RIFAMPICIN ENCAPSULATED LIPOSOMES AS DRY POWER INHALER AND ITS IMMUNOLOGICAL RESPONSED FROM ALVEOLAR MACROPHAGE CELL LINES

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Abstract

Rifampicin encapsulated liposome was formulated by dried film method and transformed to dry powder by lyophilization. Liposome dry powder formulations were characterized as aerosol by Andersen Cascade Impactor. Alveolar macrophage cell line was used to monitor cellular response (cytokines and nitric oxide production) after incubation with liposome dry powders for 24 h. ELISA kit was used to examine IL-1β and TNF-α in cell supernatant. Nitric oxide synthesis was measured by the Griess reaction. The suspension of rifampicin liposome gave 50% encapsulation which had sonicated liposome size range 150 - 200 nm. Mannitol was identified as a suitable sugar for liposome cryoprotectant since it gave a mass median aerodynamic diameter (MMAD) of 3.03 μm which is expected to deposit deep in the alveoli. More than 50% Fine Particle Fraction was obtained from liposome containing mannitol as the carrier. Liposome dry powder did not activate the immune system as compared to lipopolysaccharide (LPS) from *E.coli* (Positive control). In a similar manner to the immune response, Alveolar macrophage did not produce nitric oxide when they were challenged with liposome samples.

Keyword: liposome, aerosol, rifampicin, tuberculosis, immune response,

1. Introduction

Tuberculosis (TB) is a public health problem primarily for developing countries. It is the world's second most common cause of death from infectious disease, after HIV/AIDS (Frieden, 2003). It has been estimated that two million deaths resulted from TB in 2002. As the highest number of deaths is in the South-East Asian region. HIV has led to rapid increase in the incidence of TB and increases likelihood of dying from TB (WHO, 2004). The etiologic agent that causes tuberculosis, *Mycobacterium tuberculosis*, is an aerobic rod-shaped bacterium. The droplets containing tubercle bacilli, excreting from speaking, coughing or sneezing by active tuberculosis patients, transmitted in close contact by direct inhalation. An important component of the body's defense mechanism, alveolar macrophage (AM), appears to be immunity against mycobacterium organisms. In a few patients, problaby 10 percent, the defense mechanisms are unable to control primary infection and allow mycobaceria to survive within AM, and cause primary tuberculosis (Weinberger, 1992 : Piessens, 2000). Long-term treatment of tuberculosis with a combination of drugs is required. However, non-adherence to treatment is common because patients did not take all prescribed medicines (Frieden, 2003). These are causes of drug-resistant TB, particularly multidrug-resistant TB which is difficult to cure (Fujiwara, 2000: WHO, 2004).

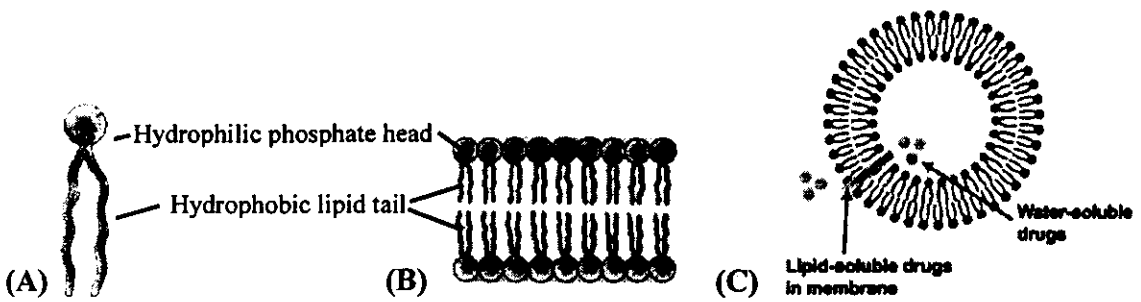


Figure 1. Phospholipid molecule (A), Bilayer membrane (B), and liposome structure (C)

Intracellular infections are difficult to eradicate because bacteria inside phagosomes are protected from antibiotic or the lower intracellular uptake of the drugs. Alternative strategy is modification of existing antibiotics to be controlled release dosage form or directly delivered drugs to intracellular infected macrophages (Couvreur, 1991). Liposomes are simply vesicles in an aqueous volume which is entirely enclosed by a membrane of lipid (usually phospholipids) bilayer (New, 1990) as shown in figure 1. Liposome is normally ingested rapidly and in large quantities by macrophages, they are exploited for macrophage specific drug delivery. This strategy may result in reduction of drug toxicity, and also slow intracellular release and produce high localized concentrations of antimicrobial drugs (Alving, 1988: Pinto-Alphandary, 2000). Effective chemotherapy for pulmonary tuberculosis can be attained by targeting drugs by tagging specific markers or a homing device to liposomes, such as *O*-stearylamylopectin (O-SAP) and administered by intravenous injection (Deol, 1997a: Deol, 1997b). However, intravenous injection of liposome posses several drawbacks such as leakage of their contents before reaching the target organ and their uptake by the macrophage of the liver and spleen (Vyas, 2004). Rifampicin (Figure 2.) is first line antituberculosis medication which shorten the course of chemotherapy to nine months or less. Rifampicin activity interferes with the synthesis of mRNA by binding to the RNA polymerase. The recommended dosage of rifampicin is 10 mg/kg body weight in daily treatment. The minimum inhibitory concentration of rifampicin for *M. tuberculosis* is about 0.25 mg/L (Rieder, 2002).

