Targeting Antituberculosis Dry Powder Inhalers to Alveoli and Pre-clinical Studies

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ชื่อเรื่องวิทยานิพนธ์ การนำส่งยาต้านวัณโรคผงแห้งชนิดสูดสู่ถุงลมปอดและการศึกษาก่อนการศึกษาทางคลินิก

ผู้เขียน นางสาวนฤมล ช่างสาน

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บทคัดย่อ

การศึกษานี้เป็นการพัฒนาสูตรตำรับลิโปโซมผงแห้งที่บรรจุยาไรแฟมปิซินและโปรลิโปโซมผงแห้งของยาไอโซไนอาซิดสำหรับการนำส่งสู่ทางเดินหายใจเพื่อใช้เป็นทางเลือกในการรักษาวัณโรค เพื่อความปลอดภัยในการใช้ผงแห้งทั้งสองชนิดจึงถูกประเมินความเป็นพิษต่อเซลล์ในระบบทางเดินหายใจและศักยภาพในการกระตุ้นการตอบสนองของระบบภูมิคุ้มกันจากเซลล์ alveolar macrophage นอกจากนี้ผงแห้งของยาทั้งสองชนิดยังถูกประเมินประสิทธิภาพในการฆ่าเชื้อ *Mycobacterium bovis* (*M. bovis*) ที่อาศัยอยู่ในเซลล์ alveolar macrophage เพื่อใช้เป็นข้อมูลเบื้องต้นก่อนเข้าสู่การศึกษาทางคลินิก

ส่วนประกอบหลักของการเตรียมลิโปโซมคือฟอสฟาทิดิลโคลีนจากถั่วเหลืองและโคเลสเตอรอลในอัตราส่วนต่าง ๆ การบรรจุยาไรแฟมปิซินในลิโปโซมอาศัยวิธีการก่อฟิล์มของคลอโรฟอร์มหลังจากนั้นเปลี่ยนเป็นผงแห้งโดยใช้เทคนิคฟรีซดราย ซึ่งน้ำตาลสามชนิดคือแมนนิทอล ทรีฮาโลส และ แลคโตส ถูกประเมินเพื่อใช้เป็นตัวพา และ สารปกป้องการทำเยือกแข็งเพื่อให้ได้ผงยาที่เหมาะสำหรับการนำส่งสู่ทางเดินหายใจส่วนล่าง ส่วนโปรลิโปโซมของยาไอโซไนอาซิดจะใช้น้ำตาลแมนนิทอลที่ผ่านการสเปรย์ดรายมาแล้วและมีขนาดประมาณ 3 ไมโครเมตรเป็นอนุภาคแกนผสมกับผงยาไอโซไนอาซิดในอัตราส่วนต่าง ๆ ผงยาจะถูกเคลือบด้วยส่วนผสมของฟอสฟาทิดิลโคลีนจากถั่วเหลืองและโคเลสเตอรอลโดยเทคนิคการสเปรย์ดราย

สารแขวนตะกอนของลิโปโซมส่วนใหญ่จะประกอบด้วยอนุภาคที่มีผนังหลายชั้นที่มีขนาดระหว่าง 200-300 นาโนเมตร และสูตรตำรับที่มีโคเลสเตอรอลเป็นส่วนประกอบในปริมาณสูงจะมีความคงตัวทางกายภาพที่สูงขึ้นดังเห็นได้จากความคงตัวของขนาดลิโปโซมเมื่อเก็บไว้นาน 4 สัปดาห์ เมื่อพิสูจน์โดย solid-state NMR พบว่าโคเลสเตอรอลส่งผลให้การจัดเรียงตัวของโมเลกุลไขมันในผนังลิโปโซมเป็นระเบียบมากขึ้นจึงลดความไม่คงตัวทางกายภาพของลิโปโซมได้ นอกจากนี้การใช้ solid-state NMR ยังสามารถทำนายตำแหน่งของไรแฟมปิซินในผนังไขมันของลิโปโซม โดยพบว่าไรแฟมปิซินแทรกตัวอยู่ระหว่างสายโซ่ของคาร์บอนในโมเลกุลไขมันและสัมพันธ์กับปริมาณของโคเลสเตอรอลเมื่อเพิ่มปริมาณไขมันรวมเป็น 20 มิลลิโมลาร์ พบว่า 50 เปอร์เซ็นต์ของปริมาณยาไรแฟมปิซินถูกเก็บกักไว้ได้ในลิโปโซม และเมื่อทำให้อยู่ในรูปลิโปโซมผงแห้งพบว่าจากคุณสมบัติความเป็นผลึกของน้ำตาลแมนนิทอลทำให้ได้ผงยาที่เหมาะสำหรับการนำส่งสู่ทางเดินหายใจคือมีค่าเส้นผ่าศูนย์กลางของอนุภาคในอากาศที่ 3.4 ไมโครเมตร นอกจากนี้ยังพบว่าความคงตัวทางเคมีของยาไรแฟมปิซินจะสูงสุดเมื่ออยู่ในรูปของลิโปโซมผงแห้ง โปรลิโปโซมของยาไอโซไนอาซิดสามารถเตรียมได้สำเร็จด้วยเทคนิคสเปรย์ดรายโดยพบว่ามีการกระจายของยาไอโซไนอาซิดอย่างสม่ำเสมอและมีค่าเปอร์เซ็นต์สัมประสิทธิ์ความแปรปรวนต่ำกว่า 6 ในทุกสูตรตำรับและสามารถกักเก็บยาไอโซไนอาซิดได้ประมาณ 10 เปอร์เซ็นต์ และพบว่าอัตราส่วนของไอโซไนอาซิดต่อน้ำตาลแมนนิทอลที่ 4 ต่อ 6 และ 6 ต่อ 4 ไอโซไนอาซิด น้ำตาลแมนนิทอล และไขมัน เกิดอันตรกิริยาต่อกันได้ดีที่สุด อย่างไรก็ตามผงยาทุกสูตรตำรับมีเส้นผ่าศูนย์กลางของอนุภาคในอากาศที่ 7-8 ไมโครเมตร ซึ่งใหญ่กว่าขนาดที่คาดว่าจะนำส่งสู่ทางเดินหายใจได้ (1-5 ไมโครเมตร) ดังนั้นการใช้น้ำตาลแมนนิทอลขนาด 3 ไมโครเมตรเป็นแกนของโปรลิโปโซมในการศึกษานี้ไม่สามารถนำส่งยาไปถึงทางเดินหายใจส่วนล่างได้ อย่างไรก็ตามเมื่อนำผงยาทั้งสองชนิดมาทดสอบความเป็นพิษกับเซลล์เยื่อบุทางเดินหายใจทั้งส่วนบนและส่วนล่าง รวมถึง alveolar macrophage พบว่าผงยาทั้งสองชนิดไม่เป็นพิษกับเซลล์ทั้งหมดใน

ทางเดินหายใจ นอกจากนี้พบว่าผงยาทั้งสองชนิดไม่กระตุ้น alveolar macrophage ให้สร้างสารก่อการอักเสบ เช่น interluekin-1β, tumor necrosis factor-α และ nitric oxide ในการตอบสนองทางภูมิคุ้มกัน ดังนั้นจึงสรุปได้ว่าผงยาทั้งสองชนิดมีความปลอดภัยต่อเซล์เพาะเลี้ยงในทางเดินหายใจ นอกจากนี้ยังพบว่าการบรรจุยาไรแฟมปิซินในอนุภาคลิโปโซมสามารถเพิ่มประสิทธิภาพในการฆ่าเชื้อ *M. bovis* ภายในเซลล์ alveolar macrophage ได้เมื่อเทียบกับยาเดี่ยวที่ความเข้มข้น 0.625 ไมโครกรัมต่อมิลลิลิตร (*p*-value น้อยกว่า 0.05) โดยที่สูตรตำรับลิโปโซมที่มีสารประจุบวกแสดงประสิทธิภาพในการฆ่าเชื้อได้สูงที่สุด (*p*-value น้อยกว่า 0.05) เช่นเดียวกันกับกรณีของโปรลิโปโซมของยาไอโซไนอาซิด พบว่ายาในรูปของโปรลิโปโซมแสดงประสิทธิภาพในการฆ่าเชื้อ *M. bovis* ภายในเซลล์ alveolar macrophage ที่เหนือกว่ายาเดี่ยวที่ความเข้มข้น 5 ไมโครกรัมต่อมิลลิลิตร (*p*-value น้อยกว่า 0.05) ดังนั้นจึงสรุปได้ว่าผงยาทั้งสองชนิดในรูปลิโปโซมมีศักยภาพและความปลอดภัยต่อเซลล์ในทางเดินหายใจและสามารถฆ่าเชื้อวัณโรคภายในเซลล์ในระยะเวลาอันสั้นดังนั้นการศึกษาก่อนการศึกษาทางคลินิกในงานวิจัยนี้จะเป็นข้อมูลยืนยันในการทดลองใช้ยาในทางคลินิกต่อไป

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ABSTRACT

Liposomes dry powder containing rifampicin (RIF-liposome) and proliposome powder containing isoniazid (INH-proliposome) were developed for delivery to the respiratory tract as an alternative tuberculosis treatment. The toxicity of both liposome powders to the respiratory associated cell lines and its potential to provoke immunological responses from AMs were determined. Studies were performed in vitro which alveolar macrophage cell lines infected with *Mycobacterium bovis* and were exposed to antituberculosis in the free drug form or RIF-liposome or INH-proliposome form. The obtained results support evidences of the potential to develop antituberculosis encapsulated liposome dry powder as an alternative tuberculosis treatment before transitioning to clinical studies in the future.

RIF liposome suspensions containing different millimole ratios of cholesterol (CH) and soybean L-∞-phosphatidylcholine (SPC) were prepared by the chloroform film method, followed by freeze-drying. Three sugars (mannitol, trehalose and lactose) were employed as a carrier and cryoprotectant at a different freeze drying

condition. Isoniazid-proliposome was prepared by using spray dried mannitol with diameter of 3 μm as a core carrier. The mixture of different ratios of isoniazid to mannitol as a powder part was coated with soybean L-∞-phosphatidylcholine (SPC) and cholesterol (CH) in ethanolic solution by spray drying method.

The RIF-liposomes were a mixture of 200-300 nm unilamellar and multilamellar vesicles. Higher CH content formulation indicated more order of lipid bilayer thus resulted in a smaller change in size distribution with time after 4 weeks storage. Furthermore, the SS-NMR results indicated that RIF was located between the acyl chains of the phospholipid bilayer and associated with CH molecules. Fifty percent encapsulation of RIF was obtained when the lipid content was high (SPC 10 mM: CH 10 mM). Mannitol was found to be a suitable cryoprotectant and carrier, which is attributed to its crystallinity, and use of mannitol gave particles with a mass median aerodynamic diameter (MMAD) of 3.4 μm. In terms of chemical stability, RIF in dry powder formulations was considerably more stable when compared to RIF aqueous solutions and RIF liposomal suspensions. INH-prolipsome was successfully prepared by spray drying method. The content uniformity was acceptable with the % RSD less than 6. Encapsulated isoniazid was 10% when isoniazid-proliposome was reconstituted. Proliposome formulation containing mannitol to INH in the ratio of 4:6 and 6:4 exhibited the greatest interaction of all compositions. A MMAD of 7-8 μm was obtained from all formulations. Therefore, the used of microparticulate mannitol as a core carrier of proliposome, in this study, did not improve the aerosolized properties of the powder. However, RIF –liposome and INH-proliposome was evidently to be safe to respiratory associated cells including bronchial epithelial, small airway epithelial and AMs. Furthermore, they did not activate AMs to produce

inflammatory mediator including interluekin 1β, tumor necrosis factor α and nitric oxide at all concentrations. The MIC against *M. bovis* was 0.16 µg/mL and 0.63 µg/mL for RIF-liposome and free rifampicin, respectively. The positively charged liposomes formulation exhibited greatest antituberculosis efficacy against *M. bovis*. The MIC against *M. bovis* was 0.4 µg/mL for both INH-proliposome and free INH. The efficacy of RIF-liposome against intracellular infection was significant higher than free RIF (p-value < 0.05) at the concentration of 0.625 µg/mL. In the similar manner, the INH-proliposome showed superior efficacy compared with free INH at the concentration of 5 µg/mL (p-value < 0.05). Therefore, the RIF-liposome and INH-proliposome demonstrated a potential to be used as an alternative tuberculosis treatment.

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Finally, I dedicate this work to my love grandfather' spirit. It is a pity that he is not here today.

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LIST OF ABBRIEVIATIONS

% RSD	=	Percentage of relative standard deviation
$^{\circ}$C	=	Degree Celcius
µL	=	microliter
µm	=	micrometer
µs	=	microsecond
ACI	=	Andersen Cascade Impactor
AM	=	alveolar macrophage
AMs	=	alveolar macrophages
ANOVA	=	analysis of variance
ATCC	=	Americal Type Cell Culture
BCG	=	bacilli Calmette-Guérin
BEGM	=	bronchial epithelial growth media
CFU	=	colony forming unit
CH	=	cholesterol
Cryo-TEM	=	Cryo- transmission electron microscopy
CSA	=	chemical shift anisotropy
DCP	=	dicetyl phosphate
DMSO	=	dimethylsulfoxide
DPBS	=	Dulbecco's phosphate buffer solution
d-POPC	=	deuterated 1-palmitoyl (D31)-2-oleoyl-*sn*-glycero-3-phosphocholine

DSC = differential scanning calorimetry

LIST OF ABBRIEVIATIONS (Continued)

ED = emitted dose

EDTA = ethylene

ELISA = enzyme-linked immunosorbent assay

EM = electron microscope

FBS = fetal bovine serum

FPF = fine particle fraction

h = hour

HPLC = high performance liqid chromatography

Hz = Hertz

IL-1β = interleukine 1β

INH = isoniazid

J/g = joule/gram

kDa = kilodalton

kHz = kilohertz

kV = kilovolt

L = litre

LPS = lipopolysaccharide

LS = liposome suspension

M = mole

M7H9 = Middlebrook 7H9

mA	=	milliampere
MDRTB	=	multidrug-resistance tuberculosis

LIST OF ABBRIEVIATIONS (Continued)

mg	=	milligram
MHz	=	millihertz
MIC	=	minimum inhibitory concentrations
mL	=	millilitre
MLV	=	multilamellar vesicle
mM	=	millimole
mm	=	millimeter
MMAD	=	mass median aerodynamic diameter
mmole	=	millimole
NHBE	=	normal human epithelial epithelial
NMR	=	nuclear magnetic resonance
NO	=	nitric oxide
OADC	=	oleic albumin dextrose catalase
PBS	=	phosphate buffer solution
ppm	=	part per million
RIF	=	rifampicin
RLS	=	reconstituted liposome suspension
rpm	=	round per minute
s	=	second

SA	=	stearylamine
SAEC	=	small airway epithelial cell
SAGM	=	small airway growth media

LIST OF ABBRIEVIATIONS (Continued)

SD	=	standard deviation
SDS	=	sodium dodecylsulfate
SEM	=	scanning electron microscope
SPC	=	soybean phosphatidylcholine
SS-NMR	=	solid-state nuclear magnetic resonance
TB	=	tuberculosis
Tg	=	glass transition temperature
TNF-α	=	tumor-necrosis factor α
UV	=	ultraviolet
v/v	=	volume/volume
XRD	=	X-ray diffractometer

CHAPTER 1

GENERAL INTRODUCTION

1.1 Rationale

The concern of conventional tuberculosis treatment regimen is the need of administration of one or more drugs for the period of six months or longer. This may lead to serious complications such as degradation of drug before reaching to target sites subsequently leading to inadequate drug concentrations at the infected sites. In addition, a potential drug may be toxic and causes hepatotoxicity over its long term administration, leading to premature termination of chemotherapy (Khuller *et al.*, 2004). Failure in compliance is the main reason for the appearance of multidrug resistance tuberculosis; MDRTB (Mitchison, 1997). The management of MDRTB needs much skill, and care and the use of reserve drugs. However, overall reserve drugs are very expensive, relative weak activity and have many side effects (Crofton, 1997). Development of new antituberculosis drug with more effective, longer acting and less toxic are still likely to be far from satisfactory (Crofton, 1997; Pinto-Alphandary *et al*., 2000; Khuller *et al.*, 2004). Therefore, there is need to improve the therapeutic index of existing drugs by employing suitable drug delivery systems (Khuller *et al.*, 2004).

Pulmonary tuberculosis is most commonly characterized by the involvement of alveolar macrophages (AMs) harboring a large number of

Mycobacterium tuberculosis (Vyas *et al*., 2004). The bacilli invade and begin their replication within AMs before spread out. Therefore, tuberculosis can be seen as a disease involving macrophages (Azarmi *et al.*, 2008). Eradication of intracellular infections is difficult due to tubercle bacilli are protected by various biological structures around the infection foci (Bakker-Woudenberg, 1995; Pinto-Alphandary *et al*., 2000). Poor penetration of antituberculosis into the cells is the major reason for the limited activity of most antibiotics in intracellular infection (Pinto-Alphandary *et al*., 2000). Thus, a critical task of TB management that is sufficient intracellulary antibiotic concentration has to be achieved (Bakker-Woudenberg, 1995, Yoshida *et al.*, 2006).

Logical strategies for using drug carrier in the treatment of certain infectious diseases involving macrophages have been developed based on the pathogenesis of the disease (Alving, 1988). AMs recognize a colloidal drug carrier such as liposomes as non-self and ingest it with its payload resulting in cell selective intracellular transfer of a pharmacological agent (Schreier *et al*., 1993; Ahsan *et al*., 2002). Hence, the rationale of this approach is based on the hypothesis that the liposomes are taken up by the cells containing the intracellular pathogens, thus increase the ability of the antibiotic to reach its target. Furthermore, from ultrastructure studies on the uptake of liposome indicate that internalized liposome could fuse with phagosome containing the infectious agent (Pinto-Alphandary *et al*., 2000).

At the present time, liposomes are the premier particulate systems over the other drug delivery systems in terms of safety and use in the clinic (Khuller *et al.,* 2004). By injectable routes, besides being a source of discomfort and inconvenience

for the patient, it may also not be very effective in the treatment of pulmonary tuberculosis infections. This is due to leakage of their contents in plasma compartment before they reach the target tissue, rapid clearance from the blood stream and their uptaken by the macrophage of the liver and spleen (Bakker-Woudenberg, 1995; Khuller *et al.,* 2004; Vyas *et al*., 2004). However, the difficulty in the treatment of lung infection can be overcome by direct targeting of drugs using an aerosol (Khuller *et al.,* 2004). Since AM is an 80% population of total macrophage in the lung and there are no natural barrier between AM and liposomes entering to the airways (Rooijen and Sanders, 1998). Therefore, direct delivery of antituberculosis to AMs generates high local intracellular drug concentrations (Schreier *et al*., 1993). This also reduced the bacterial load in the lung and prevents further development of infection organism. Less systemic toxicity and slow intracellular release are the results from this strategy (Alving, 1988). Furthermore, duration and frequency of treatment may be shorten thus improve patient compliance and lower risk of MDRTB development.

However, inhalation administration route provides approximately 80% of administered material deposited in the upper airways (McCalden, 1990), then the safety of liposome to bronchial epithelial and alveoli is questionable. Also, this dosage may cause toxic to AM since aerosolized drug produces the high local drug concentration. Several studies have been reported the *in vivo* safety of inhalation liposome both in animal (Schreier *et al.*, 1993; Deol *et al.*, 1997) and human (McCalden, 1990; Thomas *et al*., 1991; Schreier *et al.*, 1993; Gibbert *et al*., 1997; Waldrep, 1997; Whitehead *et al*., 1998; Palmer *et al.*, 2001; Ten *et al*, 2002; Verschraegen *et al*., 2004). However, different formulations may produce different safety profiles and each should be rigorously tested (McCalden, 1990). The acute

toxicity of these delivery systems must be established for the pulmonary epithelia. Furthermore, there are no evidence supports that the direct challenging AMs with the liposome containing antituberculosis do not activate the immunological response of AM to produce inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) and nitric oxide to eliminate those foreign particles. For safety reason before transferring to phase I clinical trial *(in vivo)*, this study was designed to monitor the pre-clinical studies of the liposome or proliposome containing antituberculosis, *in vitro*, potential to produce cytotoxicity and immunological activation of AMs. Cell culture models seem to be the best way to assess such effects. Standard toxicity and the release of inflammatory cytokines can be performed with this cell after particle uptake (Azarmi, 2008). However, the extent of liposome binding and subsequence ingestion by macrophage depends on a number of factors of the intended liposomes. These include composition, type, size, and surface of liposome (Ahsan *et al*., 2002). This may affect the therapeutic efficacy of liposome against intracellular Mycobacterial infection.

Another aspect is the stability of the encapsulated antibiotics in the aqueous compartment of the liposomes. As a general rule, one of the difficulties in the practical application of these products has been the long-term stability of the liposomes. Then liposomes need to be transformed to dry powders (Couvreur *et al*., 1991). In addition, due to the different physical properties of rifampicin (hydrophobicity) and isoniazid (hydrophilicity), the different methodology had been used. For rifampicin, the pre-formed liposome suspension was prepared and then transitioned to dry powder by freeze drying. Whereas pre-formed liposome suspension

is not applicable for hydrophilic agent like isoniazid due to easily leakage encapsulated drug and low encapsulation efficacy (Verumi and Rhodes, 1995), thus proliposome formulation (*in situ* forming liposome), has been formulated.

1.2 The objective of the studies

The aims of the studies are:

1. To prepare and characterize liposome containing rifampicin suspension.
2. To prepare and characterize liposome containing rifampicin dry powder as aerosol.
3. To prepare and characterize proliposome containing isoniazid dry powder for inhalation.
4. To evaluate toxicity of liposome containing rifampicin and proliposome containing isoniazid to the respiratory associated cell lines.
5. To evaluate the potential of liposome containing rifampicin and proliposome containing isoniazid to activate the immunological response from alveolar macrophage.
6. To evaluate *in vitro* efficacy of liposome containing rifampicin and proliposome containing isoniazid against *M. tuberculosis* and *M. bovis.*
7. To evaluate *in vitro* efficacy of liposome containing rifampicin and proliposome containing isoniazid against *M. bovis* infected AMs.

CHAPTER 2

REVIEW LITERATURES

2.1 Tuberculosis

Tuberculosis (TB) remains a common infectious disease worldwide. The World Health Organization (WHO) has declared TB as a 'Global Emergency' in the year 1993. Owing to increased poverty in an overpopulated world, lack of attention to tuberculosis services and the impact of the HIV/AIDS pandemic, there are more cases of tuberculosis today than at any previous time in human history (Grange and Zumla, 2002).

2.1.1 Epidemiology

TB is contagious and spreads through the air, if not treated, each active TB patient infects to other people on average of 10 to 15 people every year. There were 8.8 million new TB cases in 2005 which 80% of them found in 22 countries (World Health Organization, 2007). Three to four million cases were sputum-smear positive, the most infectious form of the disease. Most cases (5-6 million) are people aged 15-49 years (Frieden *et al*., 2003). Two billion people, one-third of the world's total population are infected with TB bacilli. People died from TB over 1.6 million in 2005, equal to an estimated 4400 deaths a day. TB is a leading killer among HIV-infected people with weakened immune systems; about 200,000 people living with HIV/AIDS die from TB every year, most of them being in Africa (World Health Organization, 2007).

2.1.2 Etiology of tuberculosis

The causative organism of tuberculosis includes in the genus *Mycobacterium,* which are *M. tuberculosis, M. bovis*, *M. africanum* and *M. microti* (Grosset *et al*., 2000 and Grange and Zumla, 2002). Human is the only reservoir for *M. tuberculosi*s. However, human infected with *M. bovis* and *M. Africanum* is uncommon (Yepes *et al*., 2004).

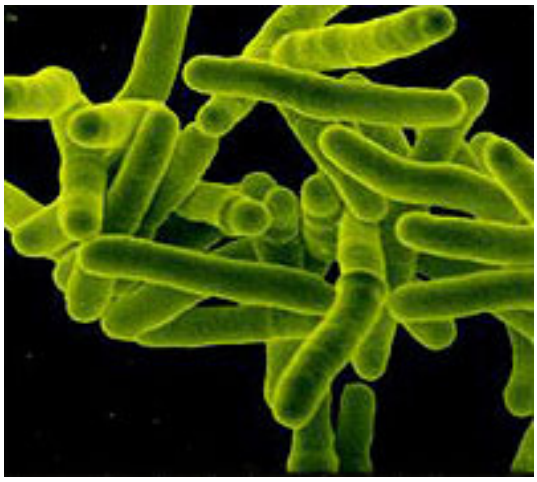


Figure 2.1 *Mycobacterium tuberculosis*

(Institute of chemical biology and drug discovery at Stony Brook University, 2005)

The organism is an aerobic, non-motile, non-spore forming bacillus. The tubercle bacilli (Figure 2.1) are often slightly thin rods 2-4 μm in length and 0.3-0.5 μm in diameter (Wienberger, 1992). *M. tuberculosis* is difficult to stain with the usual methods, although it belongs to the gram-negative bacteria (Grosset *et al*., 2000). It can be detected in sputum smears with carbon fushsine (Ziehl-Neelsen) due to the property of retaining arylmethane dyes in treatment with mineral acids (thus mycobacteria are said to be acid-fast). The bacilli appear under microscope

examination as a slightly curved or straight small red or pink rod. The organism is slow growing, with visible growth in appropriate media taking from 2 to 6 weeks (Grosset *et al*., 2000; Grange and Zumla, 2002; Yepes *et al.,* 2004).

The high lipid content of the cell wall is responsible for the resistance of mycobacteria to decontamination procedures with acid, sodium hydroxide, and/or detergents. However, *M. tuberculosis* is as susceptible as other bacteria to heat, x-rays, UV, and alcohol. *M. tuberculosis* remains viable for weeks at +4 $^{\circ}$C and for years at -70 $^{\circ}$C (Grosset *et al*., 2000).

