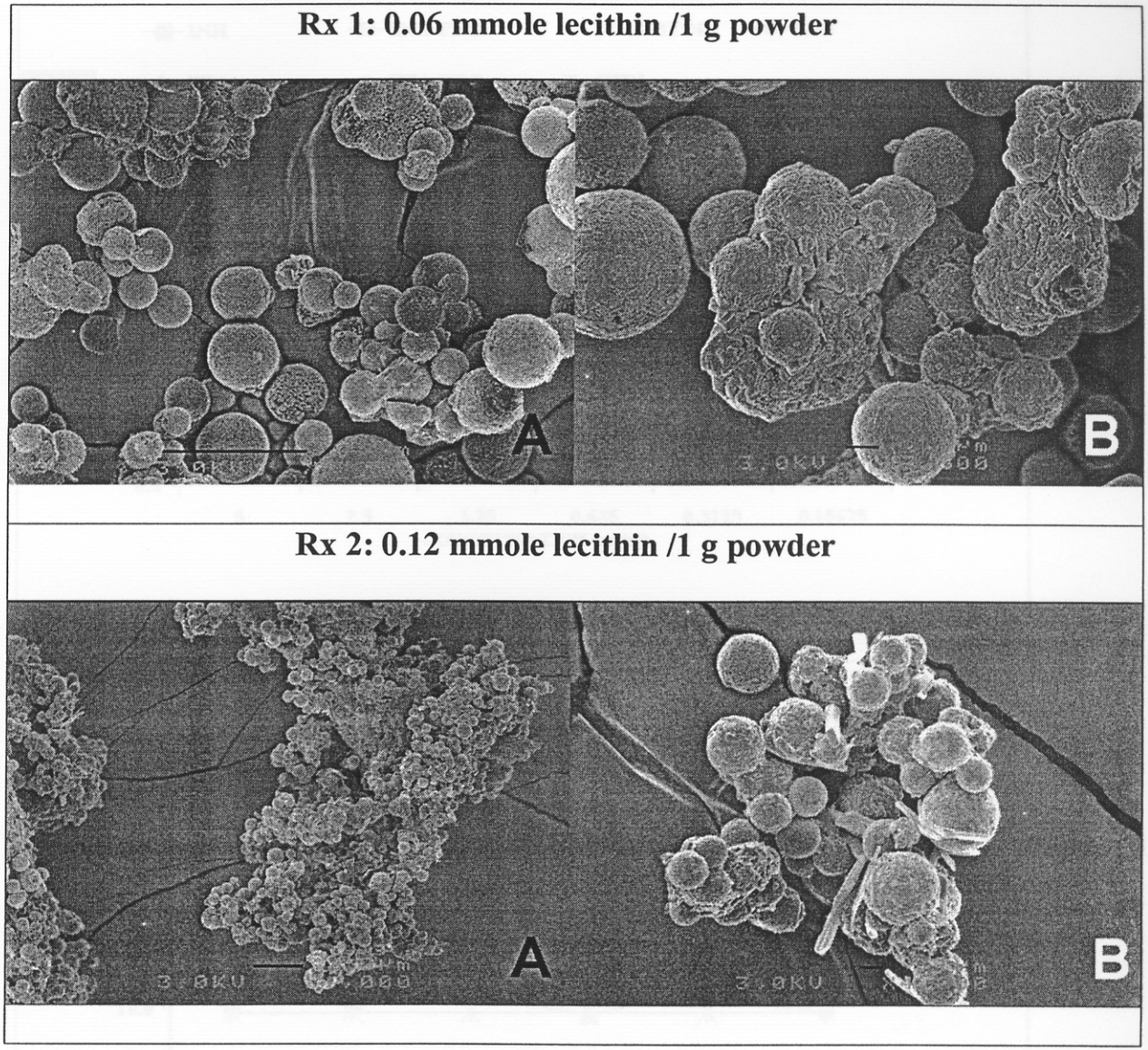


Figure 3. SEM photographs of the spray dried INH pro-liposome powder.