Aerosolization is an established method for treatment of a lung diseases. Lower dose than the oral route can be administered with similar efficacy which will minimize unwanted

side effects (Timsina, 1994). The efficacy of a therapeutic aerosol depends on the amount of drug deposited at the target site. However, an important function of the lung is to prevent deposition of particles in the airways as it dichotomously divides the airway to give progressively smaller airways. The site of the deposition of a material in the airways depends on its size and density. The particle size of aerosols may be standardized by calculation of the "aerodynamic diameter" (D_{ae}). This is the physical diameter of a unit density sphere, which settles through air with a velocity equal to the particle in question. In order to penetrate into the alveolar space, the particle must have an aerodynamic particle diameter of less than 5 μm. Such aerosols are said to be in the "respirable" size range (Taylor,1993: Suarez 2000 and Mitchell 2004). In addition, airborne particles presented to the lung are phagocyted by AMs as an efficient non-specific defense mechanism (Thepen, 1994). It is attractive to deliver rifampicin encapsulated liposome dry powders directly to alveoli where AMs reside.

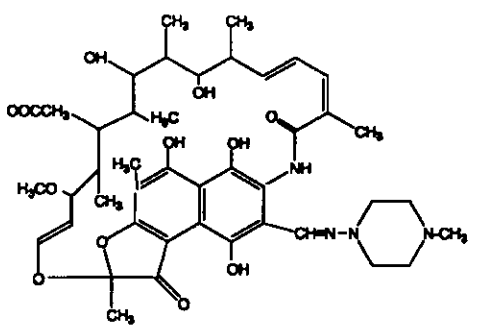


Figure 2. Chemical structure of rifampicin

However, AMs play a role in the mediation of inflammatory processes which are able to produce several pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNF-α) and Interleukine-1β (IL-1β). These cytokines evoke other defense systems (Thepen, 1994:

Abbas, 1994a). In addition to cytokines, macrophages also produce nitric oxide (Abbas, 1994b). It is realized that activated AMs produce a variety of mediators which have the potential to damage the lung tissues (Thepen, 1994: Abbas, 1994a: Abbas, 1994b). This work deals with aerosolization of rifampicin encapsulated liposome dry powder and determines immunological response of AMs to the formulations.

2. Experimentals

2.1 Materials

Rifampicin was generous gift from Siam Pharmaceuticals, Research and Development, Bangkok, Thailand. Rifampicin standard, cholesterol from lanolin, D-(+)-lactose monohydrate and L-α-phosphatidylcholine from soybean (lecithin) were purchased from Fluka, Switzerland. Trehalose dihydrate, N- (1-naphthyl)- ethylenediamine dihydrochloride, Lipopolysaccharide (LPS) from *E.coli* were obtained from Sigma Chemicals (St. Louis, MO, USA). Sodium dihydrogen phosphate was purchased from Riedel-de Haën, Germany and chloroform was purchased from VWR International Ltd., UK. Acetonitrile and methanol were obtained from J.T. Baker (NJ, USA). All of these solvents were high performance liquid chromatography (HPLC) grade. All other materials were of analytical grade and used as received. Milli-Q-water (Millipore, Watford, UK) was used in all the preparations. HAM F12 cell culture medium, Pennicillin/Streptomycin solution and heat-inactivated fetal bovine serum (FCS) were obtained from Gibco (Grand Island, NY, USA.).

2.2 Methods

2.2.1. Preparation of rifampicin encapsulated liposome suspension

Liposome suspensions were prepared by dry film method as described elsewhere (New, 1990b). Briefly, all lipid ingredients (SPC and CH) and rifampicin were dissolved in a mixture of chloroform and methanol 2:1 then the solvent was removed under vacuum rotary evaporator until lipid dried film appeared on the flask. Solution was added into the flask in order to hydrate lipid film and liposome vesicles were formed in this step. The ingredients were varied in different formulations as shown in Table 1.