2.1.3 Pathogenesis of tuberculosis

Infection of humans with *M. tuberculosis* can occur via several routes including inhalation, ingestion and inoculation (Grange and Zumla, 2002). The usual route of infection is inhalation of small aerosol droplets, generally 1-5 μm containing tubercle bacilli. The sources of these airborne droplets are from speaking, coughing, singing or laughing of the infectious tuberculosis patient (Wienberger, 1992; Piessens and Nardell, 2000; Yepes, *et al*., 2004). The small particles, lodged in the alveolar, are engulfed by AMs while large droplet land on the ciliated epithelium of the airway and then are carried up by mucociliary escalator, swallowed, and rendered harmless (Piessens and Nardell, 2000).

Traditionally, tubercle bacilli have been divided into two forms, primary and post-primary tuberculosis (Figure 2.2). It is usually assumed that post-primary tuberculosis is the result of endogenous reactivation of latent or dormant lesions (Grange and Zumla, 2002). The majority of people exposed to *M. tuberculosis*

produce a strong cell-mediated immune response that stops the progression of the infection. Thus, clinically evident tuberculosis and the primary infection may not develop and go unnoticed. As general rule, around 2-5% of infected persons develop clinically evident of primary tuberculosis and a further 2-5% subsequently develop post-primary disease (Grange and Zumla, 2002; Yepes *et al*., 2004).

Three main factors determining whether exposure to tubercle bacilli results in infection are the infecting dose, the strength of innate host immunity, and the virulence of the infecting mycobacteria. If the bacilli are inhibited or killed by the AMs that ingested it then infection aborts. If not, ingested bacilli multiply, kill AMs, and initiate the infection（Piessens and Nardell, 2000).

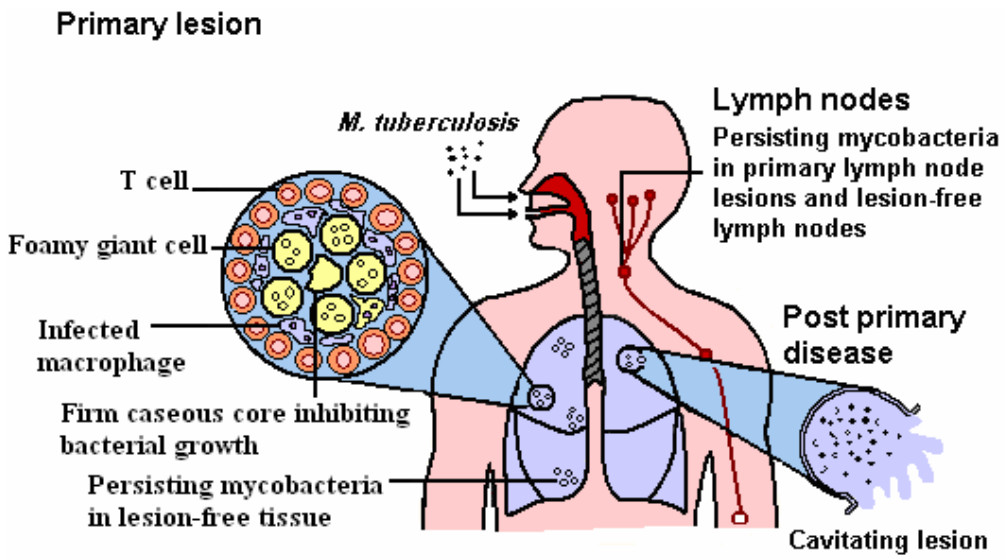


Figure 2.2 The pathogenesis of tuberculosis (Virginia bioinformatic institute, 2008)

The mechanisms of intracellular survival of tubercle bacilli are not clear. There is some evidence that *M. tuberculosis* can prevent phagosome-lysosome fusion. Other studies have demonstrated that virulent mycobacteria can prevent acidification of the phagolysosome, perhaps by modulating the activity of a membrane proton pump. In addition, some of the components of the mycobacterial cell wall (e.g. cord factor) may be directly cytotoxic to macrophages (McMurray, 2001).

2.1.4 Clinical manifestations

Patient with pulmonary tuberculosis can manifest (1) systemic symptom, (2) symptoms referable to the respiratory tract, or (3) an abnormal finding on chest radiograph but no clinical symptoms. When symptoms occur they are generally insidious rather than acute in onset (Wienberger, 1992). The systemic symptoms are often relatively nonspecific which fever is the most easily quantified (American Thoracic Society, 2000). Weight loss, anorexia, fatigue, low-grade fever, and night sweats are also common but are more difficult to quantified (Wienberger, 1992 and American Thoracic Society, 2000). The most common symptoms resulting from pulmonary involvement are cough, sputum production and hemoptysis; (Wienberger, 1992 and American Thoracic Society, 2000). Chest pain is also occasionally present (Wienberger, 1992). Many patients have neither systemic nor pulmonary symptoms but an abnormal finding on chest radiograph is found, often performed for an unrelated reason (Wienberger, 1992 and American Thoracic Society, 2000).

Patients with extrapulmonary involvement frequently have pulmonary tuberculosis, but occasional cases are limited to an extrapulmonary site. Pericardium,

pleura, kidney, peritoneum, adrenal gland, and central nervous system may be involved, with symptom resulting from the particular organ or region that is affected. With military tuberculosis, the disease is disseminated, and the patients are usually systemically ill (Wienberger, 1992).

2.1.5 Treatment of tuberculosis

The three aims of antituberculosis chemotherapy are to cure the patient, render the patient rapidly non-infectious and prevent the emergence of drug resistance.

Theoretical basis for antituberculosis treatment

Antituberculosis drugs are described into three categories based on their activity (Fujiwara *et al*., 2000):

a) Prevention of drug resistance

Drugs that are highly active suppress the growth of entire bacterial population to prevent the emergence of mutant resistant to another drug. This activity is measured by how well the drug prevents treatment failure due to the emergence of drug resistance during therapy. Isoniazid and rifampicin have the highest activity in preventing the emergence of drug resistance. The activity of pyrazinamide for prevention of TB is poor (Fujiwara *et al*., 2000).

b) Early bactericidal activity

Early bactericidal activity is the ability of a drug to rapidly kill of active growing bacilli during the initial part of therapy. Isoniazid is the most potent bactericidal agent (Mitchison, 1997 and Fujiwara *et al*., 2000).

c) Sterilizing activity

Sterilizing activity is the ability of drug to kill semidormant bacteria and is measured by the speed which the last few viable bacteria are killed (Fujiwara *et al*., 2000). Drug with potent sterilizing activity have enabled regimens as short as 6 months in duration to be used (Mitchison, 1997 and Fujiwara *et al*., 2000). Rifampicin and pyrazinamide are the most potent sterilizing agents (Mitchison, 1997).

Conceptually, effective treatment regimens are divided into two phases, initial phase and continuous phase. The initial, intensive phase contains bactericidal agents used in combination to kill large, rapidly multiplying populations of *M. tuberculosis* and to prevent the emergence of drug resistance. This is followed by the consolidating, continuation phase containing sterilizing agents to kill the less active, intermittent dividing populations (Fujiwara *et al*., 2000). A nine months regimen of isoniazid and rifampicin, with ethambutol or streptomycin in the first 2 months, provides excellent bactericidal and sterilizing activity (Fujiwara *et al*., 2000). Furthermore, addition of the either rifampicin or pyrazinamide to a basic 9 months regimen substantially reduce the duration of treatment to 6 months with a low relapse rate (Mitchison, 1997 and Fujiwara *et al*., 2000). Ethambutol and streptomycin are added initially before susceptibility results are available to prevent the emergence of rifampicin resistance if there is unrecognized initial isoniazid resistance (Fujiwara *et al*., 2000).

Antituberculosis Treatment Regimens

World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) have developed empiric treatment regimens based on case definitions. The cases are divided into four treatment

categories (Table 2.1) by several criteria. First, those with acid-fast bacillus smear positive pulmonary tuberculosis are the most potent source of infection and are given high priority for treatment. Second, person with certain severe forms of disease that may cause a threat to life (tuberculosis meningitis, pericarditis, or miliary disease) or significant handicap (disease of the spine or kidney) are also given high priority. Finally, those who have had previous treatment are at increased risk for drug resistance and need a prolonged, more costly re-treatment regimen (Grange and Zumla, 2002; Fujiwara *et al.,* 2000).

Table 2.1 The WHO-recommended short-course antituberculosis drug regimens in four categories of patients.

Treatment category	Definition	Initial intensive phase (daily or three times each week)	Continuation phase
I	New smear-positive pulmonary TB; new smear negative pulmonary TB with extensive lung involvement; new severe forms of extrapulmonary TB	2 EHRZ (SHRZ) 2 EHRZ (SHRZ) 2 EHRZ (SHRZ)	6 HE 4 HR 4 H_3R_3
II	Smear-positive pulmonary TB: relapse, treatment failure or treatment after interruption	2 SHRZE/1 HRZE 2 SHRZE/1 HRZE	5 HRE 5 $H_3R_3E_3$
III	New smear-negative pulmonary TB (other than Category I; new less severe forms of extrapulmonary TB)	2 HRZ 2 HRZ 2 HRZ	6 HE 4 HR 4 H_3R_3

IV	Chronic cases, i.e. still bacteriologically positive after supervised re-treatment	Second line drugs required according to WHO guidelines in specialized centers

H=isoniazid, R=rifampicin, Z=pyrazinamide, E=Ethambutol, S= Streptomycin

The number preceding the drugs indicates the number of months of administration; subscripts following individual drugs indicate that the drugs are given intermittently, [twice (2) or thrice (3) weekly] instead of daily.

2.1.6 Antituberculosis medications

As referred to above, the antituberculosis drugs used in the WHO-recommended short-course regimens (the first line drugs) include isoniazid, rifampicin, pyrazinamide and either ethambutol or in some cases, streptomycin. The mechanism of action of antituberculosis medications are summarised in Figure 2.3. Other drugs are available for the treatment of disease resistant to the first line drugs. These include ethionamide and the closely related prothionamide, thiacetazone, cycloserine, capreomycin, p-aminosalicylic acid, fluoroquinolones such as ofloxacin and sparfloxacin and the newer macrolides including clarithromycin and azithromycin. These drugs are generally more toxic, more expensive and less active than the first line drugs and treatment is often prolonged and therefore very costly (Grange and Zumla, 2002).

2.1.6.1 Isoniazid

Isoniazid or isonicotinic acid hydrazide (INH) has specific potent bactericidal against growing *M. tuberculosis*. No other single antimycobacterial agent has proved to be active against the *M. tuberculosis* complex, which such

comparatively low toxicity, as isoniazid. However, the activity of isoniazid is less for other mycobacteria and has no activity against non acid-fast bacteria and eukaryotic cells (Inderlied, 1991). The minimal inhibitory concentration (MIC) of *M. tuberculosis* is 0.025-0.05 µg/mL in broth and 0.1 to 0.2 µg/mL in agar plates (Rieder, 2000).

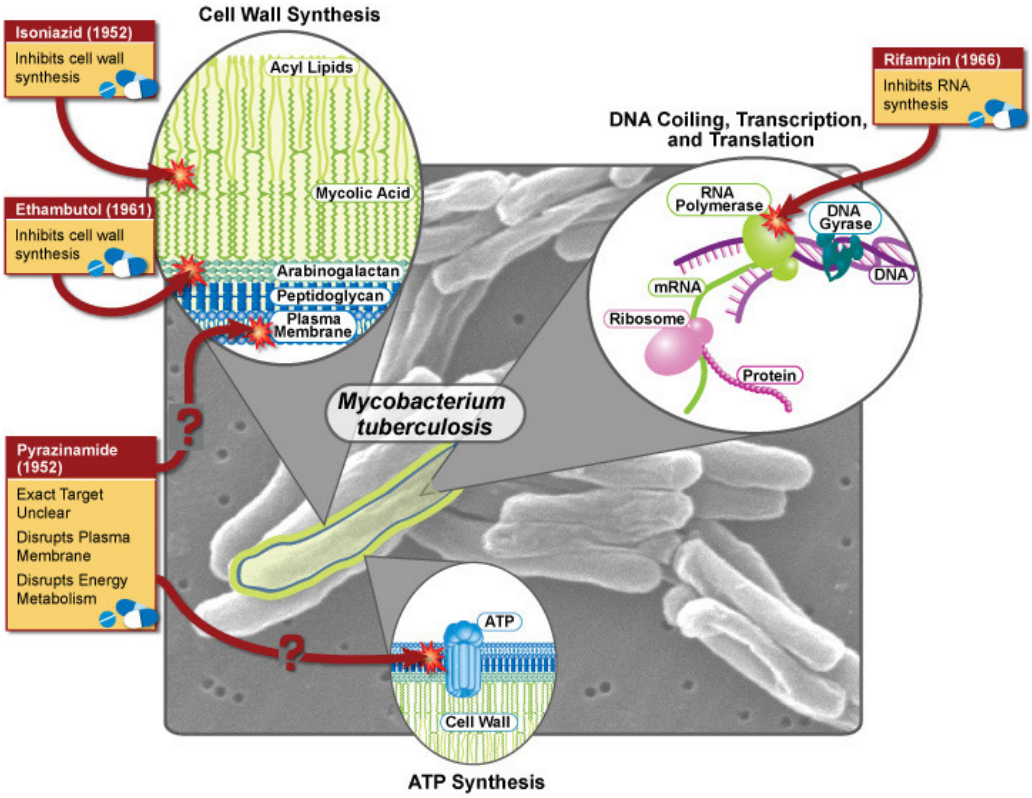


Figure 2.3 Mode of actions of antituberculosis medications (National Institution of Allergy and Infectious Disease; National Institutes of Health., 2007)

Isoniazid leads to a loss of cell wall structural integrity and physiological function which results in cell death (Inderlied, 1991). The action of isoniazid is on the mycolic acid biosynthetic pathway. Apparently, isoniazid blocks the synthesis of saturated fatty acid longer than C26 in the chain length (Sensi and

Grassi, 1996) and the synthesis of monosaturated long chain fatty acid *in vivo* (Rieder, 2000). Isonicotinic acid is responsible for the inhibitory effect of isoniazid on mycobacteria. Isoniazid penetrates the cell, where it is oxidized enzymatically to isonicotinic acid. The intracellular pH may cause isoniazid completely ionized and cannot permeate across the membrane. Consequently, it accumulates inside the cell (Sensi and Grassi, 1996).

The spontaneous mutation rate to isoniazid resistance may be high with *M. tuberculosis* and *M. bovis*, ranging from 10^{-5} to 10^{-6} and strains that become isoniazid-resistant usually simultaneously lose catalase and peroxidase activity (Inderlied, 1991). Approximately 60 to 70% of isoniazid resistant strains carry mutations, *kat G* and possibly *ahp C* or *inh A* (Rieder, 2000).

The peak serum concentrations levels of 5 μg/mL are obtained in 1-2 h after administering 300 mg isoniazid (recommended dosage of 5 mg/kg body weight daily) are well above the MIC (Rieder, 2000). Isoniazid is readily absorbed from the gastrointestinal tract in humans (AHFS, 1999 and Sensi and Grassi 1996). The primary metabolic route is acetylation in the liver to acetyl isoniazid. The elimination of isoniazid from serum is determined by the acetylator status of the individual under genetic control and individuals can be slow or rapid acetylator of the drug (Sensi and Grassi 1996).

Isoniazid is usually tolerated for a long period of treatment. Hepatic side effects consist of frequent subclinical asymptomatic enzyme abnormalities, and occasionally severe clinical hepatitis occurs, especially in patients with previous hepatobiliary disease and in alcoholics. Peripheral neuritis and stimulation of the central nervous system are common side effects. Isoniazid seems to compete with

pyridoxal phosphate, and the concurrent administration of pyridoxine (Vitamin B6) has been suggested to prevent isoniazid toxicity (Sensi and Grassi, 1996).

Isoniazid is an inhibitor of oxidative and demethylation metabolism as well as other cytochrome P-450 dependent microsomal pathways. It is also a monoamine and diamine oxidase inhibitor. (Rieder, 2002).

2.1.6.2 Pyrazinamide

Pyrazinamide is active only against *M. tuberculosis*. It was recognized early on that pyrazinamide acts only in an acid environment (Rieder, 2002). The antimycobacterial activity of pyrazinamide depends on conversion of the drug to pyrazinoic acid (POA), active metabolite which is preferential accumulated in an acidic pH, by amidase pyrazinamidase of *M. tuberculosis* (AHFS, 1999 and Rieder, 2002). In addition, the fact that POA lowers the pH of the environment below that which is necessary for growth of *M. tuberculosis* appears to contribute to the drug's antimycobacterial activity *in vitro* (AHFS, 1999). However, the exact mechanism of action of pyrazinamide or pyrazinoic acid is unknown (Sensi and Grassi, 1996). Some strains of pyrazinamide-resistant *M. tuberculosis* do not appear to produce pyrazinamidase due to mutations in *pnc A*, a gene encoding pyrazinamidase, therefore cannot convert the parent drug to POA (AHFS, 1999; Rieder, 2000).

MICs of PZA reported for *M. tuberculosis* were reported to be 50 μg/mL at pH 5.5 and 400 μg/mL at pH 5.95 (AHFS, 1999).

Pyrazinamide is absorbed from the gastrointestinal tract. Plasma concentrations following recommended doses of 20-25 mg/kg were from 30-50

μg/mL. Plasma concentration of POA, is generally greater than those of the parent drug and peak within 4-8 h after an oral dose of the drug (AHFS, 1999).

The two major adverse drug event of pyrazinamide are hepatotoxicity and interference purine metabolism. The latter leads to decrease excretion and accumulation of uric acid, occasionally accompanied by gout-like arthralgia (Rieder, 2000).

2.1.6.3 Rifampicin

Rifampicin is active against *M. tuberculosis*, *M. bovis*, *M. marinum*, *M. kansasii* and some strains of *M. fortuitum*, *M. avium* and *M. intracellulare,* both *in vitro* and *in vivo*. It also works with many gram positive bacteria and some gram negative bacteria including *Neissria meningitidis*, *Staphylococcus aureus*, *Haemophilus influenza*, and *Legionella pneumophila* (AHFS, 1999).

Rifampicin is most active against susceptible bacteria when they are undergoing cell division. It suppresses initiation of chain formation for RNA synthesis in susceptible bacteria by inhibiting DNA-dependent RNA polymerase. The β subunit of the enzyme appears to be the site of action (AHFS, 1999). Mycobacteria develop resistance to rifampicin in a one-step process by mutations in a defined region for the RNA polymerase subunit β. Mutations in the *rpo B* gene of *M. tuberculosis* are responsible for most of the resistance (AHFS, 1999).

The MIC of rifampicin against most susceptible *M. tuberculosis* on M7H10 agar is 0.1-2 μg/mL and 0.25 μg/mL in broth. Rifampicin is well absorbed

from the gastrointestinal tract. Following a single 600 mg oral dose of rifampicin (recommended dose 10 mg/kg body weight), peak plasma concentrations of 12-14 μg/mL were attained within 2-4 h (AHFS, 1999; Rieder, 2000).

Rifampicin is an inducer of several enzymes in the cytochrome P-450 system, leading to numerous interactions with multiple drugs including isoniazid. This action leads most frequently to faster elimination and lower concentrations of the companion drug (Rieder, 2000).

2.1.7 BCG vaccine

BCG (bacilli Calmette-Guérin) was developed by attenuation of *M. bovis*, which is closely related to *M. tuberculosis* (> 90% DNA homology) (Andersen, 2001). It was reported that BCG is the world most widely used vaccine and probably the safest. However, the vaccine does produce some non-life threatening side effects. A common adverse effect is the production of indurations and ulceration at the vaccination site. It leaves a scar which is evident that the recipient has been vaccinated (Groves, 1997).

Recent data indicate that different patterns of protection have been observed around the world. The efficacy of BCG in several trials has been low or non-detectable. Under Groves review (1997), the protective efficacy has varied from below 20% to above 80%. Highly efficient (70-80%) was demonstrated in developed countries such as the UK, Denmark, and North America whereas in some developing countries demonstrated no detectable protection against pulmonary TB (Andersen, 2001).

The reasons for this are multifactorial. Factors are likely to be divided into two sections, the BCG itself and the environment in which vaccine is used. BCG is now obtained from a number of sources but different cultural conditions, variable viability due to storage conditions and other characteristics, all combine to produce different growth rates, viability, morphologies and most important of all, antigen expression.

The environment in which the vaccinations themselves are carried out is likely to be even more critical. Therefore; BCG might achieve its full potential only regions where the population is not heavily exposed to mycobacterial from the environment. Based on animal experimental, it has been suggested that the protection provided by environmental mycobacteria partly masks the effect of a sequent BCG vaccination or that environmental mycobacteria have antagonistic influence and skew the immune response (Groves, 1997; Andersen, 2001). There is now some agreement that BCG should be given at birth and certainly within the first three months of life (Groves, 1997).

2.1.8 Adherence to treatment

The characteristics of tuberculosis treatment can cause a decrease in patient compliance include its length, the need to take several medications, the cost of treatment and drug toxicity. In order to maximize the chance that patients with tuberculosis complete treatment, program of directly observed therapy have been developed. These programs usually include the use of a trained health-care worker to observe a patient taken every dose of medication.

Although the gold standard for the treatment of tuberculosis is the direct observation of every dose of medication, in some tuberculosis-control programs this goal has not been reached, due to, lack of resource and program infrastructure.

Medication monitors, which indicate when pill bottles are being opened, can serve as a proxy for the intent to take medications. However, in at least one study, approximately one-third supposedly reliable patients had poor adherence to treatment (Fujiwara *et al*., 2000).

2.2 Alveolar macrophage and immunity

The alveolar macrophage (AM) was previously thought to be exclusively derived from bone marrow cells that circulate in the blood as monocyte before migrating into the lung (Thephen and Havenith, 1994 and Smith, 2007). In addition, it was postulated that interstitial macrophages are the immediate precursors for the AM population and substantial maturation or differentiation occurs subsequently to its migration into the alveolar compartment (Thephen and Havenith, 1994; Fathi *et al*., 2001).

As the AM is situated at the interface of air and tissue in the alveoli and alveolar duct, it is primarily responsible for the binding, ingestion, and clearance of inhaled particulate matter (Thephen and Havenith 1994; Kraal *et al*., 1997). AM responses ranges from abundant production of inflammatory cytokines and reactive oxygen intermediates to simple ingestion and clearance with minimal inflammation (Palecanda and Kobzik, 2000).

Phagocytic capacity of AM provides an efficient non-specific defense against inhaled particles. In addition, they play a role in the mediation of acute and

chronic inflammatory processes in the lung (Thephen and Havenith, 1994). AM posses potent antimicrobial activities via the local release of degradation enzymes, reactive oxygen metabolites and reactive nitrogen intermediates, mainly nitric oxide. (Christman *et al*., 1988 and Thephen and Havenith, 1994). The released ROIs, nitric oxide, and lysosomal enzyme can injure normal host tissue due to they do not distinguish between self tissues and stimuli (Haugen *et al.*, 1999). Moreover, AMs are primary source of several pro-inflammatory cytokine, such as tumor necrosis factor α (TNF-α) (Garner *et al*., 1994) and interleukin -1 (IL-1) (Christman *et al*.,1988). These polypeptide mediators clearly function as communication links via their ability to establish cytokine networks, whereby one cytokine can bind to an appropriate receptor on a specific cell and induce the expression of additional cytokine(s) (Kunkel *et al*., 1997). As shown in Figure 2.4, an exogenous challenge can induce the expression of IL-1 or TNF by AM which can subsequently activate non-immune lung cells, resulting in the production of additional cytokines (Kunkel *et al*., 1997).

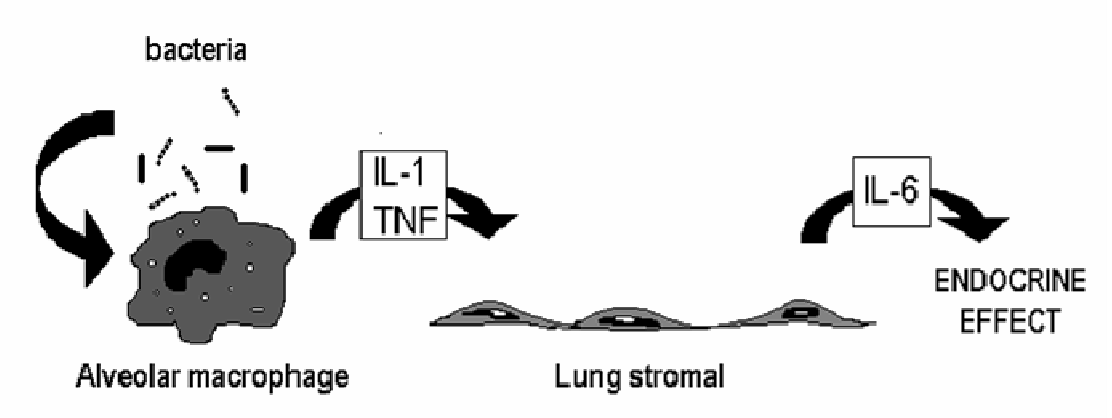


Figure 2.4 A key cytokines (IL-1 and/or TNF) as a direct cell to cell communication cytokine network (Modified from Kunkel *et al*., 1997)

AMs are present in the lower respiratory tract either attached to the wall of alveoli or as free cells in the alveolar lumen. (Thephen and Havenith, 1994). Together with oxygen, airborne antigenic material present in ambient air enters the lung and make contact with the respiratory epithelial surfaces (Thephen and Havenith 1994). However, it is remarkable that there is only a low prevalence of inflammation under normal conditions. This indicates that defense mechanisms against airborne antigens are tightly controlled and regulated. AMs also play a role in cellular immune responses and the maintenance of homeostasis on the airway surface, by inhibiting the local T cell responses to airborne antigen. To prevent constant sensitization to these ubiquitous non-pathogenic antigens and to maintain homeostasis at mucosal surfaces of the respiratory tract, an immunological suppressor has been described. Under normal circumstances, AMs act produce inhalation tolerance. The initiation of the immune system can lead to sensitization, often mediated by T-helper cell activation and the elicitation of antibody-mediated immunity dependent on the type of antigen involves. Under the steady state conditions, AM predominately function in a suppressive mode and down-regulate local pulmonary immune responses (Thephen and Havenith 1994; Kraal *et al*., 1997).