(A: low magnification, bar = 10 μm B: high magnification, bar = 1 μm)

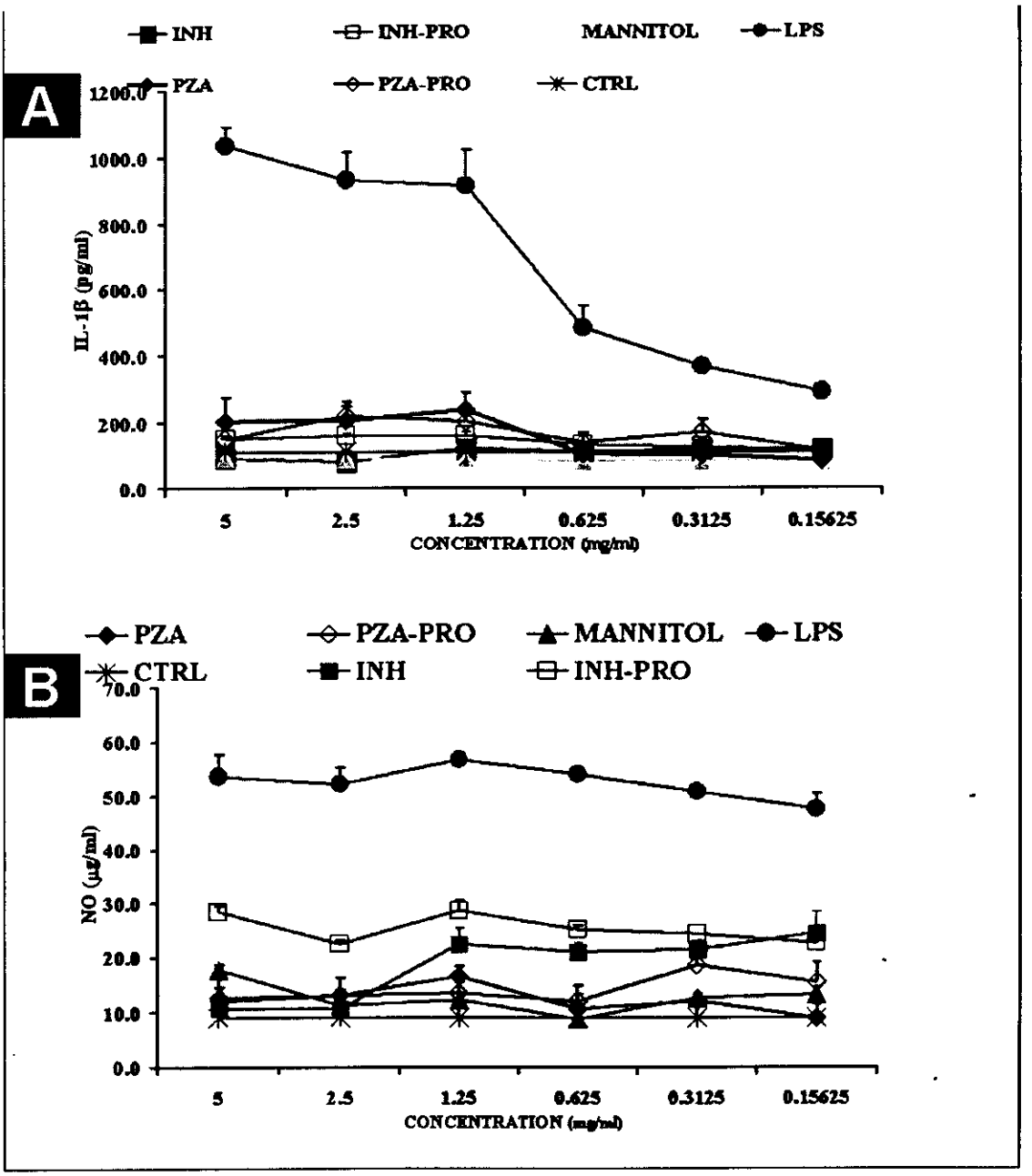


Figure 4. Immunological response of alveolar macrophage cell lines ((A) nitric oxide, (B) IL-1β) to pro-liposome formulation, active ingredient and mannitol (n=6)