2.2.2. Characterization of rifampicin encapsulated liposome suspension

The liposome suspension in each formulation was characterized in terms of vesicle size stability and percent encapsulation. The liposome vesicle sizes were measured by photon correlation spectroscopy and followed up size stability every week for 4 weeks. The unencapsulated rifampicin were removed by dialysis technique using dialysis membrane (SpectraPore®) with molecular wieght cut off 6-8000 kDa. To lyse the liposome vesicle, 0.1% Triton X-100 in methanol was used and the released rifampicin content in liposome vesicles were determined by High Performance Liquid Chromatography (HPLC). Na_2HPO_4:Acetonitrile 55:45 (v/v) was used as mobile phase with a flow rate of 1ml/min. The drug was detected at 254 nm.

2.2.3. Preparation of rifampicin encapsulated liposome dry powder

Rifampicin dry powder was obtained from lyophilized liposome suspension. Different sugars, glucose, lactose, trehalose, mannitol and sucrose were used as cryoprotectant and

carrier in the ratios as shown in Table 2. The rifampicin content of each dose in each formulation and its uniformity was determined by HPLC.

2.2.4. Determination of In vitro deposition of liposome dry powder: Andersen Cascade Impactor.

In vitro deposition of liposome dry powder was measured by Andersen Cascade Impactor (ACI) as described in USP. A glass inhaler device made in house was employed to deliver dry powder aerosols to the ACI to aerosolized liposome dry powder formulation. The flow rate of 60 ml/min was applied to the ACI. MeOH was used to wash the drug at each stage and the drugs contents were determined by HPLC. Aerodynamic diameter which provided from the particle size at 50% cumulative based on its mass, this is called Mass Median Aerodynamic Diameter (MMAD). Other important parameters of aerosol are % emitted dose (ED) which is the percentage of drug propel from the delivery aerosol device and % fine particle fraction which is the percentage of drug deposit on stage 1-7 of ACI.

2.2.5. Culture of alveolar macrophage cell line (NR 8383)

Alveolar macrophage cell line NR 8383 (ATCC : CRL-2192) was cultured in Ham's F 12K medium supplemented with 15% (v/v) FBS and 1% penicillin/ streptomycin. The cell line was incubated at 37°C in 5% CO_2 and 95% humidity. To subculture the cell, adherent cell was scrapted and re-attached to additional flasks at concentration of $4x10^5$ cells/ml.

2.2.6. Determination of immunological response of alveolar macrophage to liposome formulations

6.1 Cytokine analysis

The tumor necrosis factor α and Interleukin-1β in the cell supernatant were analyzed using commercial ELISA kits (Quantikine® RTA00 and Quantikine® RLB00 for rat TNF-α and IL-1β, respectively) as described in product assay procedures. The detectable dose of both TNF-α and IL-β is less than 5 pg/ml.

6.2 Nitric oxide assay

This method was used to investigate nitric oxide in the form of nitrite (NO_2^-), which is one of two primary, stable and nonvolatile breakdown products of NO. This measurement relies on a diazotization reaction of Griess reagent. Griess reagent was prepared by mixing 1% sulfanilamide, 0.1% N- (1-naphthyl)- ethylenediamine dihydrochloride and 2.5% H_3PO_4 in water. An equal volume of cell supernatant and Griess reagent were mixed. The absorbance was determined at 450 nm after mixing for 10 minutes. The nitrite concentration was calculated from the sodium nitrite standard curve.

3. Results and Discussions

3.1. Preparation of rifampicin encapsulated liposome suspension

Rifampicin encapsulated liposome prepared by lipid film method gave a similar suspension in every formulations. The liposome vesicles were observed under laser confocal microscopy as shown in Figure 3. The micrograph contained both large unilamellar vesicles and multilamellar vesicles.

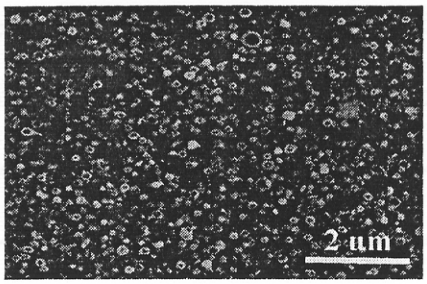


Figure 3. Confocal microscopic image of liposome suspension when staining with nile blue A

3.2. Characterization of rifampicin encapsulated liposome suspension

The effect of CH content on liposome size stability was compared in formulation #1 and #2 after 4 weeks storage (figure 4). It was found that the high CH content formulation (SPC:CH =1:1, formulation No.1) gave better size stability than that of SPC:CH (2:1).