2.2.1 Lung macrophage cell lines

A number of alveolar macrophage cell lines have been developed and used experimentally. These cell lines have arisen from spontaneous transformation or by intentional application of immortalizing retroviruses. These cell lines share the advantage of providing large numbers of cells grown under define condition and

purity. The best characterized and most used of these cell lines is the rat NR 8383 cell line. This cell resembles normal AM in many respects, including morphology, sensitivity to endotoxin, phagocytic function, and expression of cytokines. Like AM, they show substantial heterogeneity while growing in culture and include a range of cell size and adherent and non-adherent subpopulations (generally 50% each). Murine cell lines have been described including the MH-S and C11 (Kobzik, 1997).

2.3 Liposome and its characteristics

2.3.1 Liposome

2.3.1.1 Definition

Liposomes are submicron vesicles of which structure is closed by one or more concentric of an amphiphile molecules (usually phospholipids) surrounding an aqueous compartment (Figure 2.5). A liposome population of vesicles may range in size from ten nanometers to ten microns in diameter (New, 1990 a).

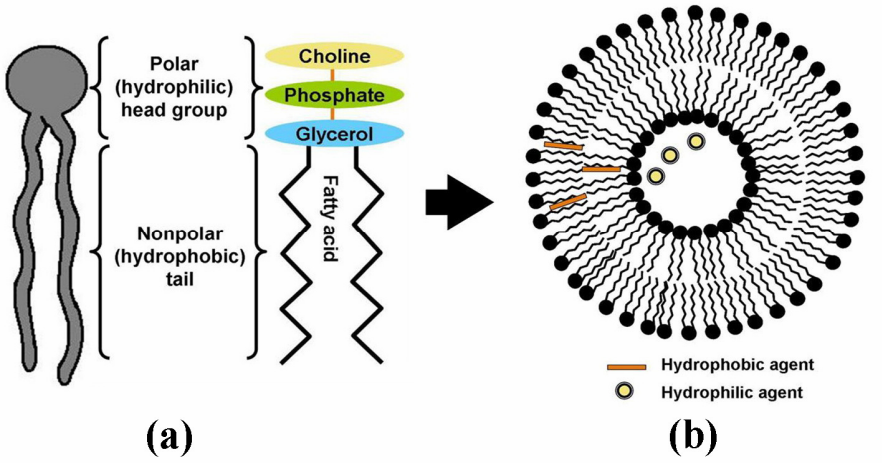


Figure 2.5 Structure of (a) phospholipid molecule (Azaya Therapeutics, 2008) and (b) liposome vesicle (HBC Protocol Inc., 2008)

The phospholipids molecule composed of defined polar and apolar regions. These molecules do not soluble in aqueous medium but they aligned themselves in planar bilayer sheets when they are dispersed in aqueous medium. Hydrophobic moiety of phospholipids form an apolar core of those bilayer while hydrophilic moiety faces the aqueous phase, in order to minimize the unfavorable interactions between the bulk aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheet fold on themselves results in the formation of closed sealed of liposome vesicles.

Liposomes are potential drug carriers for a variety of drugs. They entrap a quantities of materials both within their aqueous compartment (for hydrophilic molecules) and within the membrane (for hydrophobic molecules). Therefore, liposomes have attracted considerable attention use in drug delivery application (New, 1990a; Swarbrick and Boyland, 1994; Verumi and Rhodes, 1995; Malmsten, 2002).

2.3.1.2 Liposome composition

a) phospholipids

Phospholipids (Figure 2.6), liposomes bilayer structure, are the major structural components of biological membrane. It is biocompatible and biodegradable material therefore this is valuable for liposome using as drug delivery carrier (Sharma and Sharma, 1997; New, 1990a).

The general chemical structure of phospholipids has a glycerol backbone which the hydroxyl is esterified to phosphoric acid. The hydroxyl groups at

position 1 and 2 of the glycerol are usually esterified with long chain fatty acids. One of the remaining oxygen groups of phosphoric acid may further esterified to a variety of organic molecules including glycerol, choline, ethanolamine, serine and inositol (Figrue 2.6). The phosphate moiety represents the head group of phospholipid (Verumi and Rhodes, 1995).

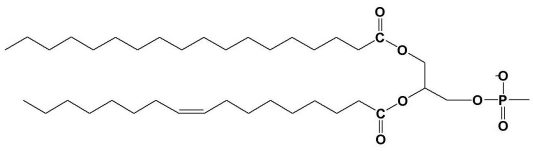
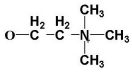
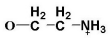
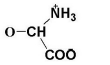
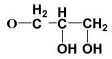
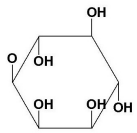
Phosphatidyl moiety	Head group	Common name	abbreviation
		choline	PC
		ethanolamine	PE
		serine	PS
		glycerol	PG
		acid	PA
		inositol	PI

Figure 2.6 Natural phospholipids (Adapted from New, 1990a)

Phospholipids are mostly used for preparing liposomes for pharmaceutical applications. They can be divided into 4 groups (Swarbrick and Boyland, 1994).

1. Phospholipid of natural sources; the main two sources are eggs and soy beans.
2. Modified natural phospholipids; these are natural phospholipid which chemically modified by hydrogenation in order to reduce the degree of unsaturation to different extents to improve the appearance and the resistance to peroxidation.

3. Semisynthetic phospholipids; the acyl chains are removed from natural phospholipids and chemically replaced by defined acyl chains.
4. Fully synthetic phospholipids; these compounds are prepared via complete chemical synthetic pathways.

The most common phospholipids are phosphatidylcholine (PC), amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl carbon chain, with a hydrophilic polar headgroup, phosphocholine (Verumi and Rhodes, 1995). However, regarding to the further development of a liposome formulation, the price of fully or semisynthetic, high quality lipids is still relatively high. They can be replaced by partially hydrogenated soybean or egg PC which is less sensitive to oxidation than the original PC from natural sources (New, 1990a; Swarbrick and Boyland, 1994; Verumi and Rhodes, 1995).

Phosphatidylcholine, also known as 'lecithin', are readily extracted from egg yolk or soy bean. They are used in as the principal phospholipid in liposome for a wide range of applications, both because of low cost relative to other phospholipids, their neutral charge and chemical inertness. Lecithin from natural source is in fact a mixture of PC, with chains of different in length and varying degree of unsaturation. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contain a high proportion of fully saturated chains (New, 1990a).

b) cholesterol

Cholesterol (Figure 2.7) does not by itself form bilayer structure but it can incorporate into phospholipid membranes to make a major changes in the properties of these membranes therefore improve the bilayer characteristics of the

liposomes (Verumi and Rhodes, 1995). Being an amphipathic molecule, cholesterol inserts into the membrane with its hydroxyl group oriented towards the aqueous surface, and aliphatic chain aligned parallel to the acyl chain in the center of the bilayer. The 3β-hydroxyl group is positioned level with the carboxyl residues of the ester linkages in the phospholipids, with little vertical freedom of movement. Thus, cholesterol improves the fluidity of the bilayer membrane, reduces the permeability of water soluble molecules through the membrane, and improves the stability of bilayer membrane in the presence biological fluids (New, 1990a).

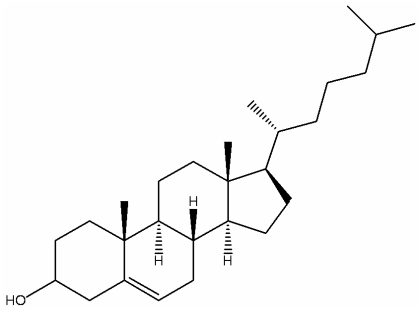


Figure 2.7 Cholesterol chemical structure

2.3.1.3 Classification of liposome

There are various classes of liposomes. Liposomes are classified either by the method of their preparation or by the number of bilayers present in the vesicle, or by their size as shown in Table 2.2. The description of liposomes by the lamellarity and size are common than by the method of their preparation (Verumi and Rhodes, 1995).

Table 2.2 Nomenclature and approximate sizes of various liposomes (Verumi and Rhodes, 1995)

Liposome	Classfication	Approximate size (nm)

By size	Small unilamellar vesicle (SUV)	25-50
	Large unilamellar vesicle (LUV)	100
By lamellarity	Multilamellar vesicle (MLV)	5-10000
	Unilamellar vesicle (ULV)	25-100
By method	Reverse phase evaporation vesicle (REV)	500
	French press vesicle (FPV)	50
	Ether injection vesicle (EIV)	20

2.3.1.4 Preparation of liposome

All methods of making liposomes involve four basic stages: drying down of lipid from organic solvent, dispersion of the lipids in aqueous media, purification of resultant liposome, and analysis of the final products. The main difference between the various methods of manufacturing is in the way in which the membrane components are dispersed in aqueous media, before being allowed to coalesce in the form of bilayer sheets.

The solvent most widely used is a mixture of chloroform and methanol in a ratio of 2:1 by volume. Compounds to be incorporated which are lipid soluble will be added to the organic solution, while compound to be entrapped in the aqueous compartment of liposomes will be dissolved in the aqueous starting solution (New, 1990b).

2.3.2 Characterization of liposome

The great interests in the use of liposome as drug carrier require the necessity of pharmaceutically acceptable procedures for the preparation and characterization of liposome vesicles.

2.3.2.1 Lamellarity determination

The lamellarity of liposomes made from different lipids or preparation procedures varies widely. Most method involves the measurement the proportion of phospholipid exposed on the outside surface of the vesicles. It detects a change of signal using ^{31}P NMR, UV absorbance or fluorescence after the active group of external bilayer reacts with the adding reagent compared to the total signal obtained from the whole liposome bilayer structures. This ratio is 1:1 for large unilamellar vesicles. For SUVs the proportion will is even higher, since the lipid distribution is asymmetric, while for MLVs the value is lower than 50% (Edward and Baeumner, 2006). In addition, other techniques are available include electron microscopy and small angle X-ray scattering (Edward and Baeumner, 2006).

2.3.2.2 Size determination

The average size and size distribution of liposome are important parameters when the liposomes are intended for therapeutic used (Verumi and Rhodes, 1995). Several techniques are available for assessing submicrometer liposome size and size distribution which vary in complexity and degree of sophistication (New, 1990c). These include static and dynamic light scattering, several types of microscopy techniques, size-exclusion chromatography, field flow fractionation, and analytical centrifugation (Edward and Baeumner, 2006). Direct observation by electron microscopy and laser light scattering are discussed here.

Electron microscopy such as transmission EM using negative staining, freeze-fracture TEM, and cryo-EM, is the most precise method since it provides

valuable information of view morphology of each individual liposome and can resolve particles of varying sizes (Edward and Baeumner, 2006). However, this procedure is not suitable for stability monitoring because it is a laborious and is regarded as a time consuming method (New, 1990c; Verumi and Rhodes, 1995).

In contrast, laser light scattering is simple, rapid to perform and suitable method to monitor the stability liposome vesicles (Verumi and Rhodes, 1995). However, this technique is suffer from the disadvantage of measuring an average property of the bulk of the liposomes, and even with the most advances refinements it may not pick up or describe in any detail small deviations from a mean value or the nature of residual peaks at extremes of the size range (New, 1990c).

2.3.2.3 Encapsulation determination

The liposome suspension are a mixture of encapsulated and unencapsulated (free drug) fractions. In a majority of procedures the free and encapsulated drug fractions are separated. The encapsulated fraction of the drug is treated with a detergent to lyse the liposome. The entrapped drug discharges from the liposomes into surrounding aqueous media (Verumi and Rhodes, 1995). The extent of drug entrapped in liposome vesicles can be determined by column chromatography technique or other methods.

The drug candidates that are best suited for encapsulation are those that have high oil/water coefficient that (a) do not leak out of the liposomes. The entrapped drugs are expected to remain encapsulated by the phospholipid bilayers and slowly diffuse out. (b) provide prolonged activity and minimal drug loss during lyophilization. The efficacy of a liposome dosage form will be judged ultimately by its

ability to deliver the drug to the desired site of action over a prolonged period of time. The liposome drug delivery system could render drugs to the biological system while reducing the toxic effects (Verumi and Rhodes, 1995).

2.3.2.4 Determination of residual organic phase in phospholipid mixtures

Chloroform is a good solvent to disperse the lipids, its complete removal from the lipid mixture is imperative when the liposomes are intended for human use. The contaminant of chloroform is carbon tetrachloride (CCl_4) and it is also a known carcinogenic material. A process that uses chloroform as a solvent in the preparation of liposomes should be included a test method (Verumi and Rhodes, 1995).

2.3.3 Stability of liposome

A stable dosage form maintains its physical integrity and does not adversely influence the chemical integrity of the active ingredient during its life on the shelf (Swarbrick and Boyland, 1994).

2.3.3.1 Chemical stability

Hydrolytic reactions of the ester bonds give the degradation products of 2-lysophosphatidylcholine (lyso-PC) and free acid. The presence of lyso-PC enhances the permeability of liposomes and thus it is essential to keep its level to a minimum in a given preparation and during storage. The hydrolysis kinetics of PC to lyso-PC (ester hydrolysis) are strongly pH dependent which the rate increased under acid or basic conditions. Finally, the chemical stability profile of a drug molecule may entirely be

different from the stability profile of it in a liposome preparation (Verumi and Rhodes, 1995).

Peroxidation reactions occur primarily at unsaturated bonds in the acyl chains of the phospholipids. Peroxidation can be induced by autooxidation or photooxidation. Peroxidation reaction can be minimized by selection of lipids with only saturated bonds; protecting the lipid preparations from light; adding antioxidant such as alpha-tocopherol or BHT; by adding EDTA to lipid formulations to remove heavy metal; and by producing the product under nitrogen or argon environment (New, 1990b, Swarbrick and Boyland, 1994; Verumi, 1995).

However, both hydrolysis reactions and peroxidation are presumably solved when using (freeze) dried liposome formulations (Swarbrick and Boyland, 1994).

2.3.3.2 Physical stability

All liposome preparations are heterogeneous in size which average size distribution of the liposome being determined at the time upon their storage. Liposomes tend to fuse and grow into bigger vesicles, which is a thermodynamically more favorable state. Fusion and breakage of liposomes on storage also pose more important problems of drug leakage from the vesicles (Swarbrick and Boyland, 1994). The visual appearance, average size, and size distribution are important parameters to monitor. If sedimentation or flotation in a liposome preparation occurs during shelf life this often indicates aggregation (Verumi and Rhodes, 1995). Vesicles bearing a net charge on their surface are generally regarded less susceptible to aggregation due to electrostatic repulsion (New, 1990b; and Brandl, 2001).

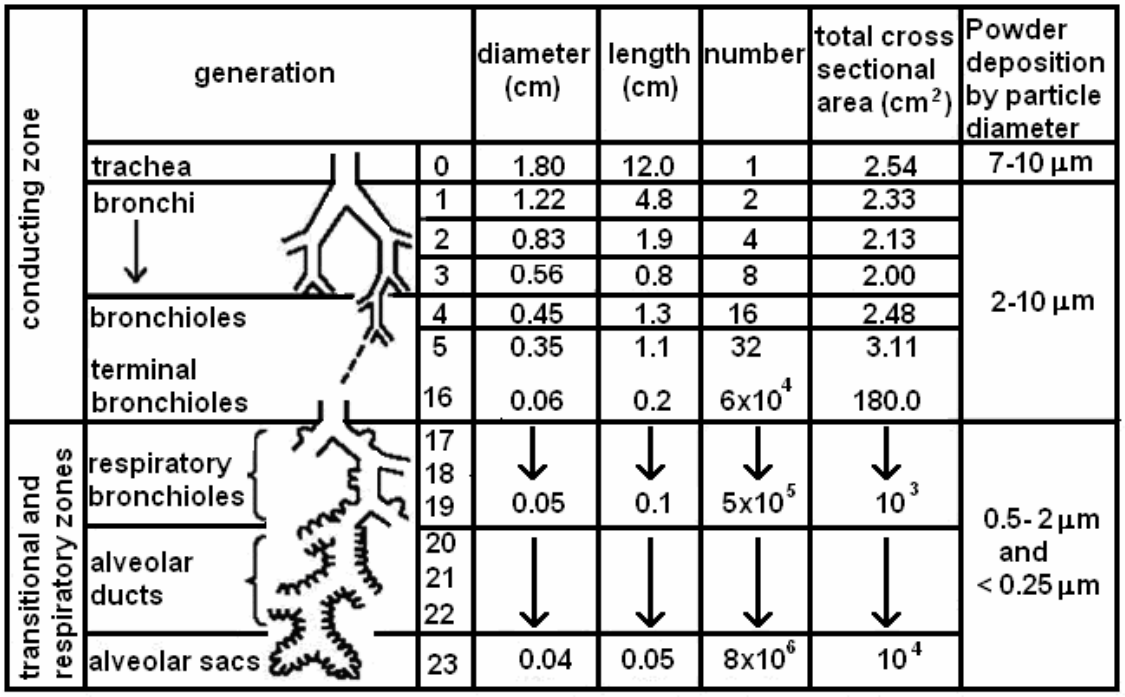
For leakage problem, rate and extent of leakage depends on both type of drug and lipid formulation. If the drug has a high tendency to leak out from liposomes the preparation may be stabilized by using a lipid formula which results in tighter membranes such as long chain saturated phospholipids in combination with cholesterol (Brandl, 2001).

2.4 Drug targeting to respiratory tract

2.4.1 Physiology of the respiratory tract

The airways are often described as the pulmonary tree (Figure 2.8). The tree trunk is analogous to the trachea of the airways that bifurcates to form main bronchi. These divide to form smaller bronchi that lead to individual lung lobes. Inside each lobe, the bronchi undergo further divisions to form new generations of smaller caliber airways: the bronchioles. This process continues through the terminal bronchioles, the respiratory bronchiole, alveolar ducts and terminates with alveolar sacs. The model proposes the existence of 24 airway generations in total, with the trachea being generation 0 and the alveolar sacs being generation 23 (Hickey and Thompson, 1992).

In passing from the trachea to the alveolar sac, two physical changes occur in the airways that are important in influencing airway function. Firstly, the airway caliber decreases with increasing generations. Secondly, the surface area of the airway increases with each generation to the extent that the total area at the level of the human alveolus is in the order of 140 m^2. Various levels of the airways may be categorized functionally as being either conducting or respiratory airways. Conducting airways do not participate in gas exchange extend from the trachea to the terminal bronchioles. The respiratory zone includes airways involved with the gas exchange and comprises respiratory bronchiole, alveolar duct, and the alveolar sacs (Hickey and Thompson, 1992).



	generation		diameter (cm)	length (cm)	number	total cross sectional area (cm^2)	Powder deposition by particle diameter
conducting zone	trachea	0	1.80	12.0	1	2.54	7-10 μm
	bronchi	1	1.22	4.8	2	2.33	2-10 μm
		2	0.83	1.9	4	2.13	
		3	0.56	0.8	8	2.00	
	bronchioles	4	0.45	1.3	16	2.48	
		5	0.35	1.1	32	3.11	
	terminal bronchioles	16	0.06	0.2	$6x10^4$	180.0	
transitional and respiratory zones	respiratory bronchioles	17 18 19	↓ 0.05	↓ 0.1	↓ $5x10^5$	↓ 10^3	0.5- 2 μm and < 0.25 μm
	alveolar ducts	20 21 22	↓	↓	↓	↓	
	alveolar sacs	23	0.04	0.05	$8x10^6$	10^4	

Figure 2.8 A schematic representation of airway branching in the human lung (Koning, 2001.).

2.4.2 Factors affecting particle deposition in the lung

Accurate assessments of site of drug deposition in respiratory tract are critical parameters in evaluating all inhalation drug delivery systems. The factor affecting these can divide into 2 groups: the properties of the aerosol themselves and the patient (Timsina *et al*., 1994).

1) The physical properties of the aerosol formulation (Gonda, 2002 and Taylor, 2002)

There are three mains mechanisms responsible for particulate deposition in the lung: gravitational sedimentation, impaction and diffusion. Keys factors that contribute to the aerodynamic properties are found in Stoke's law. These factors may be monitored or controlled to optimize drug delivery to the lungs (Crowder, 2002; Gonda, 2002 and Taylor, 2002).

2) The patient; patient-dependent factors, such as breathing pattern, and lung physiology and lung disease state making different particle aerodynamic behavior and hence affecting deposition.

Flow rate, a periods of breath-holding after inhalation, greater volume of air inhaled enhance the deposition in the peripheral lung. Deposition by gravitational sedimentation increases as the airflow velocity decreases, so that forceful inhalation followed by a period of breath-holding at total lung capacity maximizes aerosol deposition in the lungs. However, it should be noted that changes in the airways resulting from the disease state (i.e. airway obstruction) may affect the deposition profile of an inhaled aerosols (Timsina *et al.*, 1994 and Gonda, 2002).

2.4.3 Dry powder inhaler

Aerosol drug delivery has become well established in the treatment of lung disease since the administration of a drug to a site of action can result in a rapid onset of activity, which may be highly desirable (Timsina *et al.*, 1994). Smaller doses are required by this route of administration compared to oral or parenteral routes, thereby reducing the potential incidence of adverse systemic effects and reducing drug costs. Furthermore, the first-pass effect, degradation within the gastrointestinal tract, and drug interaction is avoided (Timsina *et al.*, 1994).

Dry powder inhalers (DPIs) are propellant free and do not contain any excipient other than carrier. The drug is inhaled as a cloud of fine particles (Timsina *et al.*, 1994). They are breath actuated; avoiding the problems of inhalation/actuation coordination encountered with metered dosed inhaler (MDIs), and subsequently they are particularly useful for children. DPIs can also deliver larger drug dose than MDIs.

However, the energy for drug powder dispersion and generation of the aerosols is derived from the patient's inhalation which in the case of respiratory disease, lung function may be impaired (Timsina *et al.*, 1994; Ashurst *et al*., 2000; Taylor, 2002).

2.4.4 Particle size analysis of aerosol

To deliver drug to the lower respiratory tract, the particle size is recognized to play an important role in defining the location of aerosol particles deposition (Hickey and Jones, 2000). Therefore, a reliable technique is required in order to measure the particle size of inhaled aerosols and assess the drug deposition profiles both in terms of the quantity of the drug reaching the respiratory tract and its depth of penetration. When considering lung deposition, the aerodynamic diameter (D_{ae}) is of great interest. This is defined as the diameter of a unit density sphere with the same terminal setting velocity as the particle being studied (Hickey and Jones, 2000; Mitchell and Nagel, 2004).

There are 3 mains techniques: direct imaging, optical sizing, and inertial impaction, are used to analyze the particles size of aerosol (Hickey and Jones, 2000).

a) Direct imaging based on microscopy

This method measures physical diameters and particles can be viewed. However, the subjective nature of the dimension and the largely unrealistic and unrepresentative of airway deposition are the limitation of this technique (Hickey and Jones, 2000).

b) Optical sizing

Light scattering (using laser particle sizing equipment) is a non-invasive technique which provides an estimate of volume median diameter and some index of polydispersity. The most notable of these methods have been phase doppler anemometry, time-of-flight laser vecilometry and laser diffraction. Each of this method has an advantage of describing particles in a very broad range of sizes and therefore gives more information about the distributions in the absence of a chemical detection method. This is a drawback because the mass of a drug relate to its therapeutic effect. In addition, this technique does not take into account of the anatomical structure of the human respiratory and aerodynamic behavior of the particles (Hickey and Jones, 2000).

c) Inertial sampling

This is the most widely used particle-size analysis method for inhaler output. Various instruments have been used to determine the particle size distribution of aerosols within a model respiratory tract designed to reproduce the anatomical dimension of an average healthy human airway. The instrument vary among inertial samplers, air inlet dimensions, the sampling airflow rate (12.5 to 120 L/min), the number of collecting stages (i.e. particle size ranges for collection within the distribution which varies from 2 to 8 stages), and the nature of the collection surface (liquid for impinger and uncoated or coated solid surfaces for impactor). The drug is collected and washed from these stages and analyzed by chromatographic and spectrophotometric means to determine its mass (Hickey and Jones, 2000).

Two stages device were developed as a quality control tool to indicate the proportion of the fine particles present in the distribution. The most frequency used

in vitro method is multiple-stage impactor because more information is recovered about the range of particle sizes within the distribution. The cascade impactor utilized the relationship between velocity and mass where larger particles with sufficient inertia are impacted on the upper stages whereas finer particles penetrate to the lower stages of the separator. Cascade impactors provide a useful aerodynamic measure of particle size distribution which can be used to compare devices and formulations. However, the disadvantages of this method are the impaction discriminated sizes in a narrow range, typically at values of D_{ae} from 0.2 to 10 μm; and requires considerable labor to perform time-consuming analyses, which are subject to operator variability and error (Hickey and Jones, 2000).

There are no pharmacopoeial monographs to fit the specific quality assurance requirements of dry powder inhalers. However, *in vitro* tests for DPIs should include uniformity of dose and respirable fraction in the aerosol cloud (Hickey and Jones, 2000).

2.5 Targeted delivery of antibiotic to infected macrophage

2.5.1 Alternative treatment of tuberculosis

Conventional tuberculosis treatment regimen deals with prolonged combined antituberculosis agents. These cause patient non-compliance and resulting in tuberculosis drug resistant or multidrug resistant (Mitchison, 1997; Khuller, 2004). By an oral route, most of the antituberculosis drugs are unable to penetrate into macrophages where the bacilli reside (Vyas *et al.*, 2004). Despite the discovery of new

antibiotics, the treatment of intracellular infections often fails completely eradicate the pathogens (Pinto-Alphandary *et al.*, 2000). Sufficient antibiotic concentration in the intracellular location of pathogen has to be achieved (Bakker-Woudenberg, 1995). To enhance the intracellular concentration of antituberculosis, directly targeting antituberculosis to macrophage is the promising way as an alternative treatment of tuberculosis. Thus, modification of the currently available antituberculosis drugs in such a way of slow and sustained released manner would be possible to reduce the dosing frequency thereby improving compliance. Many articles of those modified dosage were reviewed and important observations are summarized in Table 2.3.

In summary from the review literatures, microparticles containing antituberculosis drug is benefit strategy in order to manage tuberculosis infection since they can reduce drug dose, drug frequency, improve bioavailability and pharmacokinetics. The drug sustained release in the organ was obtained for several days and also reduce their toxicity. Liposomes have been used by several workers as will be detailed in the following section.