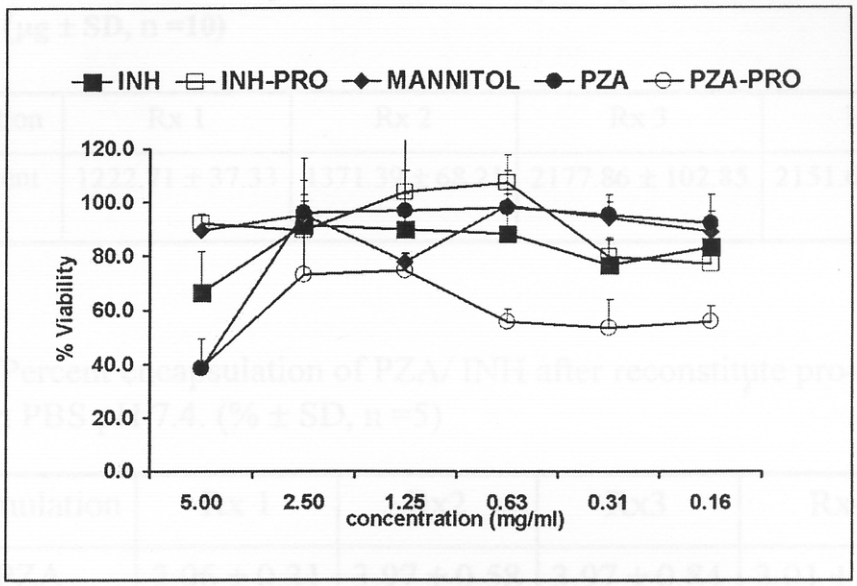


Figure 5. Viability of alveolar macrophage cell lines when composed to pro-liposome formulation, active ingredient and mannitol (n=6) for 48 h.

Table 1. Pro-liposome composition ingredients.

Formulation	Lipid part		Powder part	
	SPC (mmole)	CH (mmole)	3 μm Mannitol (g)	3μm INH/PZA (g)
Rx 1	0.06	0.06	0.9	0.1
Rx 2	0.12	0.12	0.9	0.1
Rx 3	0.18	0.18	0.9	0.1
Rx 4	0.24	0.24	0.9	0.1

Table 2. Spray dry condition of pro-liposome dry powder.

condition	concentration (mg/ml)	Inlet temperature (°C)	Atomizer pressure (Kpa)	Flow rate (ml/min)
1	10	50	800	2.8
2	10	50	600	1.4

Table 3. Content uniformity of INH/ PZA in pro-liposome formulation (μg ± SD, n =10)

Formulation	Rx 1	Rx 2	Rx 3	Rx 4
PZA content (μg)	1222.71 ± 37.33	1371.39 ± 68.21	2177.86 ± 102.85	2151.61 ± 79.80

Table 4. Percent encapsulation of PZA/ INH after reconstitute pro-liposome powder in PBS pH 7.4. (% ± SD, n =5)

Formulation	Rx 1	Rx2	Rx3	Rx4
PZA	2.06 ± 0.31	3.97 ± 0.58	3.97 ± 0.84	3.01 ± 0.33
INH	1.47 ± 0.18	ND	1.30 ± 0.49	ND

Table 5. Aerosolised parameter of pro-liposome (n =3)

(A: PZA spray dry condition 1, B: PZA spray dry condition 2, C: PZA spray dry condition 1)

A

Formulation	MMAD (μm)	% FPF	%ED
Rx 1	9.07 ± 1.24	28.24 ± 4.46	76.48 ± 5.73
Rx 2	10.21	33.05	85.52
Rx 3	9.15 ± 1.44	38.71 ± 3.68	92.71 ± 3.07
Rx 4	8.97 ± 0.59	38.48 ± 4.33	92.21 ± 0.18

B

Formulation	MMAD (μm)	% FPF	%ED
Rx 1	ND	ND	ND
Rx 2	7.53	35.72	80.65
Rx 3	7.98	34.28	78.77
Rx 4	9.04	36.61	92.33

C

Formulation	MMAD (μm)	% FPF	%ED
Rx 1	8.73	24.95	74.46
Rx 2	8.92	26.90	87.96
Rx 3	7.80	32.26	78.71
Rx 4	ND	ND	ND