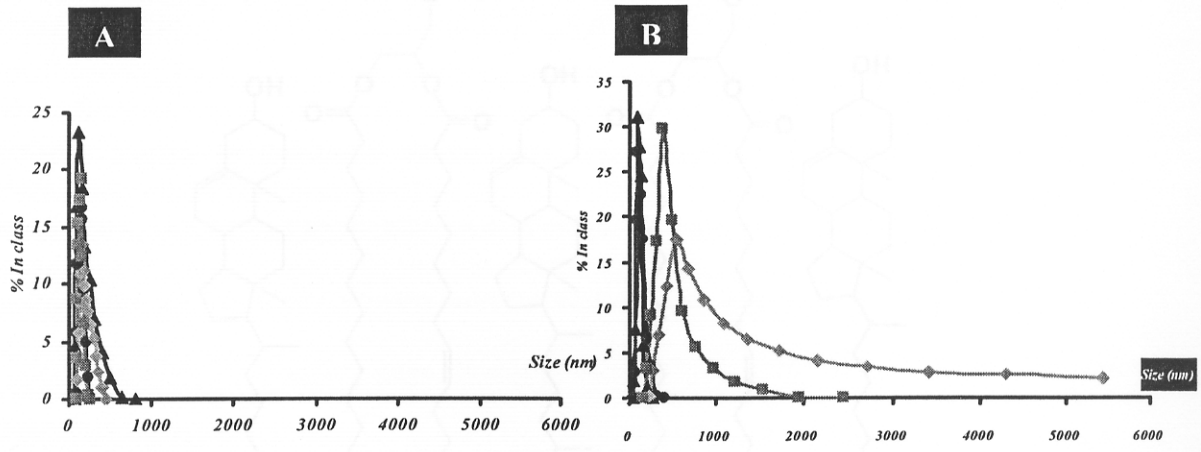


Figure 4. The size stability of high CH formulation (**A** :formulation No.1) compared to low CH formulation (**B**: formulation No.2)

● Week 1 ▲ Week 2 ◆ Week 3 ■ Week 4

This is described by CH orientation into the phospholipids membrane in which its hydroxyl group located toward the aqueous surface of PC while the aliphatic chain aligned parallel to the acyl chain of the bilayer as Figure 5 (New 1990a). These caused

alteration of the membrane fluidity and improved their rigidity therefore formulation No.1 has a size stability longer than 4 weeks. Liposome formulation No. 2 aggregation occurred after 2 weeks storage as shown in figure 4A. The encapsulation efficacy of formulations No.3, 4 and 5 with various lipid concentrations were compared. Due to hydrophobicity of rifampicin, it inserted between lipid bilayer membrane. Increasing the lipid concentration from 0.2 mM to 0.6 mM resulted in an increasing percentage of rifampicin encapsulation. Lipid concentration of 0.6 mM was chosen for further investigation dry powder as aerosol since its showed good stability and highest encapsulated rifampicin content.

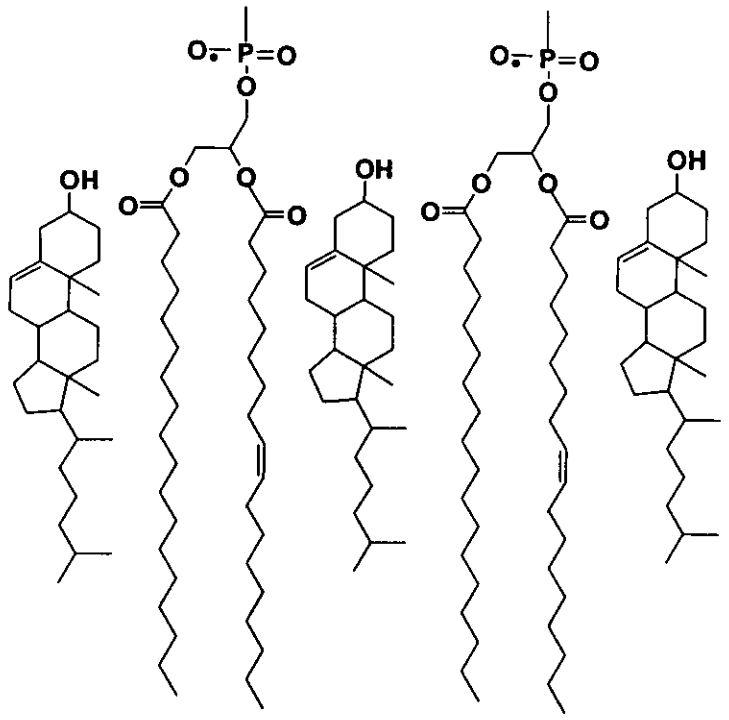


Figure 5. Orientation of cholesterol molecule between phospholipids molecule

Table 1. The composition of liposome and their percent encapsulation. (mean ± S.D., n=3)

Formulation No.	Ingredient (mmol)		% Encapsulation
	SPC	CH	
1	0.2	0.2	36.09 ± 0.44
2	0.2	0.1	35.38 ± 5.13
3	0.4	0.4	46.645 ± 2.91
4	0.6	0.6	60.845 ± 1.68