Table 2.3 Summary of alternative treatment of tuberculosis in experimental animal models

Drug(s)	Method	Route of administration and experimental model	Observations	References
RIF	PLG microspheres	Murine J 774.2 and human MM6 cell line	Greater effective to kill *M. tuberculosis* intracellular growth compared to free drug	Barrow *et al.,* 1998.
RIF	PLG microparticles	Subcutaneous injection to mouse	One or two dose of microparticles able to produce similar results from daily orally free drug	Quenelle *et al.,* 1999
INH+RIF	PLG microparticles	Subcutaneous injection to mice	Sustained release for several days compared to 24 h of free drug and higher efficacy of bacterial clearance.	Dutt and Khuller, 2001a; Dutt and Khuller, 2001b
RIF microsphere + oral INH	PLG microparticles	Subcutaneous injection to mouse + Oral INH	Complete elimination of CFU was obtained from the combination therapy	Quenelle *et al.,* 2001
RIF	PLGA microspheres	Insufflation to a guinea pig	Significantly reduced CFU inflammatory and lung damage compared to free drug	Saurez *et al.*, 2001

Table 2.3 Summaries of alternative treatments of tuberculosis in experimental animal models (continued)

Drug(s)	Method	Route of administration and experimental model	Observations	References
INH+RIF+PZA	PLG microsphere	A sustained oral administered to murine	Greater bioavailability and better bacterial clearance compared to free drug	Ain *et al.*, 2003a
INH+RIF+PZA	Alginate-based microparticle	Orally administered to guinea pig	Greater bioavailability and better bacterial clearance compared to free drug	Ain *et al*., 2003b
INH+RIF+PZA	PLG nanoparticles	Orally administered to mice	Bacterial clearance by 5 oral doses of every 10 days dosing	Pandey, *et al*., 2003
INH+RIF+PZA	Alginate-chitosam microsphere	A half dose was orally administered to guinea pig	There is a therapeutic benefit with a half dose of weekly administered	Pandey and Khuller, 2004
INH+RIF+PZA	PLG nanoparticles	A subtherapeutic dose was orally administered to guinea pig	5 doses of subtherapeutic dose gave the comparable results with 46 conventional doses.	Sharma *et al*., 2004
INH+RIF+PZA	Alginate nanoparticles	Nebulised to guinea pigs	Greater bioavailability and better bacterial clearance compared to free drug	Zahoor, *et al*., 2005

Table 2.3 Summaries of alternative treatments of tuberculosis in experimental animal models (continued)

Drug(s)	Method	Route of administration and experimental model	Observations	References
INHMS (ionizable prodrug of INH)	PLA microparticle	Intratracheal to Sprague-dawley rat and testing in NR 8383 cell line	Provide sustained release both *in vitro* and *in vivo*. Reduction of potential toxic metabolite of INH therefore the meaning of bypassing the first pass metabolism	Zhou *et al*., 2005
INH+RIF+PZA+ETB	Alginate nanoparticles	Oral administration to mice	Alginate nanoparticles hold great potential in reducing dosing frequency of antitubercular drug	Zahoor, *et al*., 2006
INH + RIF	L-PLA microparticle	dry powder inhalation to mice	20 times higher drug concentration in macrophage compared to free drug	Muttil *et al.*, 2007

Note; PLG: Poly (lactide-co-glycolide), PLA: Polylactic acid, CFU: colony forming unit, INH: Isoniazid, RIF: Rifampicin, PZA: Pyrazinamide

2.5.2 Targeted delivery of antibiotic using liposome to infected macrophage

The modification of the conventional dosage form to obtain controlled release and targeting to specific sites is an alternative strategy to the search for new antibiotics with inherent intracellular efficiency (Couvreur *et al.*, 1991). By entrapment of the antibiotic within liposomes, this may improve of the activity of antibiotics against microorganisms located inside cells (Bakker-Woudenberg, 1995).

Due to the capability of macrophages to clear non-self (e.g. microorganisms) and altered-self particles represent an important mechanism of homeostasis. This capability of macrophages can be advantages when particulate-drug carriers such as liposomes are to be targeted to macrophages (Rooijen and Sander, 1998). Such liposome particles are normally ingested rapidly and in large quantities by macrophages (Alving, 1988; Couvreur, *et al.*, 1991; Bakker-Woudenberg, 1995; Rooijen and Sander, 1998). This is expected to improve delivery to infected cells with hinding and protecting the molecule from degradation and reach to inaccessible target cells in a controlled property (Pinto-Alphandary *et al.*, 2000).

The interaction of liposomal drug carrier with macrophages and the potential fates of liposomal drugs are summarized in Figure 2.9. Theoretical strategies are as follows (Alving, 1988).

a) The drug might be released from the liposomes outside macrophages and enter the macrophage from the extracellular environment.

b) The liposome can enter a phagocytic vesicle and the vesicle will then fuse with lysosomes, degradation of liposomes by lysosomal enzymes can release drug into macrophage (into the cytoplasm or into the phago-lysosome itself).

c) The drug may be able to escape from the macrophage, either by diffusion or exocytosis.

d) The macrophage may migrate to a different site during the above processes and thereby enhance the drug distribution in the body.

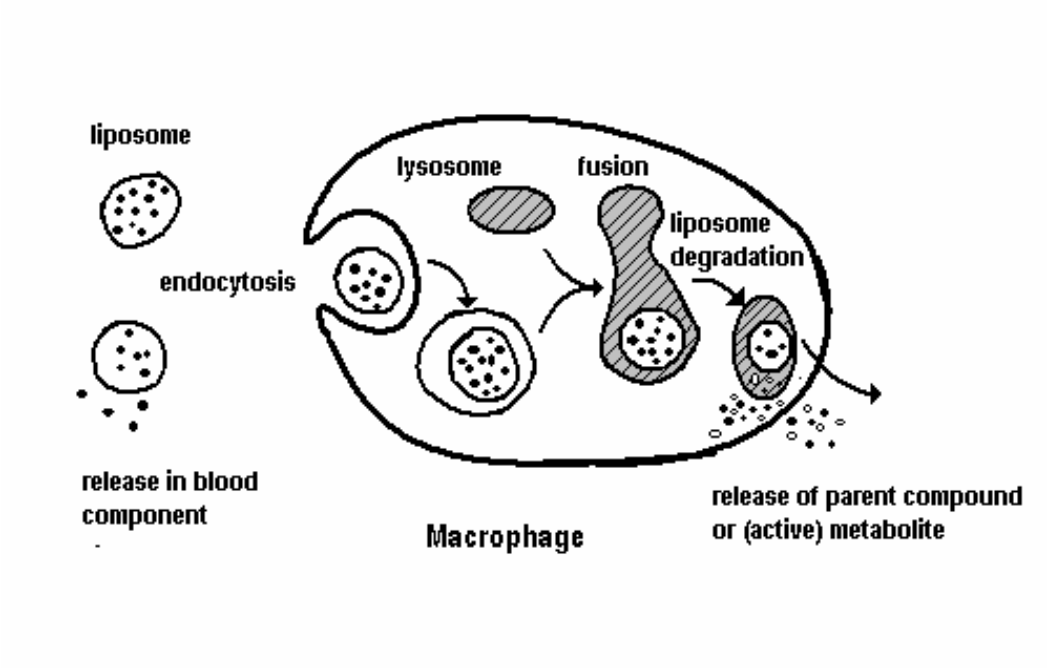


Figure 2.9 Proposed locations and mechanisms by which liposome-encapsulated drugs can exert antimicrobial effects (Alving, 1988).

Thus, targeting macrophages can result in reduction of drug toxicity to other parts of the body and also in a slow intracellular release manner while high localized concentrations of antimicrobial drugs is obtained (Alving, 1988).

There are several research articles involving with liposome containing antituberculosis. Most of them are from Khuller group (Deol and Khuller, 1997; Deol *et al.* 1997; Dutt and Khuller 2001c; Labana *et al.*, 2002, Pandey *et al.*, 2004). From our reviewed area, Deol and Khuller (1997) prepared the sterically stable liposomal

formulation with high affinity to lungs and target antitubercular drugs for achieving better tuberculosis chemotherapy. The inclusion of polyethyleneglycol and specific glycolipid into liposome resulted in prolonged drugs in the circulation. Furthermore, they modified the surface of stealth liposome by tagging the *O*-stearylamylopectin (*O*-SAP) in order to increase the affinity of this liposome towards the lung tissue. They found that after intravenously injection of lung specific stealth liposome more accumulations of drug in the lungs than in reticuloendothelial systems (RES) of normal and tuberculous mice. In addition, pre-administration of plain liposome (containing only PC and CH) prior to injection lung specific stealth liposome injection further enhanced their uptake in the lungs. The liposome demonstrated less toxicity both *in vitro* against peritoneal macrophage and *in vivo* comparison to free drugs. Also, Deol *et al.* (1997) evaluated the efficacy of the lung specific stealth liposome at subtherapeutic concentrations given by intravenous injection twice a week for 6 weeks. The results demonstrated that more effective treatment against tuberculosis than free drug. Dutt and Khuller (2001) compared the efficacy of lung specific stealth liposome and PLG microsphere in terms of the *in vitro* and *in vivo* release profile. They found that microparticles exhibited a more sustained release of rifampicin than a liposomal carrier system.

Further investigations were done by Labana *et al*. (2002) with isoniazid preparation. The co-administration of isoniazid and rifampicin encapsulated in lung specific stealth liposome intravenously to experimental mice at the one third of the recommended dose, the pharmacokinetic profiles were more than that observed with free drugs. Chemotherapeutic efficacy of once-weekly administered liposomal drugs for 6 week still significantly reduced the mycobacterial load in the lungs, liver and

spleen of infected mice compared with untreated animal. Pandey *et al*. (2004) compared efficacy of the orally, intravenously free drug (isoniazid and rifampicin) with intravenously liposomal (containing isoniazid and rifampicin) at therapeutic dose given to the guinea pigs. Liposome drug also exhibited a marked enhancement in the relative/absolute bioavailability. Equivalent results of CFU reduction was obtained from 46 daily free drug administered doses and 7 liposomal drugs dose given once weekly.

Vyas *et al*. (2004) prepared, characterized and evaluated the rifampicin-loaded aerosolized liposome. Maleylated bovine serum albumin (MBSA) and *O*-SAP liposomes were used in this study in order to target AM and prepared as the pressurized packed system metered dose inhaler. The MBSA and *O*-SAP coated formulation exhibit greater reduction of *M. smegmatis* inside macrophage (*in vivo*) than the plain drug did. Furthermore, this study suggest that the ligand-anchored liposomal aerosols are not only effective in rapid attainment of high drug concentration in lung but also maintain over prolonged period of time.

Recently, Zaru *et al*. (2007) developed the drug loaded freeze-dried liposome which designed to deliver rehydrated liposome containing antituberculosis drug to the lungs by nebulization. They found that distearoylglycerol-phosphatidylcholine to cholesterol at a ratio 2:1 was the best composition for rifampicin delivery in vesicular forms to lung.

CHAPTER 3

RIFAMPICIN-ENCAPSULATED LIPOSOMES DRY POWDER AEROSOL

3.1 Introduction

Rifampicin (RIF, Figure 3.1) is one of the first line anti-tuberculosis drugs. It is active *in vitro* and *in vivo* against *Mycobacterium tuberculosis*, *M. bovis* and *M. avium intracellulare* complex (AHFS, 1999). However, the necessity of using a high oral RIF dose (10 mg/kg daily) is still a problem today, since RIF causes serious side effects including hepatotoxicity. Additionally, rifampicin is also a strong inducer of various drug metabolizing enzymes in cytochrome P-450 system. Therefore, co-administration of RIF with other drugs may result in decreased plasma concentration of those drugs.

However, RIF, an amphiphilic drug, is chemically stable in the solid state, but is very unstable in aqueous solution (Gallo and Raddelli, 1976). Incorporation of RIF into liposome vesicles may improve the stability of RIF during storage but, to our knowledge, this has not been proven. Also, liposome suspensions suffer from certain limitations of both chemical and physical stability during storage, such as fusion, leakage and aggregation (Hu and Rhodes, 1999). In addition, an amphiphilic drug like RIF will presumably also interact with the lipid membrane and

may alter membrane properties and produce changes in the structural organization of the lipid bilayer (Bermudez *et al.*, 1999; Rodrigue *et al.*, 2003). Therefore, it is critical to determine a bilayer lipid composition so as to obtain stable drug liposome formulations (Rodrigue *et al.*, 2003). Also, there is evidence that increasing cholesterol (CH) content tends to increase the rigidity of the liposome bilayer (Verumi and Rhodes, 1995). A number of studies have reported the interaction of RIF with membrane bilayers (Bermudez *et al.*, 1999; Rodrigue *et al.*, 2003), but the influence of CH and RIF on liposome rigidity and stability has not to date been studied.

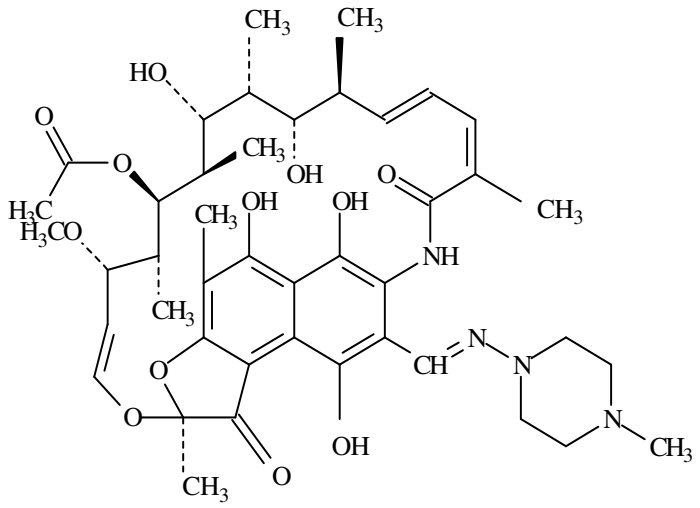


Figure 3.1 Rifampicin structure (Gallo and Raddelli, 1976)

In this chapter, the effect of liposome composition on the properties of RIF containing liposomes in suspension was determined. Using the most favorable (i.e. stable) formulation, further studies were carried out to determine the molecular orientation of cholesterol and RIF in the bilayer, and to detect changes induced by RIF in the structural organization of the lipid bilayer. These data have been used to

describe possible mechanisms responsible for the observed physical stability, since an understanding of drug-liposome interaction at the molecular level can be useful for monitoring changes in liposomes and their stability during storage. Furthermore, from the most stable suspension to dry powder, freeze-drying protocols were also evaluated to obtain a liposome powder with desired aerodynamic properties for the development of aerosolized formulations.

Targeting the liposome to AM, important point need to be concern is the immunological activation of AM by the delivering products. This is due to AM is the principal effectors cell of an innate immune system. IL-1β and TNF-α will be secreted by activated AMs in the process of innate immunity called "inflammation" in order to response to those foreign particles (Ng *et al*., 1998). Thus, the safety of the particles reaching to the airways should be determined to ensure that product does not induce the release of any toxic agent in the airways. Furthermore, normal human bronchial epithelial cell and small airway epithelial cell were also included to evaluate the cytotoxicity of the formulations to these cell lines. In addition, the antituberculosis efficacy of liposome containing rifampicin against intracellular mycobacterium growth was also determined compared with free RIF.

3.2 Materials and methods

3.2.1 Preparation and characterization of liposome containing RIF suspension

3.2.1.1 Preparation of RIF encapsulated liposome suspensions

Liposome suspensions were prepared by the chloroform-film method. Briefly, all ingredients (as shown in Table 3.1) including 2.5 mg RIF (Siam Pharmaceuticals, Bangkok, Thailand) were dissolved in a solvent mixture (10 mL) of chloroform and methanol (2:1 v/v). Six suspension formulations (numbered 1-6, Table 3.1) were prepared containing different millimole ratios of soybean phosphatidylcholine (SPC, Fluka, Buchs, Switzerland) and cholesterol from lanolin (CH, Fluka, Buchs, Switzerland). Dicetylphosphate (DCP, Fluka, Buchs, Switzerland) or stearylamine (SA, Sigma chemical company, St Louis, MO, USA) was used in a very small amount (0.17 mM) to charge the liposome surface to be negative or positive, respectively. A rotary evaporator was used with a rotation of 60 rpm and 40°C water bath in order to remove the organic solvent mixture until a dry film was formed. The dry film was allowed to vacuum further for 15 minutes to ensure that the solvent was completely removed. The 60 mL of hydrating solution consisted of 0.2 M phosphate buffer pH 7.4: 1 mM EDTA (1:1 v/v) was added to produce liposome suspensions containing 0.05 mM of RIF. The lipid film was allowed to hydrate in the rotary evaporator without vacuum at a rotation of 60 rpm for 1 h. The size reduction of

liposome vesicle was done by ultrasonication at room temperature for 1 h. The liposome suspension was kept under refrigerator until used.

3.2.1.2 Cryo-transmission electron microscopy

Cryo-TEM (TECNAI F 30, Cryo-TEM twin 300 keV field emission gun, FEI Company, Oregon, USA) was used to evaluate liposome morphology. Sample vitrification process was carried out by the robotic "Vitrobot" unit. Liposome suspension was dropped onto a carbon film supported on an EM grid. The excess liquid was blotted with filter paper and then dipped into liquid ethane for 3s (Frederik and Hubert, 2005). The vitrification action was carried out in a chamber under 100% humidity and 20°C ± 0.5°C chamber. The vitrified grid was observed under cryo-TEM. Liposome vesicle size and morphology were compared prior to and after the freeze-drying process.

3.2.1.3 Liposome size and physical size stability

Liposome size was measured by photon correlation spectroscopy with an Autosizer Lo-C (Malvern, Worcestershire, UK) at 25°C with a fixed angle of 90 degrees, in triplicate for each sample. The liposome suspensions were diluted with filtered de-ionized water to obtain a suitable rate meter reading (100,000 counts/s). The liposome suspensions were kept at 4°C and samples analyzed at weekly intervals for size stability over 4 weeks.

3.2.1.4 Measurement zeta potential of liposome surface

The zeta potential of liposome surface was measured with Zetasizer nanoseries ZEN 3500 (Malvern Instrument Ltd, Malvern, Worcestershire, UK) at pH 7.4. The instrument measured and calculated the zeta potential by determining the electrophoretic mobility which is obtained by performing an electrophoresis experiment on the sample and measuring the velocity of the particles using laser doppler vecilometry (Malvern, 2005).

3.2.1.5 Determination of encapsulated RIF in liposome vesicles

To determine the encapsulation amount of rifampicin in liposome vesicls, unencapsulated RIF was first separated from the liposome suspension system. Unencapsulated RIF was separated from liposome-entrapped drug by dialysis through a membrane with cut-off 6-8000 kDa (Spectrapore®, Millipore Corp., MA, USA). Free RIF solution was prepared by dissolved RIF (2.5 mg) in 60 mL of hydrate solution (equivalent to 0.05 mM RIF loading to liposomes). Free RIF solution (5 mL) was loaded into a dialysis bag and dialysed against 3L of distilled water to determine the time taken for complete removal of RIF. This was found to be 12 h of dialysis time. The same volume of liposomes suspension containing RIF was dialysed under the same dialysed condition. The remaining liposomes in the dialysis bag were lysed after 12 h to release RIF by adding 0.1% Triton X-100 in methanol and the RIF content was analyzed by HPLC. The HPLC system comprised a Waters™ 600 controller and pump with a Waters™ 717 plus autosampler equipped with a Waters™ 486 variable

wavelength UV detector and data collection module (Milford, MA, USA). The mobile phase was phosphate: acetonitrile (55:45 v/v), at a flow rate of 1 mL/min, and UV detection was 254 nm. The microbondapak C18 column (Phenomenex, USA) (250×4 mm i.d., 5 μm) was used in this study. The system can resolve RIF from other degradation product (Calleri *et al., 2002*). The RIF content in a same volume of liposome suspension (without dialysis) was analyzed as a total RIF loading. The percentage of drug encapsulation was calculated by dividing the amount of RIF remaining in the liposome vesicles after 12 h dialysis by the total RIF loading.

3.2.1.6 Determination of RIF and CH locations in the liposome bilayer by solid-state NMR

Deuterated 1-palmitoyl (D31)-2-oleoyl-*sn*-glycero-3-phosphocholine (*d*-POPC, Avanti® Polar Lipids, Inc, Albaster, AL, USA) was used instead of SPC for acquisition of ^{2}H NMR spectra. Liposome suspensions were formulated in a 25 mL round bottom flask prepared as previously described for preparation of RIF liposomes. The dry film was kept overnight in a desiccator before the hydration step was completed, and the amount of aqueous solution was reduced to avoid interference from the natural abundance ^{2}H in water. The liposome suspension was sonicated in a water bath and centrifuged at 10,000 rpm for 10 min to concentrate the liposome vesicles before the NMR experiment.

Solid-state NMR (SS-NMR) experiments were conducted on a Varian Inova 300 MHz spectrometer (Palo Alto, CA, USA) operating at a resonance

frequency of 300 MHz for ^{1}H. Static spectra were acquired at 28°C, using a Varian 5 mm double resonance probe. ^{31}P Broad-line spectra were collected between 2.0 s recycle delays with a proton-decoupled Hahn echo pulse sequence using a 5.8 μs π/2 pulse and 40 μs echo delay. Typically 15000 transients were acquired with a spectral width of 62.5 kHz, and processed with 100 Hz exponential line broadening. Chemical shift was referenced using 80% phosphoric acid (H_3PO_4) at 0 ppm. The chemical shift anisotropy (CSA) was estimated from the width of the ^{31}P powder pattern as the difference in chemical shift between perpendicular (or 90° edge) and parallel (or 0° edge) components taken as half height of the discontinuities (Drechsler and Separovic, 2003). ^{2}H Broad-line spectra were collected between 0.5 s recycle delays with a solid echo pulse sequence (Davis, 1983) using a 4 μs π/2 pulse and effectual 40 μs echo delay; generally 50000 transients were acquired with a spectral width of 250 kHz, and 100 Hz line broadening. The ^{2}H or quadrupolar splitting of the acyl chain terminal methyl (CD_3) were measured as the difference in frequency of the innermost peaks; and for the uppermost chain methylenes (CD_2) from the width at half-height of the 90° edge of the powder pattern (Drechsler and Separovic, 2003).

3.2.1.7 Determination of chloroform residual phospholipid mixtures

The residual chloroform in liposome suspension and freeze-dried liposome powder was detected using headspace autosampler. HP 6890 gas chromatography (GC) was operated with electron capture detector. The condition is as following:

Inlet temperature: 250 °C

Oven temperature: 40 °C, hold for 10 minutes

Detector temperature: 250 °C

Carrier gas flow rate: He, 1.0 mL/min

Injection volume: 0.5 mL

Column: Rtx-5MS, 30 m, 250 µm I.D., 0.25 µm film thickness

Sample (1 mL in 10-mL headspaces vials) were heated to 80 °C with the transfer line and valve at 90 °C. Equilibration time was 20 minutes, mixing time 7 minutes at 80% full power with 2 minutes equilibration post mixing.

3.2.2 Preparation of liposome containing RIF dry powder

3.2.2.1 Preparation of liposome dry powders from liposome suspensions

Cryoprotectants were added to the liposome suspensions to form a glassy matrix during freezing (Winden *et al.*, 1997). Three sugars, mannitol (Fluka, Buchs, Switzerland), trehalose (Fluka, Buchs, Switzerland) and lactose (Sigma Chemicals, St. Louis, MO, USA), were used in the freeze-drying systems in this study. Liposome suspensions were mixed with mannitol, trehalose or lactose in the ratio of 1:1 by weight. The liposome suspension was then slowly frozen in the chamber

containing dry ice to obtain the temperature of ~50°C and held this temperature for 1 h then immediately immersed into liquid nitrogen for 10 min before the drying processes under vacuum was initiated. The primary drying was varied to three different temperatures (-10°C, -5°C and 0°C for condition 1, 2 and 3, respectively). Secondary drying was set at 20°C for all conditions.

3.2.2.2 X-ray diffraction of freeze-dried liposome powder

X-ray diffraction (XRD) of the obtained freeze- dried powder measurements were carried out on a Siemens D 5000 (Siemens Analytical X-ray instrument, Inc., WI, USA) using Cu radiation equipped with a diffracted-beam monochromator. The freeze-dried liposome powders were spread on a glass sample holder in an area of 4 cm^2 with a depth of 1 mm. The surface of the powder was pressed and smoothed with a glass slide. The diffraction intensity was recorded at an angle of 2θ from 3 to 60° with a step size of 0.05° and step time of 1 s. The total time for the diffraction scan was 19 min and each sample was analyzed in triplicate. The voltage and current generator were set at 40 kV and 30 mA, respectively. The obtained data were analyzed by EVA software. The relative crystallinity was obtained from the powder XRD results by the ratio of the intensity of a characteristic crystalline peak to that of the amorphous halo for each sample. If the ratio of the peak to the amorphous halo around it decreases, the crystallinity is proportionately lower (Morris and Rodriguez-Hornedo, 1993).

3.2.2.3 Differential scanning calorimetry of liposome suspensions and dry powder products

DSC (DSC 2920, TA instrument, Newcastle, UK) was used to investigate the thermal properties of the liposome suspensions and dry powders. The eutectic temperature and glass temperature (T_g) was determined for each liposome-sugars suspension. The sample (20 μL) was placed in an aluminium pan, sealed hermetically, and then assessed by DSC in the cooling mode from 25 °C to -50 °C and heating mode from -50 °C to 170 °C at a rate of 10 °C/min. In addition, the dry powders were also investigated for their thermal behaviour. Each powder sample (about 5 mg) was weighed into the aluminium pan and sealed. The DSC was run from room temperature (25 °C ± 1 °C) to 250 °C at a rate of 10 °C/min. All samples were analyzed in triplicate. The DSC thermograms were analyzed using the Universal analysis 2000 program version 3.4c.