3.3. Preparation of rifampicin encapsulated liposome dry powder.

Verumi (1995) notified that liposome containing drug molecules can be lyophilized and reconstituted with significant drug retention without significant change in mean vesicle size. To protect the liposomes during lyophilizaiton, sucrose, lactose and trehalose were commonly used as a cryoprotectant. However, the report on the cryoprotection ability of different sugars in liposome formulation was debatable (Verumi, 1995). In our experiments, two other sugars, glucose and mannitol, were included as they are approved by FDA for use as a dry powder aerosol carrier. The obtained lyophilized liposome products (total to 10 samples) gave similar powder in both ratios of each sugar. The role of saccharides for lyophilization has not been fully understood (Miyajima, 1997). Wolker (2004) suggested that sugars stabilize liposome during drying involves the formation of a glassy state by the sugars as well as a direct interaction between the sugars and the phospholipid head groups. Glucose, monosaccharide with low glass transition temperature, provided very sticky products even during the lyophilization process. Sucrose, trehalose and lactose gave a free flowing powder after completion of the

lyophilization process. However, they showed low physical stability of the dried liposome after storage in desiccator at room temperature since they slowly converted to sticky products. Mannitol seems to gave the best powder since a bulky and stable dry powder was obtained. Only the formulation containing trehalose, sucrose, lactose and mannitol were determined for contents, the results revealed in Table 2. Good content uniformity were obtained from all selected formulations.

Table 2. Rifampicin content in a dose of dry powder and aerosol parameter of liposome dry powder obtained from Andersen Cascade Impactor (n=3)

Sugar	Rifampicin content (μg ± SD)	% FPF (%± SD)	% ED (%± SD)	MMAD (μm ± SD)
Trehalose 1:1	113.81 ± 2.22	31.953 ± 1.174	100 ± 0	6.653 ± 0.286
Trehalose 1:2	71.26 ± 1.97	15.186 ± 1.429	90.065 ± 2.312	6.137 ± 0.335
Sucrose 1:1	137.075 ± 0.658	12.196 ± 1.582	94.562 ± 0.276	8.007 ± 0.547
Sucrose 1:2	62. 273 ± 2.354	34.072 ± 2.052	89.169 ± 0.710	5.083 ± 0.752
Lactose 1:1	123.46 ± 3.93	27.825 ± 3.511	93.936 ± 0.296	6.183 ± 0.554
Lactose 1:2	88.02 ± 1.14	32.725 ± 0.399	91.058 ± 0.879	5.637 ± 0.752
Mannitol 1:1	132.34 ± 2.24	66.746 ± 6.222	100 ± 0	3.350 ± 0.460
Mannitol 1:2	72.37 ± 0.80	61.419 ± 4.459	100 ± 0	4.097 ± 0.652

3.4. Determination of In vitro deposition of liposome dry powder: Andersen Cascade Impactor.

From a toxicity viewpoint, liposomes are particularly appropriate systems for drug delivery to the lung. Studies in humans and animals have indicated that liposomes can

modulate the fate of lung deposited liposome, increasing the residence time of drugs in the tissue and potentially decreasing systemic side effects (Taylor,1993). Surprisingly, mannitol has not been reported as liposome cryoprotecting sugar giving desired results of all aerosol parameters, MMAD < 5μm, ED = 100% and FPF > 50%. While the other three sugars which are commonly used as cryoprotectant gave high MMAD and low % FPF, due to their rapidly change to sticky products when exposed to the inhaled air from vacuum pump. The % FPF result of sucrose lyophilized liposome dry powder is in agreement with Joshi (2001a) and Joshi (2001b) who also used sucrose as a cryoprotectant in very high ratio (lipid : sugar =1:10). They used twin stage impinger (TSI) to determine %FPF and found that sucrose gave 20-25 % FPF. However, as TSI cannot determine aerodynamic diameter, this can not be compared with our results. The dry powders containing mannitol as cryoprotectant were selected to determine the immune response of alveolar macrophage cell line.

3.5. Culture of alveolar macrophage cell line (NR 8383)

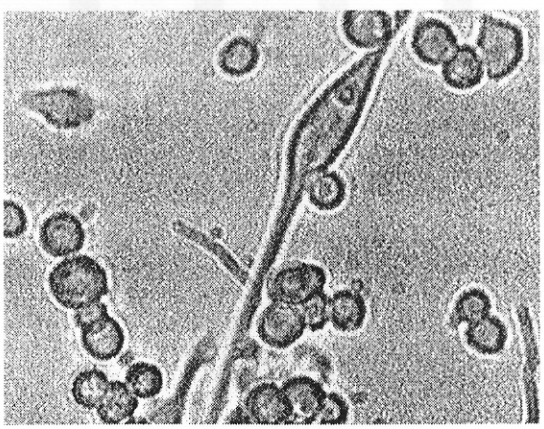
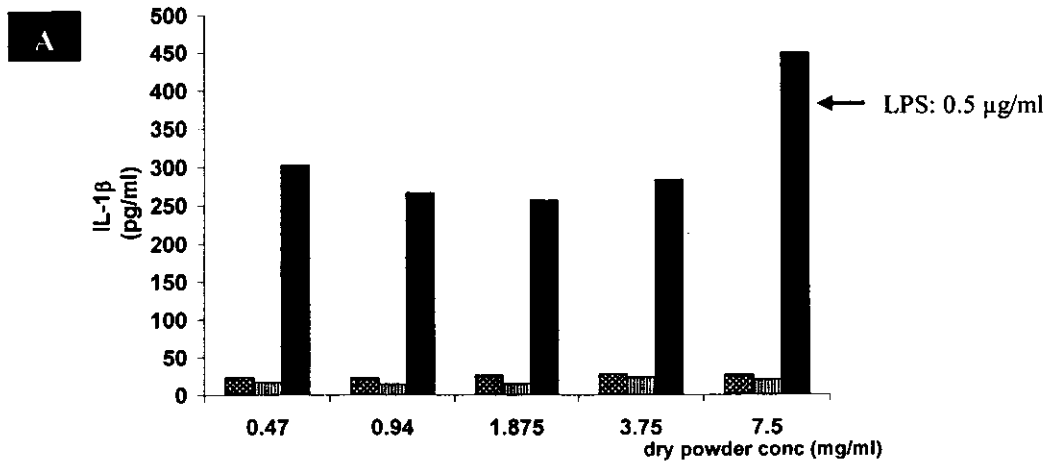


Figure 6. Morphology of cultured alveolar macrophage NR 8383 (x 20 objective lens)

Alveolar macrophage were grown in Ham F 12 k media supplemented with 15% FBS and the media was changed twice a week. Because the cell is a semi-adherent type, it does not need to be trypsinised in the subculture process. The macrophage cell lines displayed a specific characteristic of macrophage which has pseudopods as show in Figure 6.

6. Determination of immunological response of alveolar macrophage to liposome formulation

6.1 Cytokine analysis



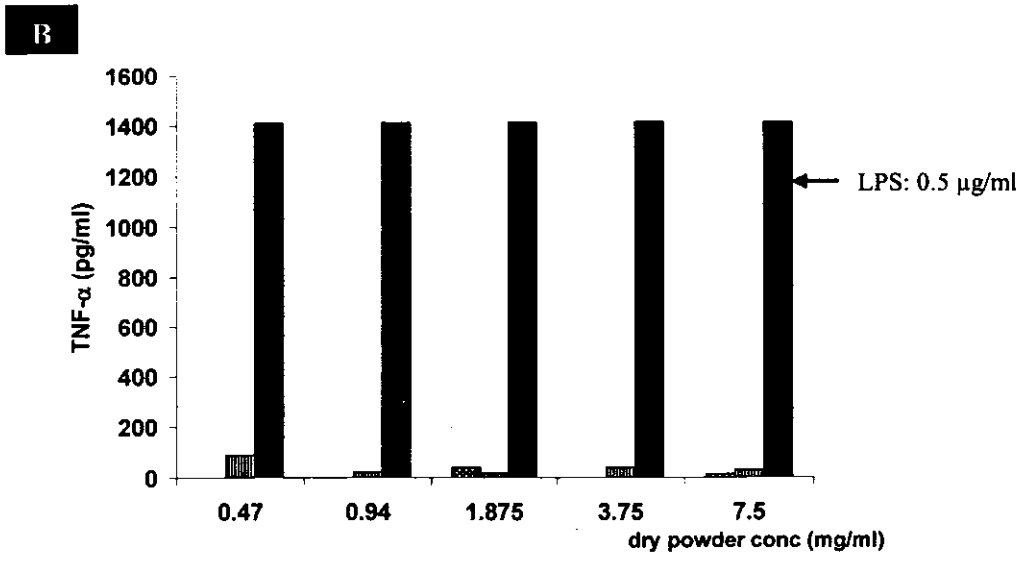


Figure 7. Cytokine production of alveolar macrophage (n=2) **A**: IL-1β and **B**: TNF-α (For LPS from *E. Coli* the used highest conc is 0.5 μg/ml)

Liposome dry powder RIF standard LPS from *E.coli*

TNF-α and IL-1β are the principal mediators of the acute inflammatory response. Lipopolysaccharide (LPS) is a potent stimulus for eliciting TNF-α and IL-1β production. It is the most potent activator of macrophage which causes significant pathologic changes in the host. For this reason, LPS was chosen to be a positive control of alveolar macrophage immune function. Negative control was a supernatant of cell culture.