3.2.2.4 RIF content and characterization of the reconstituted freeze-dried powder formulations

Each formulation was examined by sampling a dry powder formulation following the United States Pharmacopeia 24 guideline (USP 30 &NF 25, 2007). A total of 10 doses of 30 mg were collected: three doses at the top, four in the middle and three at the bottom of the bottle containing the powder blends. The sampling powder was dispersed in 5 mL of deionized water and then dissolved and adjusted to the volume with methanol and 10 minutes sonication was done to obtain the clear

solution. RIF content was analyzed by HPLC technique as described earlier. The content of RIF in freeze dried liposomes was analyzed immediately and after being kept either at 4°C or room temperature which the results compared with RIF in solution and RIF in liposome suspentsiom at time period of 6 weeks and 6 months

The freeze-dried liposomes containing RIF were reconstituted in phosphate buffer solution pH 7.4. The resulting reconstituted liposomes suspensions (RLSs) were characterized, including cryo-TEM, encapsulation efficacy, zeta potential and residual chloroform as described earlier in section 3.2.1.2, 3.2.1.4, 3.2.1.5 and 3.2.1.7 respectively.

3.2.2.5 *In vitro* deposition of liposome encapsulated RIF dry powder in an Andersen Cascade Impactor

The aerosolized parameter of the products including mass median aerodynamic diameter (MMAD), % fine particle fraction (FPF) and % emitted dose (%ED) were evaluated by Andersen cascade impactor (ACI, Atlanta, GA, USA). ACI is an apparatus using to determine the particle size distribution of aerosols within a model respiratory tract designed to reproduce the anatomical dimension of an average healthy human airway (Timsina *et al.*, 1994; Hickey and Jones, 2000). The eight stages of ACI separates the samples aerosol into nine size intervals when used with a backup filter after the last impaction stage (Stein and Olson, 1997). Each stage of an ACI has multi-orifices that display progressively smaller from top to bottom (Figure 3.2). The particles that deposit on stages 6-7 correspond to alveoli *in vivo*. The ACI

was applied with a vacuum pump under flow rate of 60 L/min for 10 s. 0.1% Triton X-100 in methanol was used to rinse particles deposited on each stage, and RIF content was determined by HPLC. The cumulative percentage of deposition was transformed to Z-value and plotted against log cut off diameter of each stage. MMAD is obtained from the particle diameter at Z-value of zero (Atkins *et al*., 1992) which means the aerodynamic particle size at which half of the total aerosol mass is contained larger particles size and half in smaller particles. Emitted dose is amount of drug was propelled from delivery device where as FPF means the fraction of particles smaller than 5 µm (Srichana *et al*., 2003) which is the percentage of drug deposited on stage 1-7.

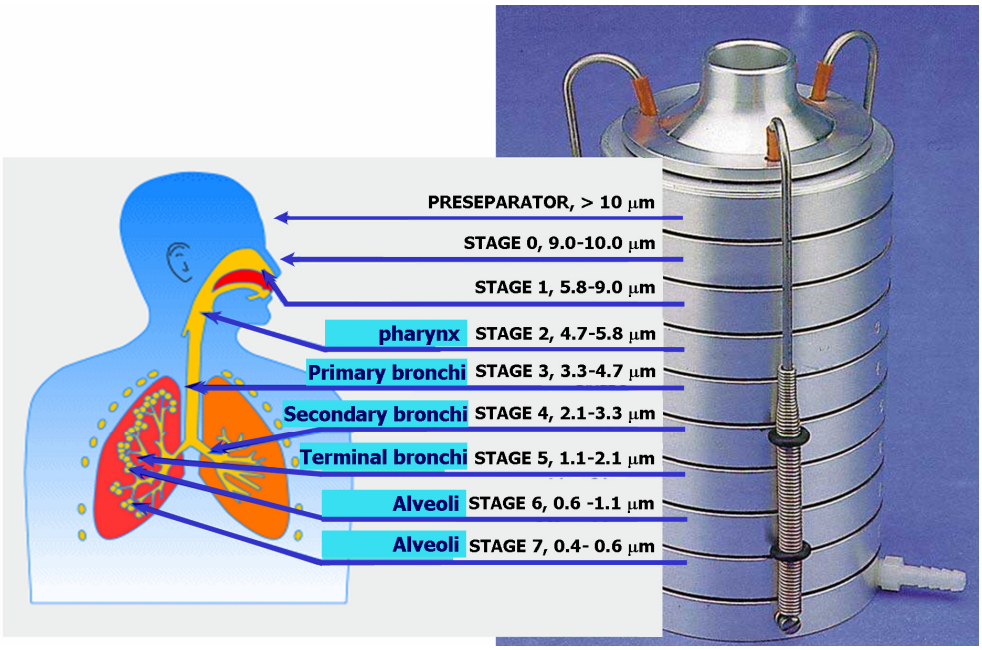


Figure 3.2 Andersen cascade impactor and respiratory airways

3.2.3 Cell culture

3.2.3.1 Normal human bronchial epithelial cell (NHBE) and small airway epithelial cell (SAEC)

Normal human bronchial epithelial cells (NHBE) and small airway epithelial cells (SAEC) were cultured in the clonetics media and its supplement (BEGM bullet kit® and SAGM bullet kit®) provided by Lonza Group Ltd. (Walkerville, MD, USA). The cell was cultured followed the Lonza recommended protocol (see appendix). Briefly, the cell was seeded density for NHBE is 3,500 cells/cm^2 and SAEC is 2,500 cells/cm^2 and incubated under 37°C, 5% CO_2 and 95% humidity incubator. To subculture, when the cells are 60%-80% confluent, rinse the cell with HEPES buffered saline solution (HEPES-BSS) to wash remaining complex protein which may neutralize trypsin activity. Aspirate HEPES-BSS and covered the cells with 2 mL of trypsin/EDTA solution. After cell released, the trypsin/EDTA activity was neutralized with trypsin neutralizing solution, centrifuge, resuspended and then transferred to new culture flask. The cell were used in early passages number since clonetics NHBE and SAEC cryopreserved cultures are assured for experimental use for fifteen population doubling. All solutions (reagent pack) used for maintenance and culture of NHBE and SAEC were from Lonza Group Ltd (Walkerville, MD, USA).

3.2.3.2 Alveolar macrophage cell line

A rat alveolar macrophage cell line NR 8383 (ATCC CRL-2192, Rockville, MD, USA) was established from normal rat lung lavage. The cell exhibit characteristics of macrophage cell: phagocytosis of zymosan, and *Pseudomonas aeruginosa*, non specific esterase activity, Fc receptors, oxidative burst, IL-1, TNF-β and IL-6 secretion, and replicative response to exogenous growth factors. The cells respond to appropriate microbial, particulate or soluble stimuli with phagocytosis and killing. The NR 8383 cell line provides a homogenous source of highly responsive alveolar macrophages which can be used *in vitro* to study macrophage related activities (http://www.atcc.org). The cell was cultured in F12 Kaighn's cell culture medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate (Gibco, Grand Island, NY, USA) supplemented with 15% (v/v) heat inactivated fetal bovine serum, 50 units/mL penicillin, 50 μg/mL of streptomycin (Gibco, Grand Island, NY, USA) and incubated under 37°C, 5% CO_2 and 95% humidity incubator. Cultures can be maintained by transferring floating cells to additional flasks. Adherent cells may be harvested by scraping. Upon reseeding, about one half of the cells will re-attach. The fresh medium was replaced two or three times weekly.

3.2.4 Determination of cytotoxicity of RIF encapsulated liposome

Viabilities of NHBE, SAEC and AMs and were determined by using MTT assay to detect functioning mitochondria. Live mitochondria transform MTT (3-(4,5- dimethylthiazole-2-yl)-2 diphenyltetrazolium bromide, Sigma Chemicals, St. Louis, MO, USA) to formazan, which can be measured with a spectrophotometer.

Briefly, 100 μL of 1 x 10^5 cells/mL was cultured in each well of 96-well plate and allow to adhere and growth overnight under 37°C, 5% CO_2 and 95% humidity incubator. The day after, the fresh media (100 μL) was replaced and 100 μL of cell culture media containing either liposome suspension (LS), reconstituted freeze dried liposome suspension (RLS) or free RIF solution was added. The plate was then incubated for 24 h. The supernatant from each well of AMs culture was removed to examine the level of generated inflammatory cytokine by ELISA method or nitric oxide by Griess reagent as described below. The remaining cell number was quantitative measured and compared with untreated control by MTT assay. Filtered sterilized stock MTT solution (50 μL of 5 mg/mL in Dulbecco's phosphate buffer saline, DPBS, Gibco, Grand Island, NY, USA) was added into each well containing 150 μL fresh media and were incubated for 4 h at 37 °C. After that, the supernatant was carefully removed and the resulting formazan crystal was dissolved by adding 200 μL of dimethylsulfoxide (DMSO, Riedel-de Haën, Seelze, Germany) and mixed thoroughly. The absorbance was recorded at 570 nm with the microplate reader (Biohit BP 800, Helsinki, Finland). The proportion of viable cells in treated well was compared to the untreated well (Huttunen *et al.*, 2000; Punturee *et al.*, 2004).

3.2.5 Determination of alveolar macrophage response to liposome formulations

3.2.5.1 Production of inflammatory cytokines

Measurement of inflammatory cytokines level of TNF-α and IL-1β generated from AMs responding to liposome, free RIF or lipopolysaccharide (LPS) from *E.coli* (positive control). This was conducted by enzyme linked immunosorbent assay (ELISA) method. Commercial ELISA kits (Quantikine® RTA00 and Quantikine® RLB00 for rat TNF-α and IL-1β, respectively, R&D systems Inc., MN, USA) were used as described in product assay procedures (see appendix). The detectable dose of both TNF-α and IL-β is less than 5 pg/mL. The assay employs the quantitative sandwich enzyme immunoassay technique (Figure 3.3). A monoclonal antibody specific for rat TNF-α or IL-β has been pre-coated on a microplate (Step 1). Standards, controls and samples are pipetted into the wells and any rat TNF-α or IL-β present is bound by the immobilized antibody (Step 2). After washing away any unbound substances (Step 3), an enzyme-linked polyclonal antibody specific for rat TNF-α or IL-β is added in to the wells (Step 4). Following a wash to remove any unbound antibody-enzyme reagent (Step 5), a substrate solution is added to the wells (Step 6). The enzyme reaction yields a blue product that turns yellow when the stop solution is added (Step 6). The intensity of the color measured is in proportion to the amount of rat TNF-α or IL-β bound in the initial step. The sample values are then read off the standard curve.

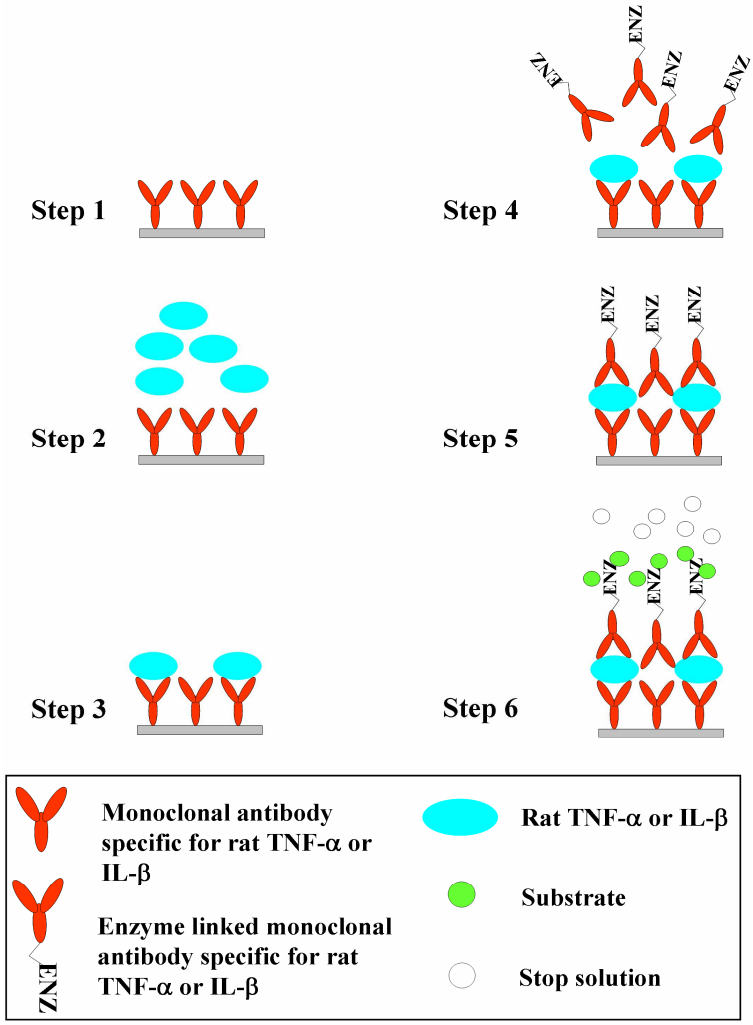


Figure 3.3 Sandwich enzyme linked immunosorbent assay (Crowther, 1995).

3.2.5.2 Nitric oxide

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 product of NO. This measurement relies on a diazotization reaction of Griess reagent as showed in Figure 3.4. Griess reagent was prepared by mixing of 1% sulfanilamide (Sigma Chemicals, St. Louis, MO, USA), 0.1% N- (1-naphthyl)- ethylenediamine

dihydrochloride (NED, Sigma Chemicals, St. Louis, MO, USA) and 2.5% phosphoric acid in water. Equal volume of cell supernatant (100 μL) and Griess reagent (100 μL) were mixed. The absorbance was determined using microplate reader (Biohit BP800, Helsinki, Finland) 10 minutes after mixing at 450 nm. The nitrite concentration was calculated from sodium nitrite standard curve (Huttunen *et al.*, 2000; Punturee *et al.*, 2004).

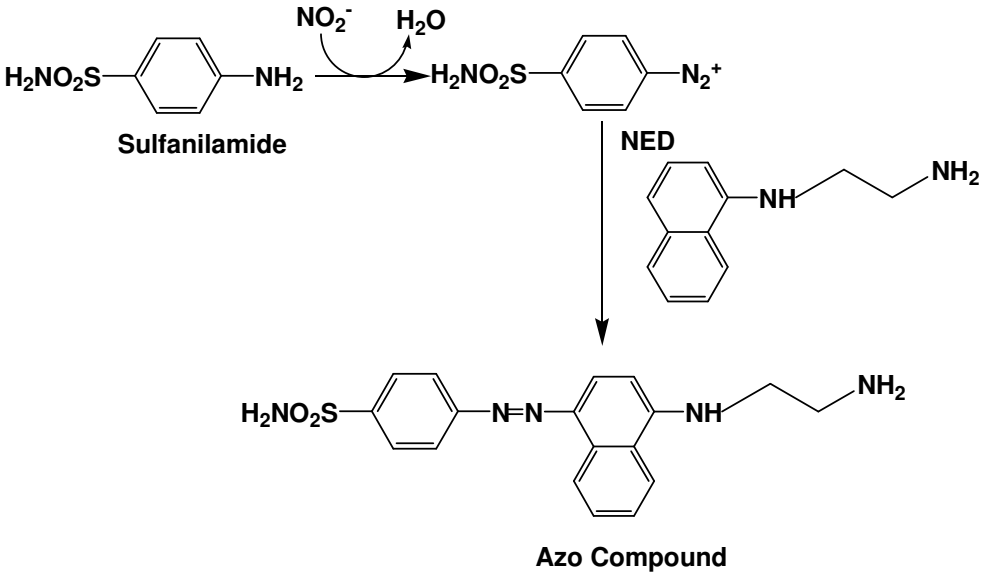


Figure 3.4 Griess reaction (Promega, 1995)

3.2.6 Assessments of antimycobacterial activity

3.2.6.1 Culture of *Mycobacterium bovis* from BCG vaccine

The lyophilized BCG vaccine of *Mycobacterium bovis* (Pasteur aventis, Paris, France) was reconstituted with 1 mL sterile deionized water for injection provided with the lyophilized vaccine. 200 μL of reconstituted BCG vaccine was

grown in Middlebrook 7H9 (Becton Dickinson and company, MD, USA) containing 0.5% Tween 80 and 10% oleic albumin dextrose-catalase (OADC) enrichment (Becton Dickinson and company, MD, USA) (Ritelli *et al*., 2003). The bacilli was incubated in the 37 °C incubator and subcultured every 3 weeks. The obtained *M. bovis* suspension at 3 weeks old was used in the further experiment.

3.2.6.2 Determination of minimum inhibitory concentration against *M. bovis*

The minimum inhibitory concentration (MIC) of liposome containing RIF and free RIF solution were determined. The inoculum of *M. bovis* was prepared from a suspension of the 3 week olds organism in M7H9 broth (Becton Dickinson and company, MD, USA) supplemented with Middlebrook OADC enrichment (Becton Dickinson and company, MD, USA) and the bacilli suspension was adjusted with normal saline solution to obtain turbidity of McFarland standard solution No. 1. Ten-fold serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of the above inoculum suspension was made in normal saline solution. Liposome containing RIF and free RIF solution was two-fold serially diluted to obtain concentration equivalent to RIF content of 0.04 – 1.25 μg/mL. Each concentration (100 μL) was added into the well containing of 10^{-4} diluted bacilli suspension (100 μL) and incubated at 37 °C. At day 1, 3 and 7, triplicate of 10 μL sample was taken from each well to drop on M7H9 agar plate (M7H9 broth with 1.5% w/v agar powder) and incubate at 37 °C for 3 weeks. Each experiment was done for triplicate. After 3 weeks, the colony was counted under the colony counting machine (Suntex colony counter, Suntex instrument company Ltd., Taipei, Taiwan).

3.2.6.3 Determination of minimum inhibitory concentration against *M. tuberculosis*

M. tuberculosis $H_{37}Ra$ ATCC 25177 were obtained from American Type Culture collection (Rockville, MD, USA). *M. tuberculosis* $H_{37}Ra$ was grown in 100 mL M7H9 broth supplemented with 10% OADC enrichment and 0.05% Tween 80 (Sigma Chemicals, St. Louis, MO, USA). Antimicrobial susceptibility testing was performed in black, clear-bottomed, 96-well microplates in order to minimize background fluorescence. Outer, perimeter wells were filled with sterile water to prevent dehydration in experimental wells. Initial drug dilutions were prepared in distilled deionized water and subsequent two fold dilutions were performed in 0.1 mL of M7H9 (without tween 80) in the microplates. The final bacterial density of 5×10^4 CFU/mL in the well was used. Wells containing drug only were used to detect autofluorescense of compounds. Plates were incubated at 37 °C. Starting at day 4 of incubation, 20 µL of 10X Alamar blue solution (Alamar Biosciences/ Accumed, Westlake, Ohio, USA) and 12.5 µL of 20% Tween 80 were added to one bacteria only and medium only control well, and plates were incubate at 37 °C. Well were observed at 12 and 24 h for a color change from blue to pink. If the bacteria only control well became pink by 24 h, reagent was added to the entire plate. If the well remained blue, additional bacteria only and medium only control well were tested daily until a color change occurred, at which time reagents were added to all remaining wells. Plates were then incubated at 37 °C, and results were recorded at 24 h post-reagent addition. Visual MICs were defined as the lowest concentration of drug that prevented a color change (Collins and Franzblau, 1997)

3.2.6.4 Determination of intracellularly antimycobacterial against *M. bovis*

The antimycobacterial effectiveness of LS, RLS and free RIF solution against intracellular growth of *M. bovis* in AMs was examined (Barrow *et al.*, 1998). Prior to infection, AM cells were plated at a concentration of 10^5 cells/well in 12 well tissue culture plate and incubated overnight in the CO_2 incubator to allow the cell adhere on the well surface. Fresh medium containing 1% fetal bovine serum was replaced in order to reduce cell proliferation and penicillin-streptomycin mixture was excluded to avoid the interferences of the antibiotics on the day after. *M. bovis* was suspended in F 12 Kaighn's medium containing 1% FBS and the suspension was dispersed into individual wells at a density of five mycobacterium per macrophage. Infected AM were then incubated at 37 °C, 5% CO_2 incubator for 4 h. Following incubation, the supernatant was aspirated and the wells were washed by 3 x 1000 μL with Dulbecco's phosphate buffer saline (DPBS) to remove unphagocytosed mycobacteria. Fresh media with and without sample was added into each well. The final concentrations in the well were 0.63, 1.25 and 2.5 μg/mL equivalent to RIF concentration. Fresh media (1 mL) was added at day 4 into the well. The well plate was incubated for 3 and 7 days. After a specified time, the media was discarded and the well was carefully washed 3 times with DPBS to remove the excess sample. Determination of CFU was conducted by lysing AM with 0.125% sodium dodecyl sulfate (SDS) in DPBS (400 μL) (w/v) and incubated at 37 °C for 15 min. After that, M7H9 broth (600 μL) was added into each well to make the total volume of 1 mL. Solution from each well (100 μL) was taken to dilute serially with M7H9 broth. Sample (10 μL) from each dilution was taken to drop on the surface of M7H9 agar

plate and incubated at 37 °C for 3 weeks. Each sample was done in triplicate. After 3 weeks, the numbers of CFU were enumerated.

3.2.7 Statistical analysis

Data, when applicable, are presented as mean ± standard deviation (SD) from at least three samples unless indicated. The size, percent encapsulation and all aerosol parameters of each liposome formulation were compared using analysis of variance (ANOVA). All statistical comparisons were calculated using the SPSS software version 11.5 (SPSS, Inc., Chicago, IL). A significance level of p-value < 0.05 was considered statistically significant.

3.3 Results and Discussions

3.3.1 Physico-chemical characteristics of liposome suspensions containing RIF

The liposome particles were in a size range 200-300 nm with an average size of 240 nm (Table 3.1). The morphology of liposome vesicles are shown in the cryo-TEM images (Figure 3.5). The suspensions comprise a mixture of unilamellar and (predominantly) multilamellar vesicles. RIF is a lipid soluble compound and may be lost from the vesicle bilayer by collision and fusion with liposome surfaces, especially in unilamellar vesicles, which have a greater surface area. In general, multilamellar vesicles (MLV) give a greater sustained release

performance than unilamellar vesicles since their concentric membranes are slowly degraded in sequence (New, 1990a). Hence, MLV may be more suitable for RIF encapsulation. Furthermore, RIF appears to be incorporated into the bilayer membranes, based on SS-NMR results described later, the maximum amount taken up into the liposome is directly proportional to the quantity of membrane components as shown in Table 3.1. The greatest percentage encapsulation of 51 was being with the highest amount of 20 mM of total lipid (Formulation No. 4-6). Therefore, liposomes having 20 mM total lipid content were chosen for further study.

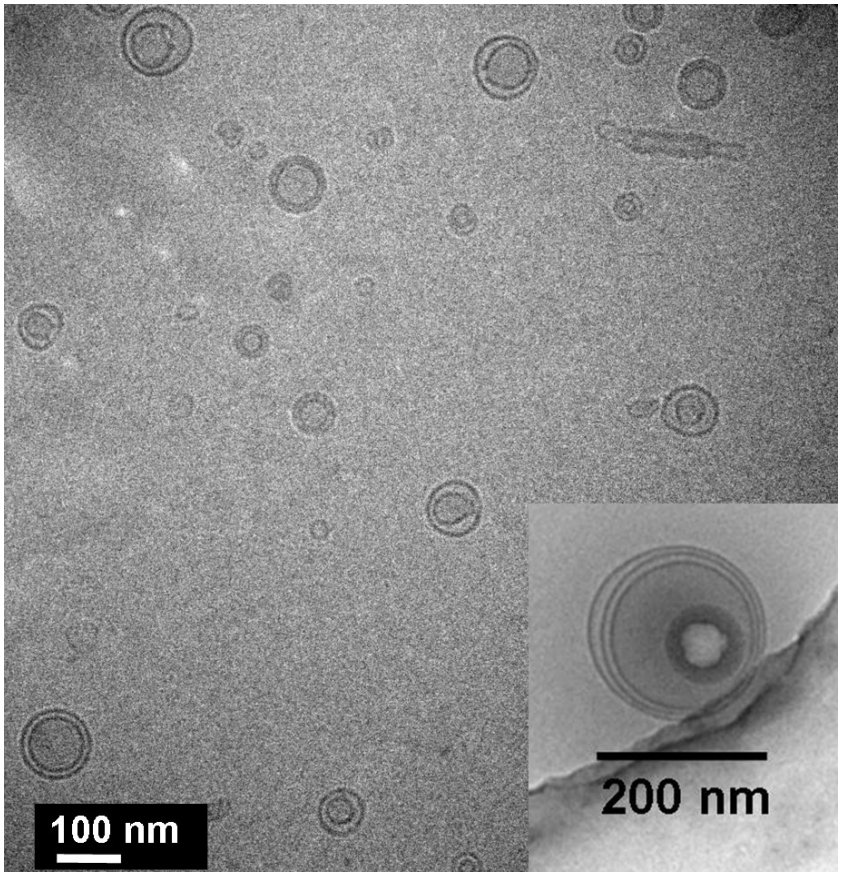


Figure 3.5 Cryo-transmission electron microscope (Cryo-TEM) image of a liposome suspension.