LPS positive control showed significant difference in cytokine levels for all concentrations. Liposome samples also displayed low cytokine level which was similar to negative control as the result show in Figure 7A and 7B.

6.2 Nitric oxide assay

Macrophage produce reactive nitrogen intermediate, nitric oxide, by the action of inducible nitric oxide synthase (iNOS). iNOS is a cytosolic enzyme that is absent in resting macrophages but can be induced in response to LPS. When macrophages are strongly activated, they can injure normal host tissue by the release of nitric oxide. These products of macrophage do not distinguish between self or non-self tissue as a result of causing tissue injury. LPS was also used as positive control while cell supernatant was used negative control. The identical result to cytokine examination was obtained as showed in figure 8. Liposome dry powder did not activated macrophage to produce nitric oxide. It is appreciated that our liposome dry powders did not activate alveolar macrophage even at the highest dose. These results proved the safety of liposome dry powder to deliver liposome directly to alveolar macrophage.

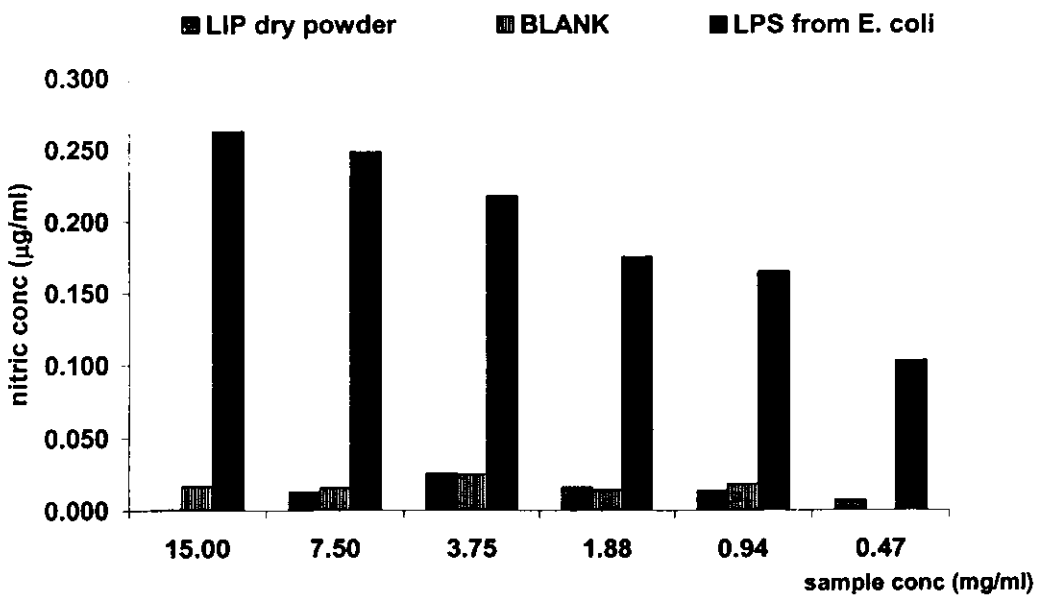


Figure 8. Nitric oxide production of alveolar macrophage (n=2) (For LPS from *E. Coli* the used highest conc is 1 µg/ml)

4. Conclusion

For liposome preparation, CH is an important ingredient which is able to improve rigidity of phospholipids bilayer as reduced the liposome aggregation. Mannitol, was proved to be the most suitable cryoprotectant for this application. While other common cryoprotecting sugars, trehalose, lactose, sucrose, tended to become sticky when exposed to inhaled air. Mannitol cryoprotected dry liposomes did not activate alveolar macrophage NR 8383 since they produce TNF α , IL-1β and nitric oxide in very low level.

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Reference

1. Abbas, A.K. and Lichtman, A.H. Cytokine. In: Abbas, A.K. and Lichtman (eds) (2000a) Cellular and Molecular Immunology, China, W.B. Saunders company, pp 243-274.
2. Abbas, A.K. and Lichtman, A.H. Innate immunity. In: Abbas, A.K. and Lichtman (eds) (2000b) Cellular and Molecular Immunology, China, W.B. Saunders company, pp 275-317.
3. Alving, C. R. (1988) Macrophages as targets for delivery of liposome-encapsulated antimicrobial agents. *Adv. Drug Deliv. Rev.*, 2, pp. 107-128.

4. Couvrer, P., Fattal, E. and Andremont, A. (1991) Liposome and nanoparticles in the treatment of intracellular bacterial infections. *Pharm. Res.*, 8, pp. 1079-1086.

5. Deol, P. and Khuller G.K. 1997a. Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice, *Biochim. Biophys. Acta.*, 1334: 161-172.