Table 3.1 Characteristics of the liposome containing RIF formulation

(mean ± SD, n ≥3)

Formulation No. (Legend)	Compositions (mM)				Size (nm)	% Encap-sulation	Zeta potential (mV)
	SPC	CH	SA	DCP			
Liposome suspension							
1	3.3	3.3	-	-	247.1 ± 14.1	33.2 ± 4.8	ND
2	3.3	1.7	-	-	221.9 ± 6.6	33.9 ± 5.1	ND
3	6.7	6.7	-	-	235.8 ± 23.0	45.8 ± 2.6	ND
4	10	10	-	-	245.4± 34.6	51.5 ± 2.8	-18.12 ± 0.64
5	10	10	-	0.17	261.3 ± 17. 5	50.4 ± 3.7	-20.56 ± 0.76
6	10	10	0.17	-	236.3 ± 11.7	50.4 ± 3.0	-15.11 ± 0.97
Reconstituted liposome suspension							
Negative charge	10	10	-	0.17	711.8 ± 88.3	23.91 ± 2.8	-26.87 ± 1.41
Uncharge	10	10	-	-	593.5 ± 99.0	23.42 ± 4.	-24.17 ± 1.02
Positive charge	10	10	0.17	-	648.9 ± 147.6	22.68 ± 2.7	-17.19 ± 1.52

SPC= Soybean phosphatidylcholine CH= Cholesterol DCP= Dicetylphosphate (negative charge substance)

SA= Stearylamine (positive charge substance) ND: Not determined

The surface zeta potential charge of all liposome vesicle displayed negative values (Table 3.1). This is due to the overall negative charge of SPC which is the phospholipid mixture of phosphatidylcholine (neutral charge, 13-18%), phosphatidylethanolamine (neutral charge, 10-15%), phosphatidylinositol (negative charge, 10-15%) and phosphatidic acid (negative charge, 5-12%) (American lecithin, 2002). This was confirmed by the negative charge value of -24.5 mV obtained from zeta potential of RLS formulated with SPC only. However, the charge containing substance significantly affected the surface charge of liposome in the uncharged formulation (*p- value* < 0.05 for both LS and RLS). The highest negative value was the DCP containing formulation (-20.56 mV and -26.87 mV for LS and RLS, respectively) whereas the lowest negative value belonged to SA containing formulation (-15.11 mV and -17.19 mV for LS and RLS respectively). This reason is also applied to describe the insignificant difference of encapsulation efficacy of RIF in each charged liposome vesicle (*p*-value >0.05). Since RIF is partially ionized (~ 40%) to the anionic form at pH 7.4 [Rodrigues *et al*., 2003], therefore the zeta potential was slightly decreased for the liposome containing RIF compared to the blank liposome as depicted in Figure 3.6. From electrostatic interaction, anionic form of RIF should be more effectively incorporated in positively charged liposome surface. However, the same encapsulation results were obtained in all formulations due to the all negative charged of liposome surface as described above. The encapsulation of RIF did not significantly increase from the positive charge of SA compared to uncharged liposome (*p*-value >0.05). The high percentage encapsulation of RIF (~50%) in the liposome was in agreement with the works of Vyas *et al*. (2004), however, they used ten times higher of lipid content compared to this study.

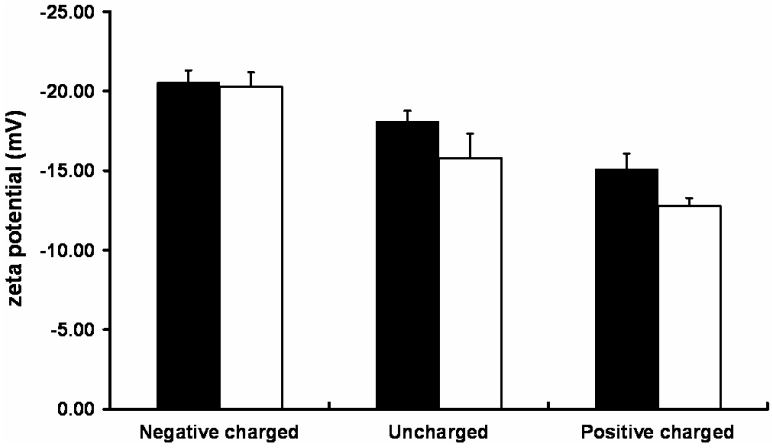


Figure 3.6 The zeta potential of liposome surface charge compared between blank liposome (□) and liposome containing RIF (■).

3.3.2 Location of RIF and CH in the liposome bilayer

The combination of ^{2}H and ^{31}P SS-NMR is able to point to the location of RIF in the lipid bilayer (Drechsler and Separovic, 2003). Figure 3.7 shows the ^{2}H SS-NMR spectra of the liposome bilayer and the quadrupolar splittings for the terminal methyls (CD_3) and methylenes (CD_2) of the acyl chain are summarized in (Table 3.2) together with the ^{31}P chemical shift anisotropy (CSA) of the phospholipid head groups (Figure 3.8). The CD_3 and CD_2 splitting increased when CH was added while the effect of RIF depended on the CH content. The results are separated into two groups: firstly when no CH is present or when CH content is low (POPC or POPC:CH 2:1), and secondly data for high CH content (POPC:CH 2:2). The similar results for samples without CH is present and when CH content is low could be due to the less ordered bilayer being able to incorporate RIF.

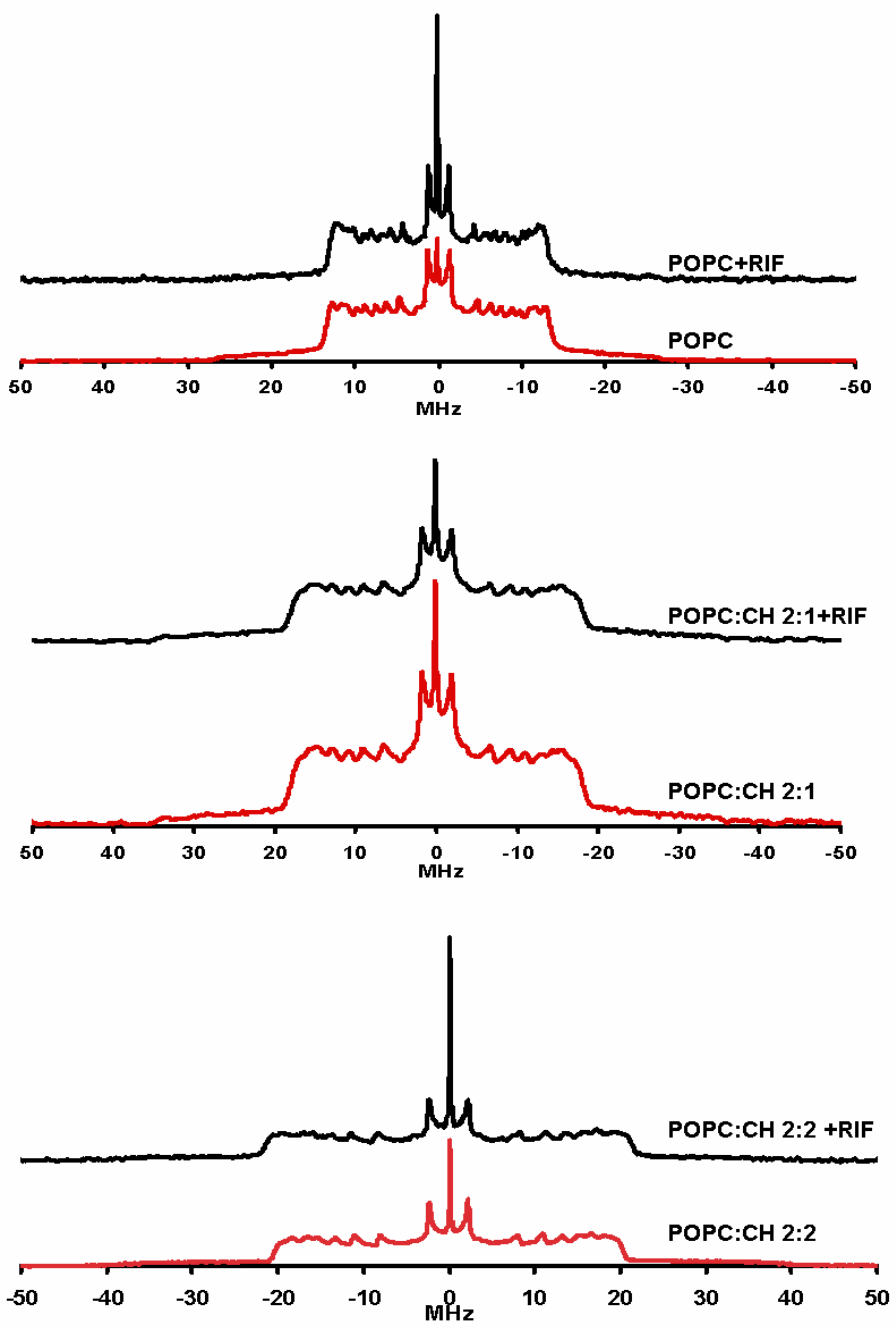


Figure 3.7 Solid-state ^{2}H NMR spectra of each liposome suspension formulated with different ratios of deuterated 1-palmitoyl (D31)-2-oleoyl-*sn*-glycero-3-phosphocholine (*d*-POPC) and cholesterol (CH) incorporated with 0.05 mmole RIF or without RIF.

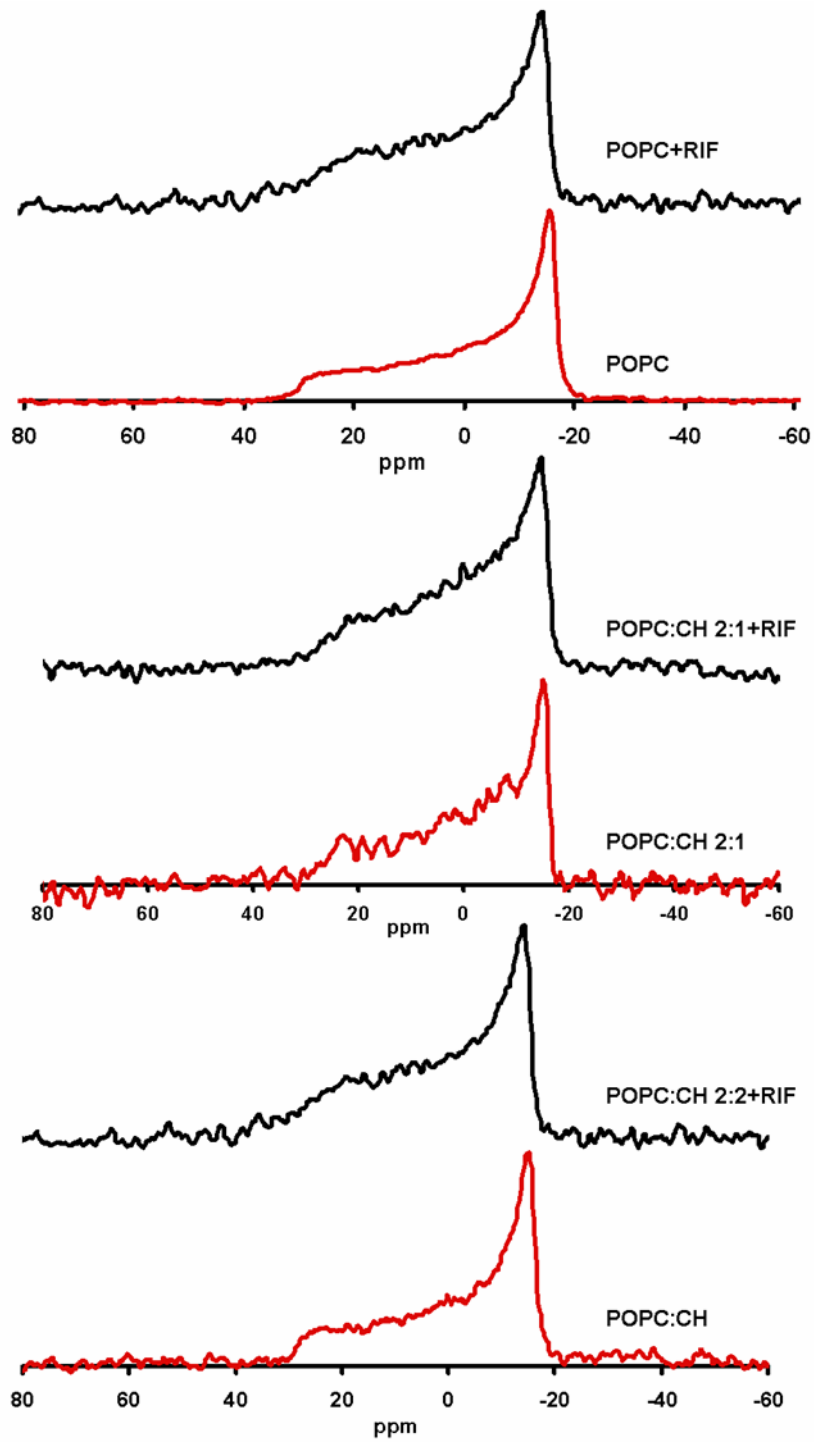


Figure 3.8 Solid-state ^{31}P NMR spectra of each liposome suspension formulated with different cholesterol (CH) content with or without rifampicin (RIF).

Table 3.2 Solid-state NMR ^{2}H quadrupolar splitting and ^{31}P chemical shift anisotropy (CSA) of the different formulations.

Formulation	CD_3 splitting	CD_2 splitting	^{31}P (CSA)
POPC	2650 Hz	26.98 kHz	-47 ppm
POPC with RIF	2440 Hz	26.25 kHz	-47 ppm
POPC : CH 2:1	4150 Hz	37.72 kHz	-43 ppm
POPC : CH 2:1 with RIF	3630 Hz	36.26 kHz	-43 ppm
POPC : CH 2:2	4520 Hz	40.77 kHz	-45 ppm
POPC : CH 2:2 with RIF	4540 Hz	43.06 kHz	-42 ppm

The addition of RIF molecules to the formulation without or with low CH content appeared to disorder the bilayer hydrocarbon chains and have no effect on the head groups as reflected by the ^{31}P CSA. These opposite effects for the ^{2}H and ^{31}P data suggest that the RIF is residing below the bilayer interface, having little effect on the head groups but disordering the lipid chains (Cornell *et al*., 1988 and Cornell and Separovic, 1988). This indicates that the incorporation of RIF in the liposome membrane without or with low CH content may disorder the methylene chain resulting in increased fluidity of the membrane and may cause vesicle leakage or fusion.

For the high CH content samples (POPC:CH 2:2), the ^{2}H splitting of the CD_2 groups significantly increased and gave the similar values as reported for POPC:CH in the liquid ordered phase. (Thewalts and Bloom 1992). For the terminal

CD_3, there was almost no change in splitting with RIF, which indicated that the RIF was not in the middle of the POPC bilayer in the more ordered liposomes. The RIF molecules when associated with high amount of CH ordered the CD_2 in the upper acyl chain area, but had the opposite effect on the head group as, confirmed by the ^{31}P NMR data (Table 3.2). The reduction in ^{31}P CSA suggests that the head group of POPC was disturbed by the RIF molecules, which inserted in the upper part of the acyl chain, increasing the order of the methylenes but disordering the head group in a similar way to the effect of cholesterol on POPC bilayers (Table 3.2) (Drechsler A and Separovic F., 2003). Thus, for the more ordered formulation with high CH content, the RIF molecules may be stacking alongside the CH molecules (Figure 3.9).

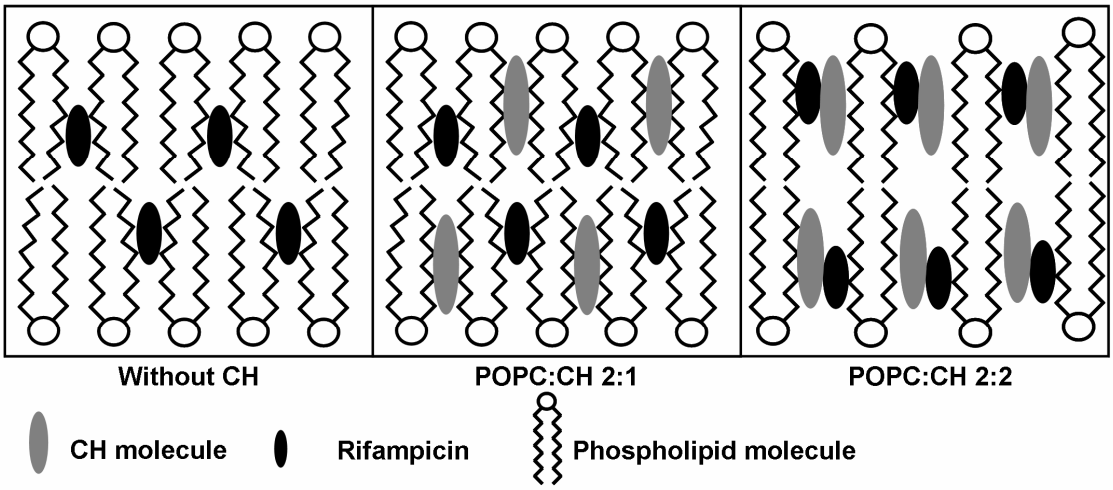


Figure 3.9 The possible location of cholesterol (CH) and rifampicin (RIF) in the lipid bilayer with different molar ratios of CH.

It is well known that increasing CH content in a liposomal system tends to increase the rigidity of the liposome bilayer (Vemuri and Rhodes, 1995 and Thewalts and Bloom 1992). However, the incorporation of the RIF molecules within the lipid bilayer may influence how CH affects the liposome rigidity and stability. From the physical stability, liposomes with a higher CH content (Formulation No. 1) gave better physical size stability (Figure 3.10), whereas the vesicles of Formulation No. 2 (lower CH content) aggregated after 2 weeks storage with the particle size increasing from 200-300 nm to 500-2500 nm. The results correlated with the SS-NMR data (Figure 3.7, 3.8 and Table 3.2). The ^{2}H quadrupolar splittings of POPC, POPC:CH 2:1 and POPC:CH 2:2, both with and without RIF, increased in order, respectively, due to a decrease in the fluidity of the lipid bilayer by the addition of CH. Cholesterol increases order and decreases fluidity within the membrane (Drechsler and Separovic, 2003; Thewalts and Bloom, 1992). So that liposomes with CH have higher rigidity and, therefore, SPC:CH 2:2 had size stability for longer than 4 weeks. This indicates that RIF did not reduce the lipid bilayer ordering property of CH when incorporated at high concentration. Moreover, RIF increased the CD_2 splitting, increasing lipid order when associated with a high CH content. In contrast, RIF with no or low CH decreased the CD_3 quadrupolar splittings, which is associated with an increase in fluidity in the centre of the lipid bilayer and may affect the stability of the liposome suspension and cause leakage or fusion of the vesicles. Therefore, CH content is an important factor in liposome formulation for RIF encapsulation.

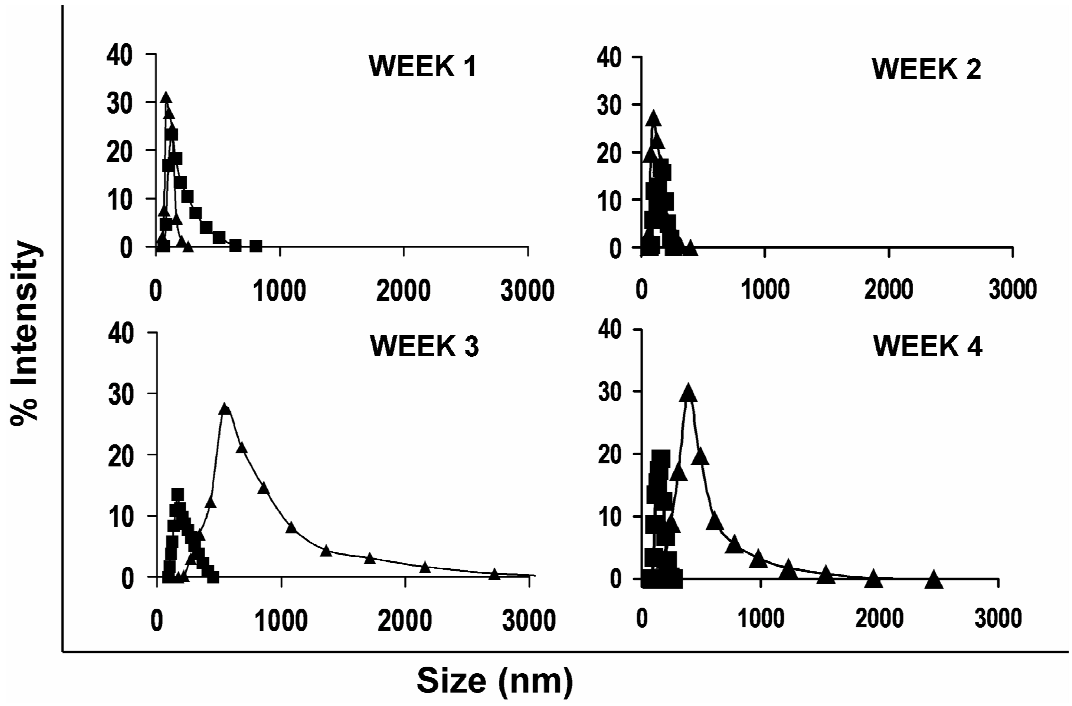


Figure 3.10. Size stability over 4 weeks of liposomes with high CH content (3.3 mM) (■ Formulation No. 1) and low CH content (1.7 mM) (▲ Formulation No. 2) (mean ± SD, n = 3).

3.3.3 The residual chloroform in the formulation

To ensure that chloroform was completely evaporated from the formulation, the qualitatively detection of chloroform content was operated. The result shows that chloroform content of this formulation was not detected (the limit of detection is 0.25 ng/mL) both in the liposome suspension and liposome freeze–dried powder. Therefore, the chloroform may be completely disappeared, evaporated, ensuring that the formulation is safe for inhalation.

3.3.4 Freeze dried liposome powder

After a suitable liposome suspension for RIF formulation was obtained, it was used to formulate a suitable dry powder for inhalation by freeze-drying under different conditions. Freeze-drying is presently used to achieve long term stability of liposome as drug carrier systems. Under appropriate cryoprotective conditions, liposomes should maintain their vesicle size and hydrophobic drugs remain associated with the bilayer following freeze-drying and rehydration.

The data obtained for the heating–cooling DSC thermograms of liposome suspension (Figure 3.11) are summarized in Table 3.3. From the supercooling temperature of sugar-liposome suspensions during the freezing process, water in the liposome systems showed a large exothermic peak where ice nucleated and crystallized (Figure 3.11) for all three formulations when the suspension temperature was at least -24 $^{\circ}$C. On heating, there was a transition temperature of liposome suspension containing lactose and mannitol at -34.9 $^{\circ}$C and -36.7 $^{\circ}$C, respectively (see expansion of Figure 3.11). In addition, the endotherm of the ice melting temperature of mannitol liposome suspension was observed at 3-4 $^{\circ}$C. In this study, the suspension was frozen in dry ice for 1 h before immersion into liquid nitrogen for 10 min to ensure that the temperature was lower than transition temperature and water crystallization temperature and that sugar are in glassy matrix and unfrozen water was not left in the frozen products. The product temperature was -50°C when placed in the freeze-dryer chamber, which was much lower than the supercooling temperature of water and transition temperature. The calculated enthalpy for the three different systems was similar (~ 300 J/g).

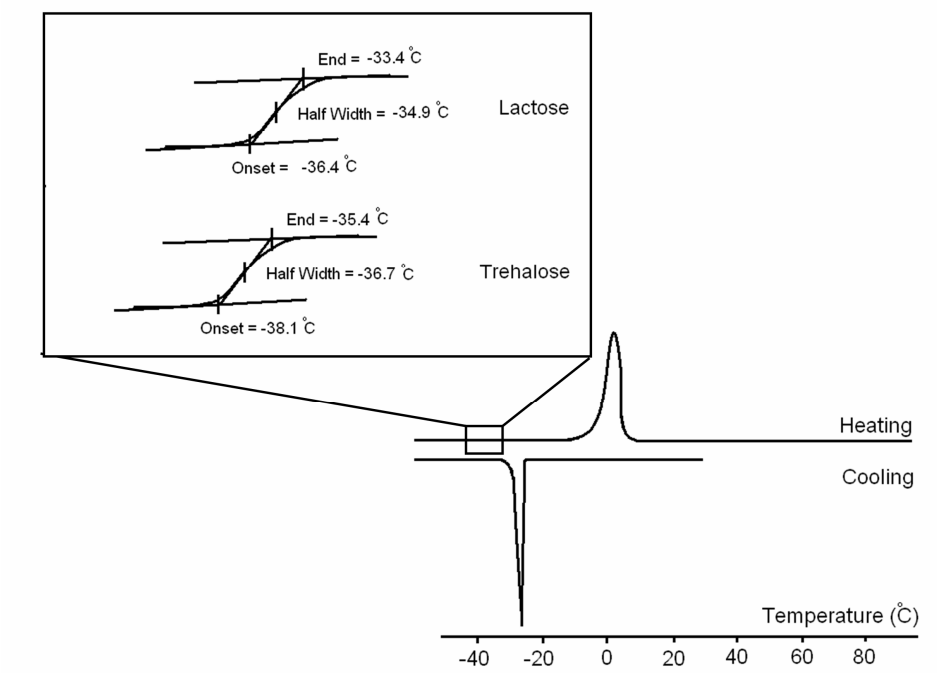


Figure 3.11 DSC thermograms in heating and cooling mode of liposome suspensions.

Table 3.3 DSC data of liposome suspensions upon cooling from room temperature following by heating.

Formulation	Exothermic peak		Endothermic peak	
	Temperature	Enthalpy (J/g)	Temperature	Enthalpy(J/g)
Liposome containing mannitol	-19.3 °C	88.8	3.4 °C	299.9
Liposome containing trehalose	-19.8 °C	77.0	3.7 °C	301.3
Liposome containing lactose	-23.0 °C	78.6	3.0 °C	302.6

Normally, drying is carried out at temperatures above -40°C in order to be practically accomplished. Below this temperature, the vapor pressure of water is so low that the drying process proceeds at an extremely low rate, or not at all. As more water is removed from the glass, the glass transition temperature increases, making it possible to increase the drying temperature above -40°C (Cortes and Caekenberghe, 1997). The freeze-drying conditions were varied for the primary drying temperatures (-10, -5 and 0°C), with a secondary drying temperature of 20°C. The primary drying was performed are all above -40°C as described above. A combination of XRD and DSC is often necessary to determine the nature of such powders (Pikal, 1999). The phase behaviour of the product was determined by DSC (Figure 3.12), the liposome powders with lactose and trehalose displayed a transition temperature of dehydrated liposome at ~20°C and 42°C without an endothermic peak, which indicates that both powders were in amorphous form and correlated with the XRD results (Figure 3.13). On the other hand, the liposome powder with mannitol showed two endothermic peaks, a small endothermic transition at 41.6°C and a melting endotherm at 136.9°C, which was lower than the literature value for pure mannitol 165°C. This is due to the mixed component system with liposomes, which may affect with the freezing point of mannitol. The XRD results showed that the relative crystallinity of the liposome powder with mannitol decreased from 11.25 to 7.25% when the primary drying temperature decreased from 0°C to -10°C. This is a consequence of a decrease in the primary drying temperature. Hence, knowledge of temperature control for the freeze-drying process is an important factor for stabilization of the amorphous glass phase.

Amorphous compounds are usually less physically stable than the crystalline form (Separovic *et al.*, 1998 and Steele, 2001).

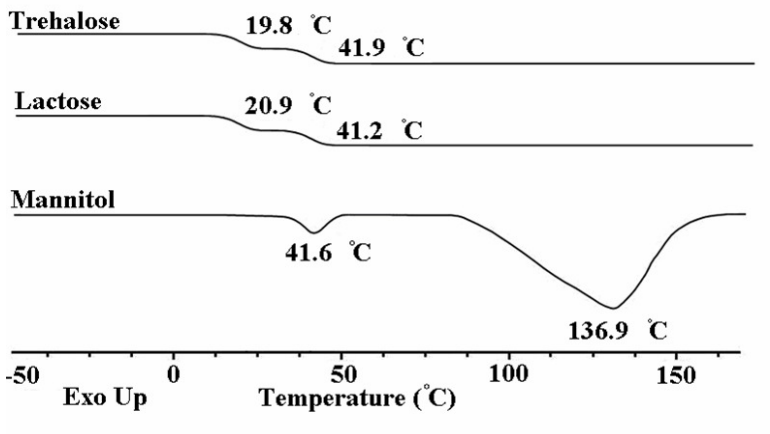


Figure 3.12 DSC thermograms of liposomes dried with different sugars. Number shows the deflection point of liposome containing trehalose or lactose and endothermic peak of liposome containing mannitol.

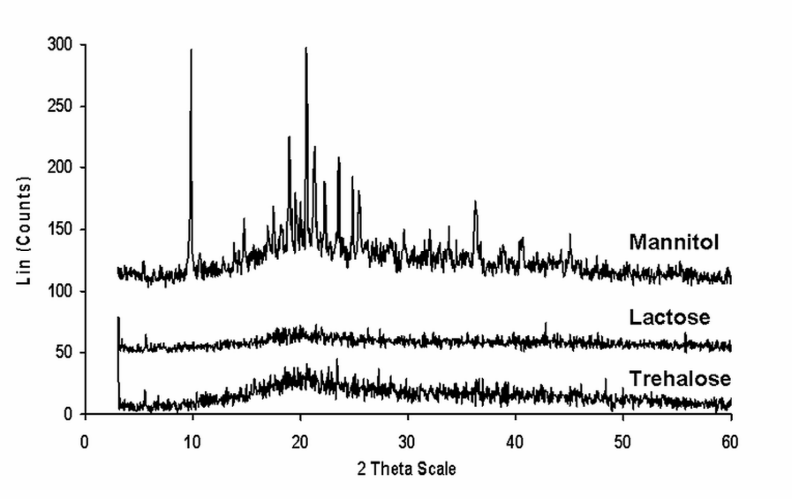


Figure 3.13 XRD pattern of dry powder of liposome encapsulated RIF.

3.3.5 Reconstituted liposome freeze dried powder

The obtained freeze-dried liposome powder (with mannitol) was reconstituted with phosphate buffer pH 7.4 and the morphology of the resulting liposomes was observed under cryo-TEM (Figure 3.14). The reconstituted liposome suspension comprised a broad distribution of liposome vesicle from 70 nm to 4.5 µm as shown in Figure 3.15. However, from the size distribution data, the most populations (70%) are in size range 200 -1000 nm with the average size of 700 nm (Table 3.1).

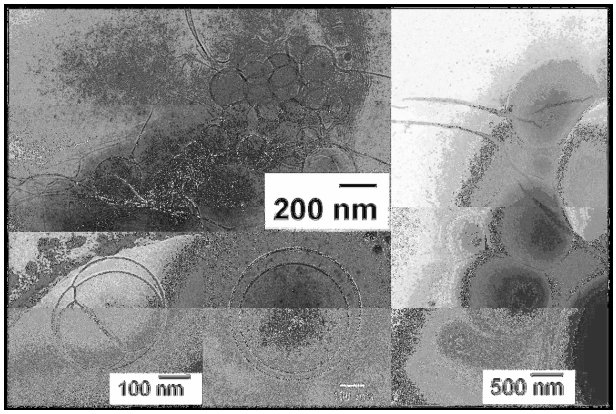


Figure 3.14 Cryo-TEM images of liposome vesicles reconstituted from freeze dried liposome powder (with mannitol).

Also, the freeze-drying process decreased the encapsulation efficiency to 20-22% (Table 3.1). An increase in liposome size most likely occurred in order to reduce the vesicle curvature because larger particles have less surface free energy and are likely to be more stable than smaller particles. The lower RIF encapsulation for the dry vesicles was observed as a result of the change in liposome size after reconstitution in comparison to the vesicles prior to drying. The fusion process involving vesicle-to-vesicle adhesion led to leakage of RIF from the liposomes. The

fusion and aggregation of liposome vesicle thus broader liposome size distribution and leakage of some RIF content may be explained by the freeze dried condition used in this study is not the most suitable to cryoprotect liposome vesicle.

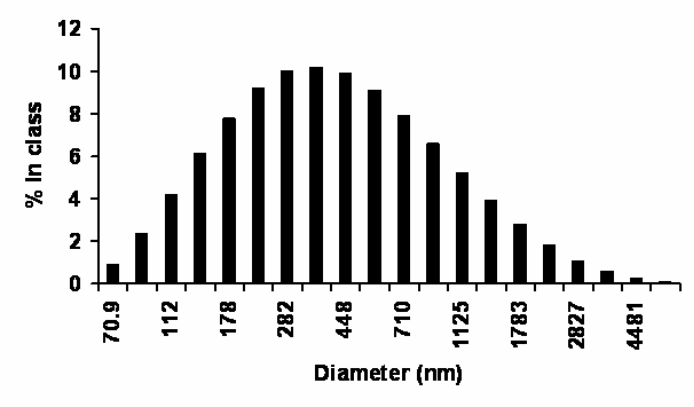


Figure 3.15 Size distribution of liposome vesicles obtained from reconstituted of freeze dried liposome powder.

As observed in Figure 3.11, transition temperature of the system was -36.7 °C and -34.9 °C for the liposome suspension containing trehalose and lactose, respectively. Below this temperature, both lactose and trehalose are able to form glassy matrix (vitrified structure) to protect close contact of liposome vesicle hence preventing both fusion and leakage of liposomes in the dry state (Hincha *et al*., 2006). However, the primary drying of this study was -10, -5 and 0 °C which are all over the transition temperature then losing of vitrified structure. In addition, lactose and trehalose were unable to effectively protect liposome from fusion and leakage. On the other hand, mannitol may be not suitable for cryoprotected liposome since mannitol is unable to form the glassy matrix and protect fusion of liposome vesicles. In order to improve the protection ability of trehalose and lactose, the liposome suspension

containing such two sugars (lactose and trehalose) should be dried at the primary temperature lower than that transition temperature (~-40 °C).

Moreover, the content of sugar may affect the prevention of liposome fusion since Sun *et al.* (1996) reported that a higher amount of sugar can help maintain the liposome size and encapsulation after the freeze-drying process. However, the use high amount of sugar may dilute the active drug content especially when applying for inhalation which only a small portion of powder is delivered to deep lung.

3.3.6 Aerosolized efficacy of freeze-dried RIF encapsulated liposome powder

Mass median aerodynamic diameter (MMAD), % fine particle fraction (FPF) and % emitted dose (%ED) data are shown in Table 3.4. Liposomes with lactose or trehalose gave MMAD larger than 5 μm (6.2 and 6.7, respectively), which is not suitable for inhalation. This was due to the secondary drying temperature (20°C) and room temperature being higher than transition temperature for both amorphous powders (liposomes with lactose or trehalose). As a result, sticky powders were obtained especially when the powders were exposed to moisture. An ED of 100% of was obtained from liposome powders with mannitol and trehalose and almost 100% with lactose.

The liposome powder with mannitol displayed the significantly lowest MMAD (3.4 μm) and the highest % FPF (66.8%) (significantly different with p-value < 0.05) which is suitable for aerosol formulation (Atkins *et al.*, 1992). The obtained powder was bulky and free flowing. Liposomes containing mannitol showed a

crystalline property and a small endothermic peak at 41.6°C, which indicated the storage condition should be lower than 40°C for aerosolized formulations. Hence, under these experimental conditions, mannitol was the most suitable sugar as a carrier of freeze-dried liposome powder aerosol. However, by the used of mannitol as a carrier, third component may be useful to maintain liposome structure upon drying process.

Table 3.4 RIF content for a single dose of liposome dry powder and aerosol parameters obtained from Andersen Cascade Impactor (mean±sd, = 5).

Sugar	RIF content (μg)	% FPF*	% ED	MMAD (μm)*
Trehalose	49.9 ± 0.6	32.0 ± 1.2	100 ± 0.1	6.7 ± 0.3
Lactose	49.9 ± 0.6	27.8 ± 3.5	94.0 ± 0.3	6.2 ± 0.6
Mannitol	50.1 ± 2.2	66.8 ± 6.2	100 ± 0.1	3.4 ± 0.5

* significantly different (p-value < *0.05*) only between formulations containing trehalose/lactose and mannitol.

3.3.7 Stability of RIF-encapsulated liposome

The RIF content in different formulations was monitored for 6 months and compared with their starting content (Figure 3.16). RIF in aqueous solution and RIF encapsulated in liposome suspensions was very unstable which the remaining RIF was 20% when they was kept at room temperature while RIF in liposome dry powder form displayed better stability of 80 % RIF remaining after being kept for 6 weeks.

Longer storage time of 6 months rendered to reduce the RIF content of liposome dry powder to 63.5 ± 8.0 % compared to the initial time when it was kept in desiccator. However, 58. 6 ± 0.6 % and 17.4 ± 4.0 % RIF remaining was obtained from liposome suspension and free RIF being kept at 4°C for 6 months. This indicated that RIF encapsulated in the liposome bilayer was protected from degradation since more than 50% of RIF (correlated to the percentage RIF encapsulation) was detected from the 6 months storage of liposome suspension at 4°C. However, when liposome suspension was kept at room temperature, only 20% of RIF was detected this may be due to the physical instability of liposome suspension at room temperature which render to leakeage and fusion of liposome vesicle then leakage RIF was degraded rapidly by 6 weeks. However, some RIF was degraded when kept at room temperature as compared to that in the refrigerator (4°C). This indicates that storage of RIF liposomes in the dry powder form at 4°C is a suitable condition for long term stability.

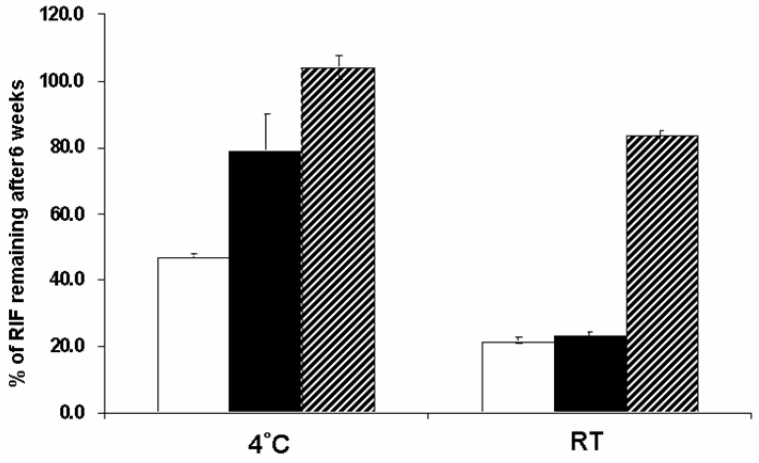


Figure 3.16 Chemical stability of RIF as a solution (□), RIF encapsulated in liposome suspension (■), and RIF in dry liposomes (▨) after 6 weeks storage at 4°C and RT (mean ± sd, n = 6).

3.3.8 Cytotoxicity of RIF encapsulated liposome to the cell in respiratory tract

The viability of NHBE, SAEC and AMs were estimated after challenged the cells with LS, RLS and equivalent concentration of free RIF solution for 24 h. NHBE (Figure 3.17A and 3.18A) and SAEC (Figure 3.17B and 3.18B) showed the percentage of viability nearly to 100 at all concentrations. Also with the % viability of AMs cell line which was nearly to 100 (Figure 3.17C and 3.18C) when it was exposed to different concentrations of all charged LS or RLS except at the highest concentration (10 μM of total lipid) at which a cell viability slightly decreased (~ 20%). This may be due to a very high density of liposome vesicle provided in the limited well surface area then it covered the cell and prevented the air from penetrating deeply into the cells, which may thus caused cell death. This result is in parallel with Zaru *et al* (2007) works which phosphatidylcholine (PC) caused a slightly cell death due to the higher lipid concentration incubated with the cells. The lower viability of NHBE, SAEC and AM was induced by free RIF solution compared to that induced by LS or RLS at the equivalent amount. Similar observation was noted by Zaru *et al* (2007). Lower uptake of RIF liposome by the cells (compared to free RIF) may due to the different types of interaction of the cells with free RIF and liposome-associated RIF that resulting in different intracellular distribution of RIF and rendering the drug less toxic in liposomal form. From our hypothesis, RIF molecule itself produces toxicity to the cell at the high concentration. Incorporation RIF into the liposome bilyer may prevent the direct contact of the drug to the cell and, therefore, decrease it toxicity. In a final remarks, the most considerable is the low cytotoxicity to the cell in airway system with RIF liposomal. This is an advantage to apply RIF liposome in the respiratory tract.

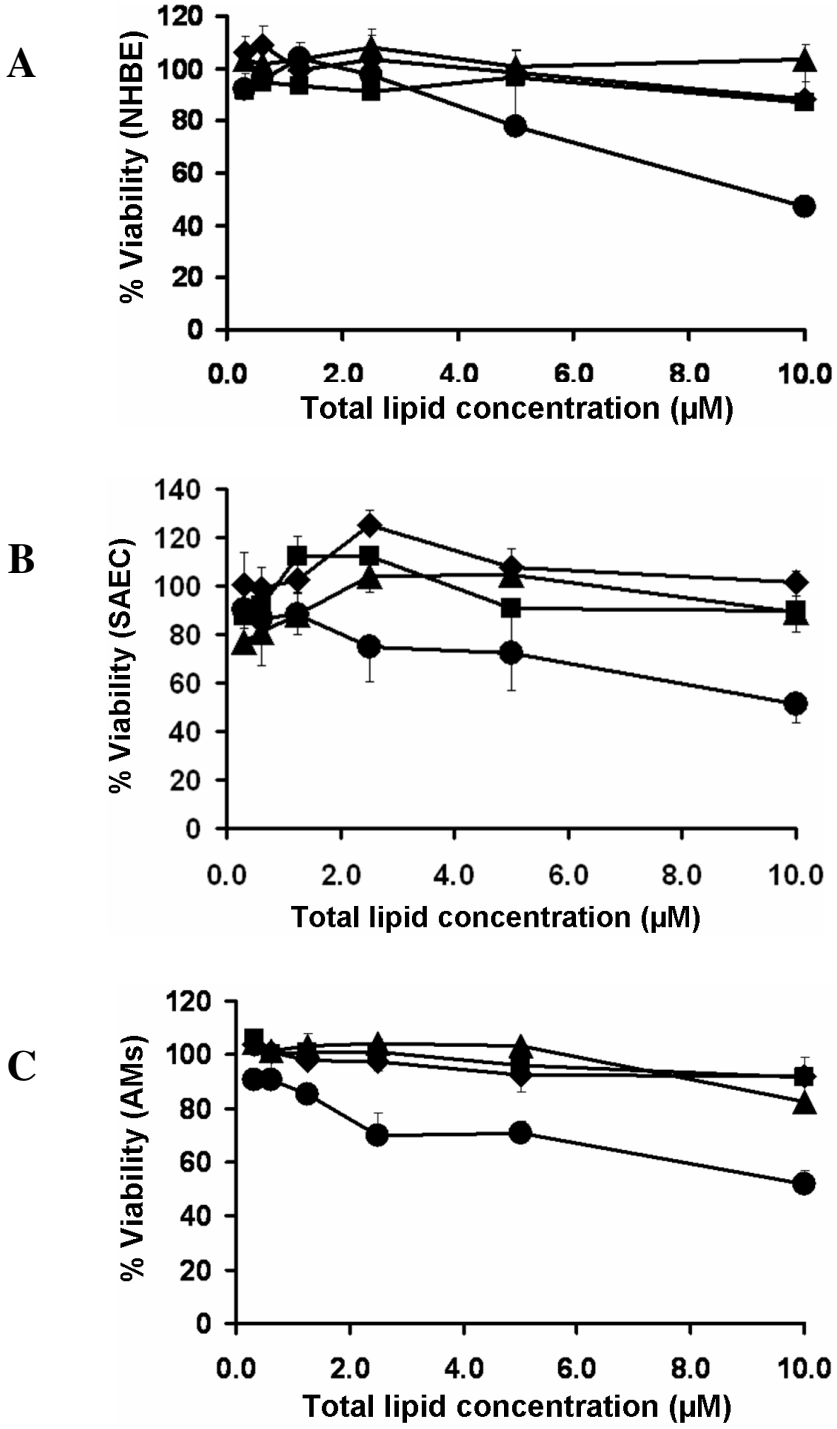


Figure 3.17 Viability of NHBE (A), SAEC (B) and AMs (C) after they have been incubated with negative charged LS (◆), neutral charged LS (■), positive charge LS (▲) and free RIF (●).

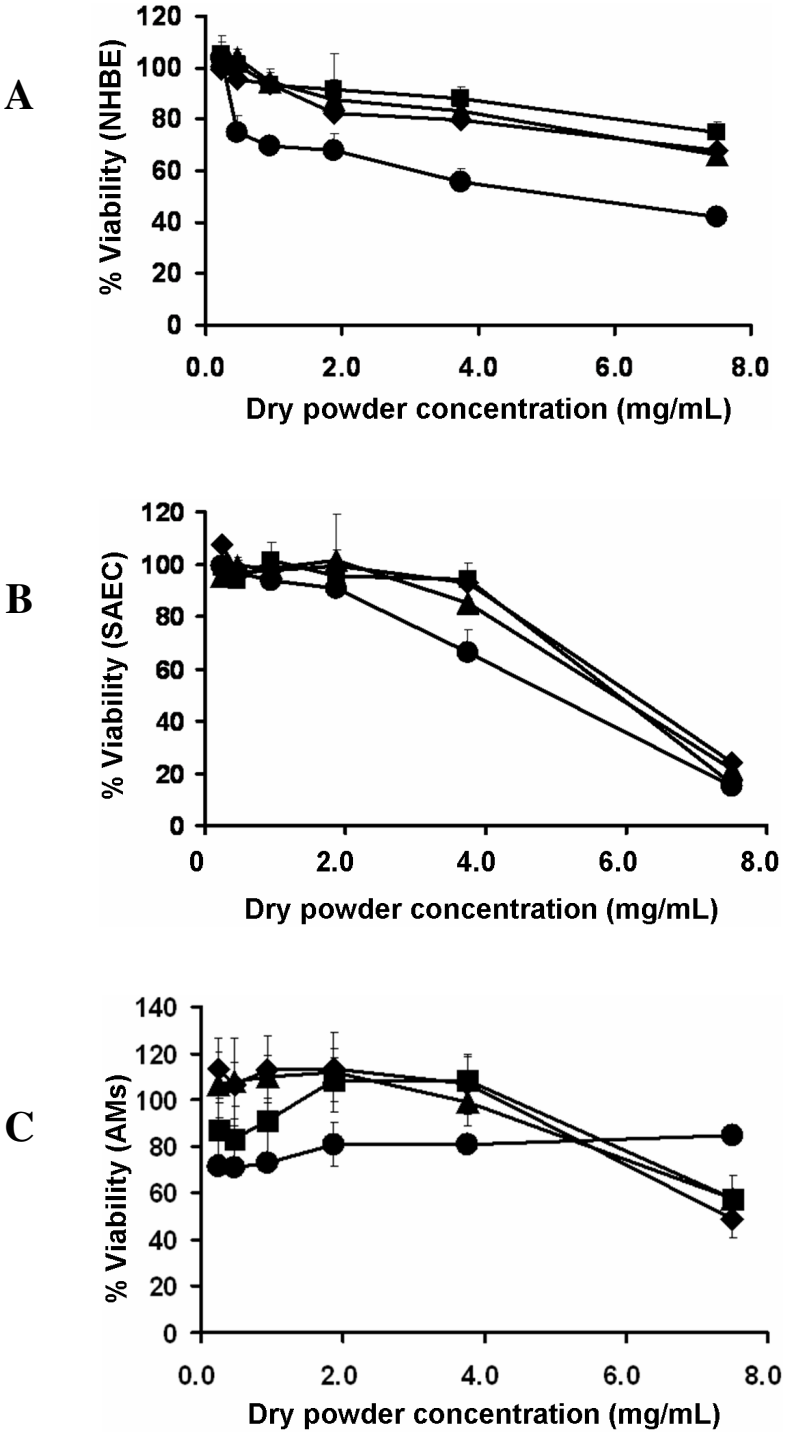


Figure 3.18 Viability of NHBE (A), SAEC (B), and AMs cell line (C) after they have been incubated with negative charged RLS (◆), neutral charged RLS (■), positive charge RLS (▲), free RIF (●).

3.3.9 Response of AM to RIF encapsulated liposome

Liposome are recognized as foreign materials by AM, thereby stimulating phagocytic cells undergoing phagocytosis and inducing host defense reactions. Thus, the most concern is the potential of AMs to generate oxidative burst and inflammatory cytokines (IL-1 and TNF-α) should be negligible in order to be used clinically. Even though, Ng *et al.* (1998) reported that poly (L-lactide) microsphere did not activate AMs to produce the significant magnitude of those inflammatory cytokines. However, this study is worked with liposome which prepared from different compositions and display different physicochemical properties. For safety reason, the pre-clinical studies *in vitro* with cell line has been evaluated.

From the results, after AMs were incubated with different concentration of LS or RLS for 24 h, IL-1β, TNF-α and nitric oxide were measured from the cell culture supernatant as shown in Figure 3.19 and 3.20 A-C, respectively. Production of IL-1β, TNF -α and nitric oxide by AMs were detected at very low level while very high level of IL-1β, TNF -α and nitric oxide was generated from AMs exposed to LPS. LPS which is a potent stimulator of innate immune responses was used as a positive control in this study at the concentration range of 15.63- 500 ng/mL. LPS induced immune response from AMs significantly different from when AMs was challenged with the liposome. However, LPS was used at a much lower concentration of 15,000 times compared to samples. It is clearly indicated that liposome containing RIF with all charges (negative, uncharged and positive) did not activate the AMs to produce the immune reaction even at high concentration.

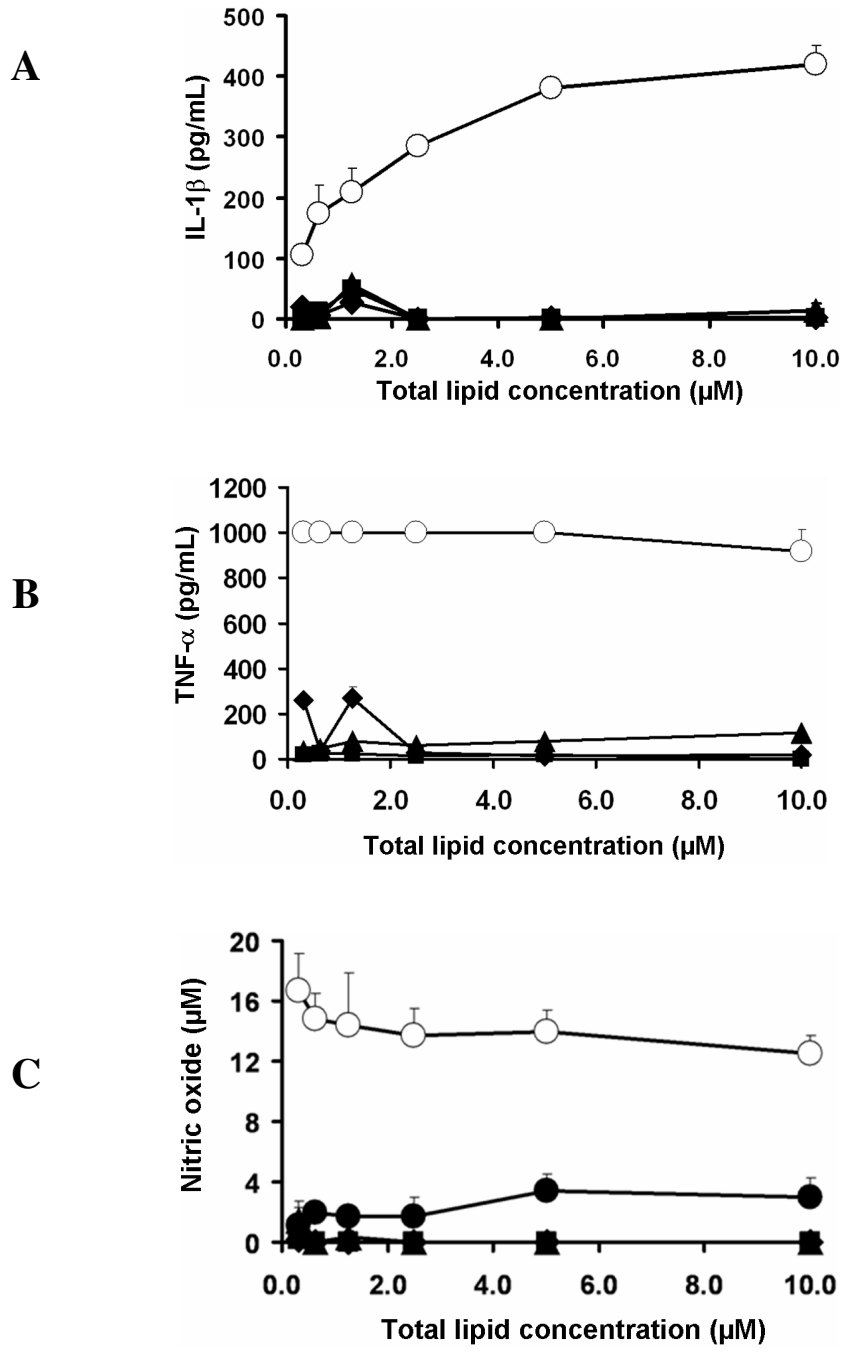


Figure 3.19 The level of inflammatory cytokine (A: IL-1β, B: TNF-α) and C: nitric oxide produced after exposed AMs with negative charged LS (◆), neutral charged LS (■), positive charge LS (▲), free rifampicin (●) and LPS from *E.coli* (○) for 24 h.