6. Deol, P., Khuller, G.K. and Joshi, K. 1997b. Therapeutic efficacies of isoniazid and rifampicin encapsulated in lung-specific stealth liposomes against Mycobacterium tuberculosis infection induced in mice, *Antimicrob. Agents Chemother.*, 41(6): 1211-1214.

7. Freshney,R.I. Cytotoxicity. In: Freshney, R.I. (ed) (1994) Culture of animal cells: A manual of basic technique, New York, Wiley-Liss Inc., pp 329-344.

8. Frieden, T.R., Sterling, T.R., Munstiff, S.S., Watt, C.J. and Dye, C. 2003. Tuberculosis, *Lancet.*, 362: 887-899.

9. Fujiwara, P.I., Simone, P. M. and Munsiff, S. S. Treatment of tuberculosis. In: Reichman, L. B. and Hershfield, E. S. (eds) (2000) Tuberculosis: A comprehensive international approach, New York, Marcel Dekker, pp 401-443.

10. Joshi, M.R. and Misra, A. 2001a. Dry powder of liposomal ketotifen fumarate: formulation and characterization, *Int. J. Pharm.*, 223: 15-27.

11. Joshi, M.R. and Misra, A. 2001b. Liposomal budesonide for dry powder inhaler: preparation and stabilization, *AAPS. PharmSciTech.*, 2(4): 1-10.

12. Mitchell, J.P. and Nagel, M.W. 2004. Particle size Analysis of aerosols from medicinal inhalers, *Kona.*, 22: 32-65.

13. Miyajima, K. 1997. Role of saccharides for the freeze-thawing and freeze drying of liposome, *Adv Drug Deliv Rev.*, 24: 151-159.

14. New, R.R.C., Introduction, In New, R.R.C. (ed) (1990a) Liposomes; a practical approach, New York, IRL press, pp. 1-32

15. New, R.R.C., Preparation of liposomes, In New, R.R.C. (ed) (1990 b) Liposomes; a practical approach, New York, IRL press, pp. 1-32

16. Piessens, W.F. and Nardell, E. A. Pathogenesis of tuberculosis. In: Reichman, L. B. and Hershfield, E. S. (eds) (2000) Tuberculosis: A comprehensive international approach, New York, Marcel Dekker, pp 241-259.

17. Pinto-Alphandrary, H., Andremont, A. Couvrer, P. (2000) Targeting delivery of antibiotics using liposomes and nanoparticles: research and applications. *Int. J. Antimicrob. Agents*, 13, pp. 155-168.

18. Rieder, H. L. Interventions for tuberculosis control and Elimination, Paris, International Union against tuberculosis and lung disease, pp 276-284.

19. Suarez, S. and Hickey, A.J. 2000. Drug properties affecting aerosol behavior, *Respiratory care.*, 45(6): 652-664.

20. Swarbrick, J., and Boyland, J. C., Encyclopedia of pharmaceutical technology vol. 9 : liposome as pharmaceutical dosage forms to microencapsulation. New York : Marcel Dekker Inc. 1-39.

21. Taylor, K.M.G. and Farr, S.J. Preparation of liposomes for pulmonary drug delivery. In: Gregoriadis, G. (ed) (1992) Liposome technology: liposome preparation and related techniques vol. I, Florida, CRC Press Inc., pp 177-195.

22. Thepen, T. and Havenith, C.E.G. Funtion of alveolar macrophage. In: Bruijnzeel-Koomen, C.A.F.M. and Hoefsmit, E.C.M. (eds) (1994) Immunopharmacology of macrophages and other antigen-presenting cells, London, Academic Press Inc., pp 35-43.

23. Timsina, M.P., Martin, G.P., Marriott, C.D., Ganderton, D., Yianneskis, M. 1994. Drug delivery to the respiratory tract using dry powder inhalers, *Int. J. Pharm.*, 101: 1-13.

24. Verumi, S. and Rhodes,C.T. 1995. Preparation and characterization of liposomes as therapeutic delivery systems: a review, *Pharm. Acta. Helv.*, 70: 95-111.

25. Vyas, S.P., Kannan, M.E., Jain, S., Mishra, V., Singh, P. 2004. Design of liposomal aerosols for improved delivery of rifampicin to alveolar macrophage, *Int. J. Pharm.*, 269: 37-49.

26. Weinberger, S.E. Tuberculosis and nontuberculosous mycobacteria. In: Weinberger, S.E. (ed) (1992) Principles of pulmonary medicine, Mexico, W.B. Saunders company, pp 276-284.

27. Wolker, W.F., Oldenhof, H., Tablin, F., Crowe, J.H. 2004. Preservation of dried liposomes in the presence of sugar and phosphate, *Biochim Biophys Acta.*, 1661: 125-134.

28. www.who.int