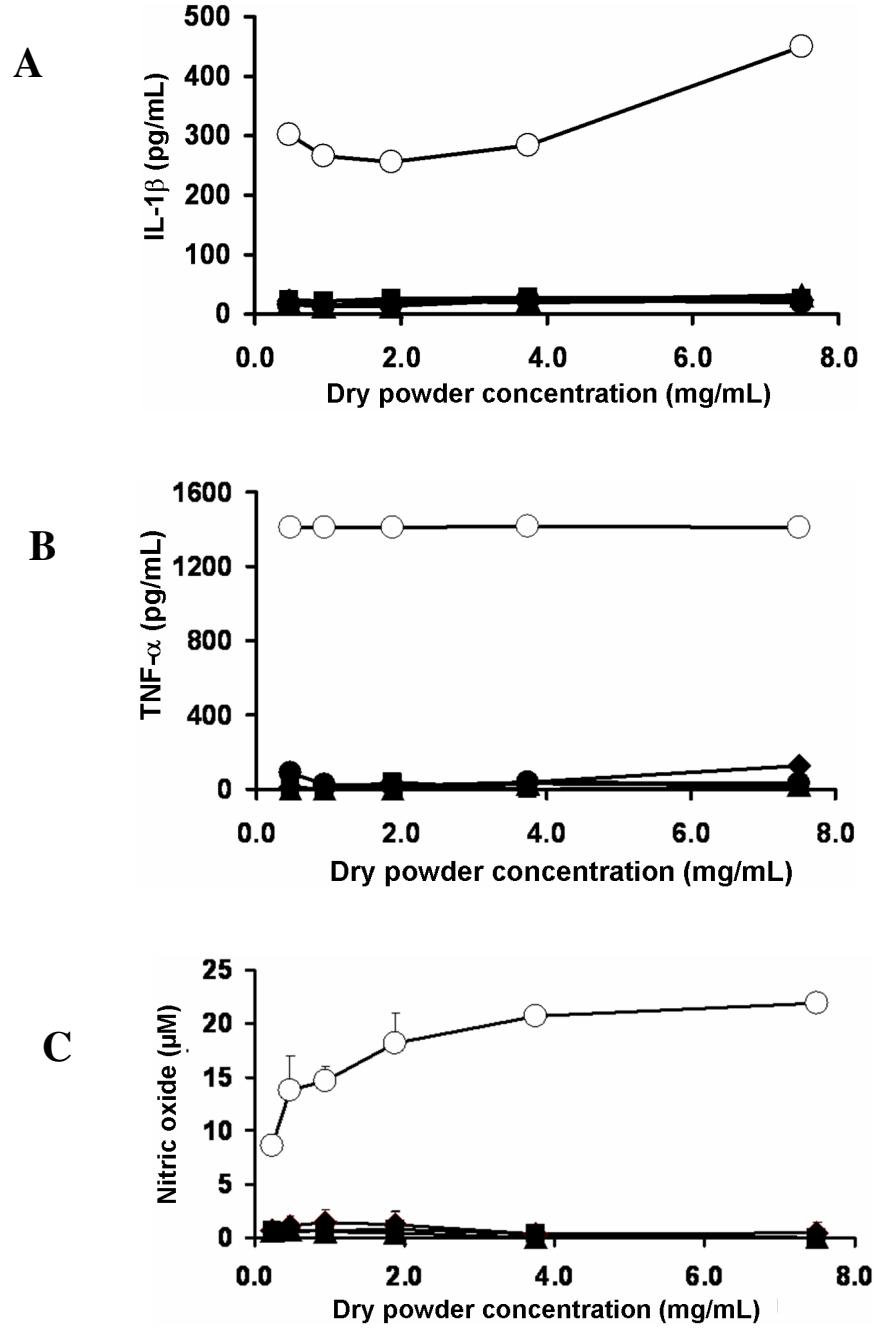


Figure 3.20 The level of inflammatory cytokine (A: IL-1β, B: TNF-α) and C: nitric oxide produced after exposed AMs with negative charged RLS (◆), neutral charged RLS (■), positive charge RLS (▲), free RIF (●), and LPS from *E.coli* (○)for 24 h.

3.3.10 Activity of liposome containing RIF and free RIF against *M. bovis*

This experiment section was to determine the MIC of liposome containing RIF compared to free RIF solution. The bacterial was sampling at the day 1, 3 and 7 to determine the remaining CFU after incubation with liposome containing RIF and free RIF at specified period. The bacilli did not reduce in their number of CFU after they were exposed with either liposome containing RIF or free RIF for only 24 h (data not shown).

However, significant CFU reduction was obtained in the day 3 and day 7 as shown in Figure 3.21. At day 3, the CFU was clearly eliminated at the concentration of 0.16 μg/mL (MIC) for all charged RIF-encapsulated liposome whereas the four times higher MIC of 0.63 μg/mL was obtained from free RIF. This indicates the superior ability of liposome containing RIF-encapsulated liposome than free RIF in term of antimycobacterial activity against *M. bovis*. This observation was similar to that of Mehta *et al.*, 1993 which they found MICs of free rifampicin against *M. avium- M. intracellulare* complex was four times higher than the liposome incorporated RIF.

It is more important to know whether the intracellular activity is also maintained or is enhanced after liposome encapsulation. For longer incubation period at day 7 the complete reduction of CFU number was found in all formulations of liposome including to RIF solution which indicated the MIC was less than 0.02 μg/mL.

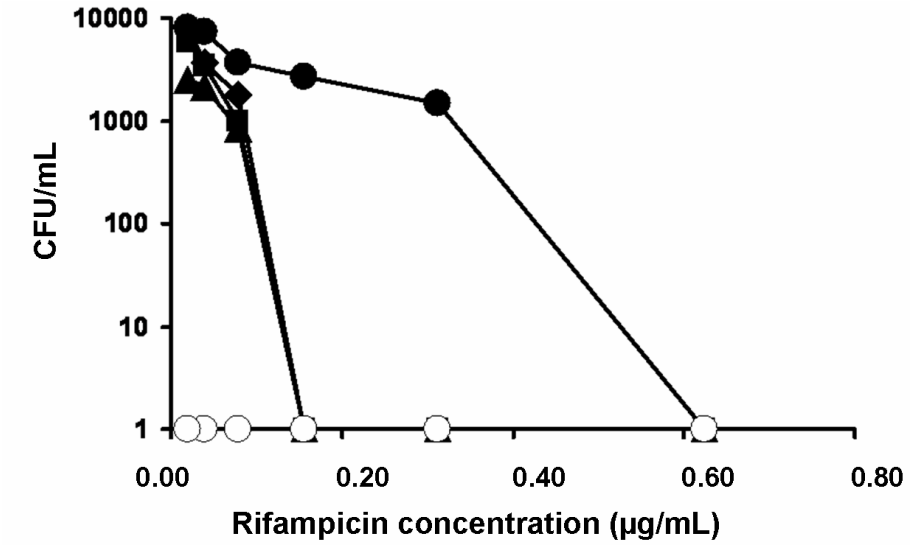


Figure 3.21 Effectiveness of negative charged (◆), uncharged (■), positive charged (▲) liposome containing RIF and free RIF solution (●) at various concentrations in reduction of *M. bovis* at day 3 (dark symbol) and day 7 (blank symbol).

3.3.11 Determination of minimum inhibitory concentration against *M. tuberculosis*

The summary of MIC values of the LS and free RIF against *M. tuberculosis* is shown in Table 3.5. The MIC in range of 0.006-0.06 µg/mL, which is in MIC range of RIF against *M. tuberculosis*, was obtained. However, the incubation time of the Alamar blue method is more than 3 days then the MIC against *M. bovis* at day 7 was used to be compared. The MIC of free RIF against *M. tuberculosis* is 3 times lower compared the the MICs of free RIF against *M. bovis* whereas for LS, the higher MIC against *M. tuberculosis* of 3 times was obtained. This means the use of *M.*

bovis to determine the antimycobacterial activity of RIF is comparable to *M. tuberculosis*.

Table 3.5 The minimum inhibitory concentration (MIC) of RIF liposome formulation against *M. tuberculosis*.

Formulation	MIC $_{H37Ra}$ (µg/mL)
Free RIF	0.006
Negative charged LS	0.06
Uncharged LS	0.06
Positive Charged LS	0.06

3.3.12 Activity of liposome containing RIF on intracellular growth of *M. bovis*

The activity of the LS, RLS of all charges compared with free rifampicin solution against *M. bovis* infected alveolar macrophage intracellularly was examined for various incubation times and concentrations. AMs (Figure 3.22A) was infected with *M. bovis* (as the arrow pointed in Figure 3.22B) for 4 h before exposed to the drug. Based on the results of the previous section the highest MIC against *M. bovis* was obtained from free RIF at 3 days (MIC = 0.625 µg/mL), the 10, 20 and 40 times of the MIC were used to treat AMs infected by *M. bovis*. As such, the final concentration of 0.625, 1.25 and 2.5 µg/mL of RIF (or equivalent in case of liposome formulation) in the cell culture media were obtained.

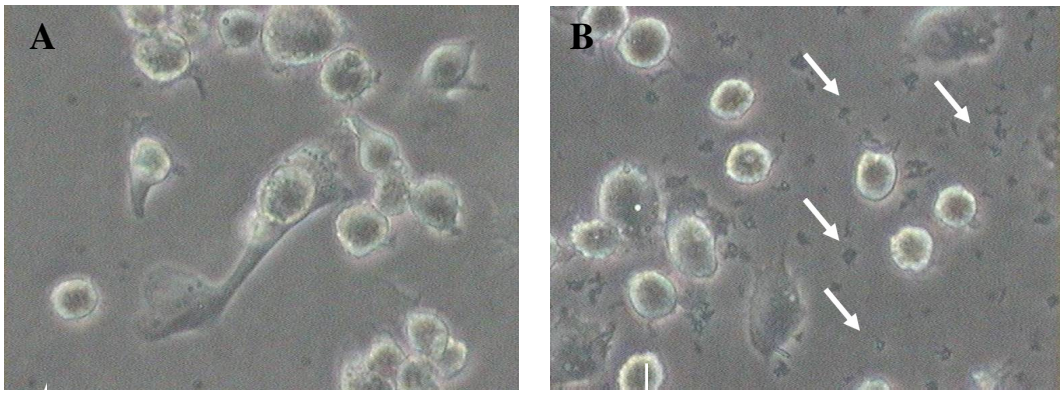


Figure 3.22 Alveolar macrophage cell line before infection (A) and during infection (B) (magnification 200x).

At the concentration of 1.25 and 2.5 μg/mL, infection was completely clear by all charged formulation both for LS and RLS containing rifampicin including free RIF solution at day 3 and day 7. There were not significantly different in the charged effects or formulation type of RIF in these high concentrations. However, at the lowest concentration of 0.625 μg/mL, there were differences among formulations and charges effects. The effects of the liposome surface charge, size, and formulation were discussed as follows. The RLS, broader size distribution of their vesicle size as previously described, was able to completely decrease the CFU number of *M. bovis* at day 3 and day 7. In contrast, for LS, at the concentration of 0.63 μg/mL the CFU number in 1 mL at day 3 was significantly reduced by positive charged LS (100 times reduction) compared to other charged formulation (*p-value* <0.05) (10 times reduction for negative charged, uncharged and free RIF) as shown in Figure 3.23. This indicated that positively charged liposome are likely to be more effective than other formulation.

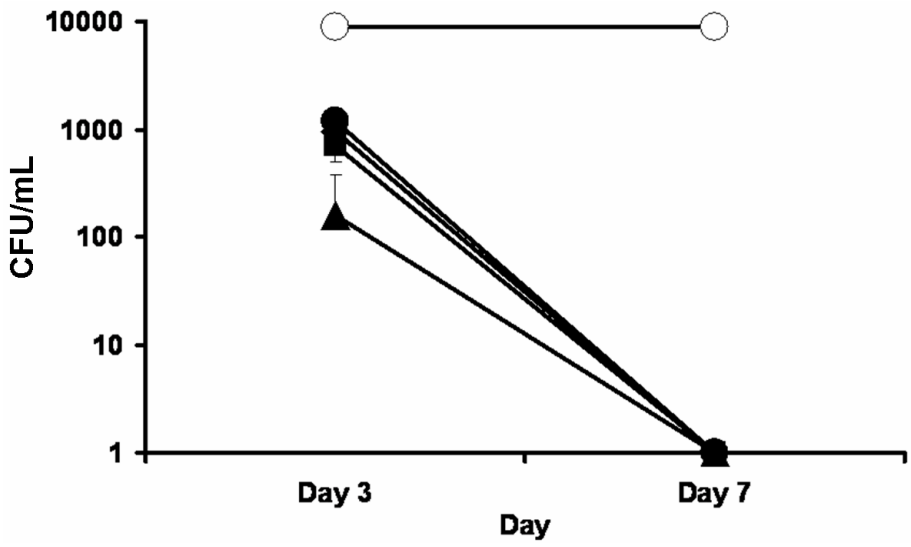


Figure 3.23 Effectiveness of negative charged (◆), uncharged (■), positive charged (▲) LS containing RIF and free RIF solution (●) against intracellular growth of *M. bovis* (○).

This may due to positive charged liposome are phagocytosed more effectively compared to negative charged and uncharged liposome vesicle as observed qualitatively by light microscopy images of AMs (Figure 3.24) after exposed to the liposome suspension for 7 days. From Figure 3.24, the vesicle associated with the AMs was found to be qualitatively highest with the positive charged liposome compared to others. The surface charge of the AMs is considered to be negative, it is suggested that liposome vesicle having positive charge are easily accessible to AMs (Makino *et al.*, 2003). However, Ahsan *et al.* (2002) reviewed and summarized that the potency of liposome encapsulated drug is affected by liposome surface charge, size, and varies among different cell lines. The role of surface charge and size in particle uptake is controversial as its alteration and modified other characteristics of the liposome (Ahsan *et al.*, 2002).

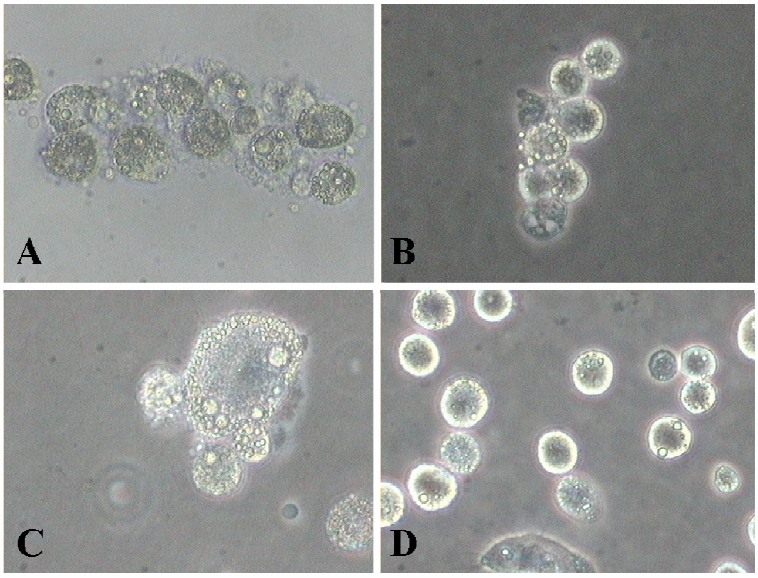


Figure 3.24 The digital images of AMs after exposed to LS containing RIF with negative charged (A), uncharged (B), positive charged (C) and free RIF solution (D) for 7 days (magnification 200x).

3.4 Conclusions

In conclusion, the 0.05 mM RIF liposome suspension formulated with 10 mM SPC and 10 mM CH is suitable formulation with the good physical stability due to the presence of high cholesterol and a high encapsulation efficacy (51%). Mannitol used as a cryoprotectant for the liposomes produced a crystalline powder with good aerosol characteristics (MMAD of 3.4 μm). However, the leakage of RIF and fusion of the liposome suspension was found with this freeze-dried protocol. To overcome these problems, the extensive study on the freeze-dried protocol has to be further investigated. The used of liposome vesicle, including negative charged, uncharged and positive charged, as a carrier of RIF in order to target to AM is safety

for the target cell itself and the epithelium cell in respiratory tract whereas free RIF suspension induced cytotoxicity at the equivalent concentration. In addition, the liposome containing RIF did not activate AM to produce inflammatory cytokines and nitric oxide. Incorporation of RIF with liposomes has been shown to enhance antimycobacterial activity when compared to free drug while broad size distribution of reconstitute liposome containing RIF are likely to be more effective than liposome suspension and free RIF. From the very low concentration effective to completely kill the bacilli (0.63 μg/mL), it is expected that the much lower dosage of liposome freeze-dried powder should be given by inhalation compared to a conventional oral administration (600 mg daily).

CHAPTER 4

ISONIAZID PROLIPOSOME DRY POWDER AEROSOLS

4.1 Introduction

Isoniazid (INH, Figure 4.1), the first line hydrophilic antituberculosis agent, is difficult to obtain high encapsulation efficacy liposome (Verumi and Rhodes, 1995). Furthermore, liposome in aqueous systems remain suffer from the physical stability problems such as hydrolysis or oxidation of the starting material, loss of encapsulated active component to the external phase especially for hydrophilic agent, sedimentation, aggregation or fusion of liposomes during storage (Payne, 1986; Hu and Rhodes, 1999; Song *et al*., 2002).

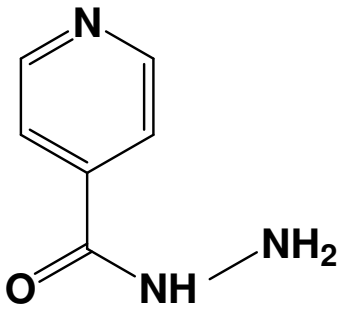


Figure 4.1 Chemical structure of INH (Merck, 1997)

Proliposome, a dry free-flowing powder, could be hydrated immediately to form liposomal dispersion on contact with water or biological fluid (Payne *et al.,* 1986). The purpose of this modified dosage is to overcome the problems associated with aqueous liposome dispersions. In general, they are prepared by penetrating a solution of drugs and phospholipids in volatile organic solvents into

the microporous matrix of water soluble carrier particles, normally sorbitol, followed by the evaporation of the organic solvents by vacuum rotary evaporator. Drugs and phospholipids are then deposited in the microporous structure of the carrier materials, thus maintaining the free flowing surface characteristics of the carrier material (Ahn *et al*., 1995; Hu and Rhodes, 1999; Song *et al*., 2002). However, this method is not suitable to prepare powder for inhalation since aerosol formulation requires the particles of nearly spherical in shape and monodisperse with aerodynamic diameter of 3 to 5 μm (Chougule *et al.*, 2007). The sorbitol particle used as a carrier material is in range of 125-500 μm (Payne *et al*., 1986) resulting in a large proliposome particle which is unable to deliver the drug deeply to alveoli. Using of the particle less than 125 μm is unsuitable due to excessive agglomeration during manufacture (Payne *et al*., 1986) therefore the free flowing particle of fine particles are impossible (Chogule *et al*., 2007). Also, the manufacturing procedures appear to be tedious and difficult to control, since the operation required (1) a discontinuous steps of solvent addition and evaporation; (2) close monitoring so that to ensure that neither the powder is allowed to become overly wet, nor the unit is operated in the absence of vacuum; and (3) overnight drying of the proliposome in a desiccator under reduced pressure (Chen and Alli, 1987).

Spray drying has also been considered as a one-step process for the production of small particles for pulmonary administration (< 5 μm) and has better control on particle formation and hence can be easily scaled up. Spray drying is not only limited to aqueous solution but also non-aqueous systems to prepare porous particles suitable for aerosol delivery (Chogule *et al*., 2007). Blank phospholipid dry powder prepared by spray drying for inhalation has been developed by Alves and

Santana (2004). The obtained lipid particles were spherical with aerodynamic diameter less than 5 µm.

Hence, it is interested to produce proliposome powder by using of spray drying technique in order to obtain the particle suitable for drug delivery to the lung. In this study, proliposome containing INH was prepared by different spray drying conditions. Spray dried mannitol (3 µm) was used as a core carrier and soybean phosphatidylcholine was coated on to its surface. Mannitol and INH were varied to different ratios to obtain the most suitable proliposome particles with adequate mannitol-INH-phospholipid interaction with suitable deep lung delivery.

The safety of the proliposome particles reaching to the airways was determined to ensure that products did not induce the release of any inflammatory substance in the airways and did not induce the cytotoxicity of respiratory associated cells. Similar methodology was employed as mentioned on the previous chapter.

4.2 Materials and Methods

4.2.1 Production of microparticulated mannitol

Mannitol (Fluka, Buchs, Switzerland) was dissolved in distilled water to obtain the concentration of 10 mg/mL. The mannitol solution was spray dried through the spray nozzle (0.4 mm) of the spray dryer model SD-1 (Eyela, Tokyo, Japan) under the inlet temperature of 110 $^{\circ}$C, atomizer pressure at 800 kPa and feeding rate at 3 mL/min. The outlet temperature was 90 $^{\circ}$C ± 5 $^{\circ}$C. The product was collected from the cyclone into the collecting chamber. The obtained microparticles were used as a core carrier of proliposome preparations.

4.2.2 Production of INH-proliposome by spray drying technique

The scheme of proliposome production is shown in Figure 4.2 and the ingredients of the formulations are shown in Table 4.1. Briefly, the microparticulate mannitol particle (size ~ 3 μm) was used as a core material. The lipid solution containing INH and microparticulate mannitol was sprayed dried and then the proliposomes obtained.

Table 4.1. Formulation ingredients of proliposome powder containing INH

	Lipid part		**Powder part**	
Formulation	SPC (mmole)	CH (mmole)	Mannitol (g)	INH (g)
No. 1	0.06	0.06	0.9	0.1
No. 2	0.12	0.12	0.9	0.1
No. 3	0.06	0.06	0	1.0
No. 4	0.06	0.06	0.2	0.8
No. 5	0.06	0.06	0.4	0.6
No. 6	0.06	0.06	0.6	0.4
No. 7	0.06	0.06	0.8	0.2

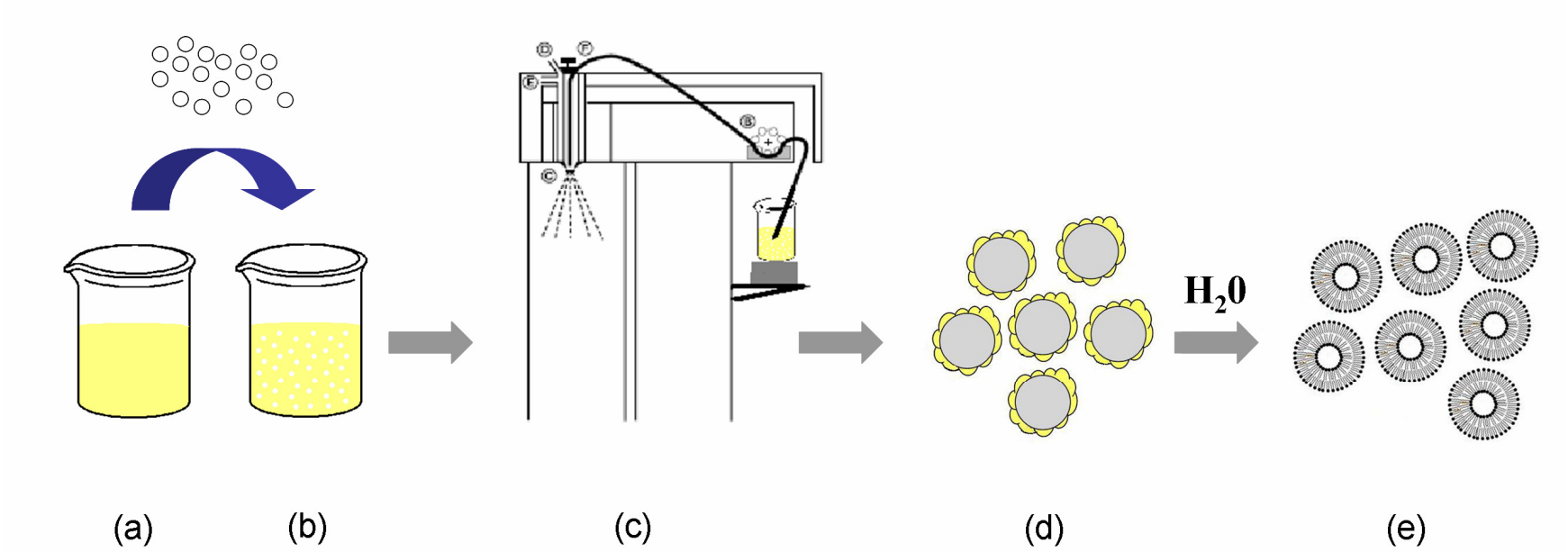


Figure 4.2 The scheme of proliposome production by spray drying technique; (a) ethanolic lipid solution containing INH, (b) dispersion of fine mannitol in ethanolic lipid solution containing INH, (c) spray drying processes, (d) proliposome powder product, (e) reconstituted proliposome powder.

Lipid part containing mixture of L-α soybean phosphatidylcholine (SPC, Fluka, Buchs, Switzerland) and cholesterol from lanolin (CH, Fluka, Buchs, Switzerland) in the mole ratio of 1:1 were weighed and dissolved in 100 mL of 95 % ethanol to obtain the ethanolic lipid solution. INH (Sigma Chemical company, St Louis, MO, USA) was added in ethanolic lipid solution and sonicated until the clear solution obtained. Due to solubility of INH in ethanol is 2% (Merck, 1997) and the maximum concentration of INH used in this study was only 1%, therefore clear solution was obtained in this step. The microparticulate mannitol particles were dispersed in a clear ethanolic lipid solution (Formulation No. 3 – No.7). The suspension of microparticulate mannitol in ethanolic lipid solution containing INH was obtained in this step because of co-dissolving of INH and mannitol may decrease solubility of mannitol in ethanol. The suspension was sonicated for 15 min in order to deaggregate of mannitol particles before spray drying processes. The suspension (or solution in case of Formulation No. 3) was spray dried with continuously stirred during a spraying period to provide the homogeneity of the suspension. The inlet temperature was 90 $^{\circ}$C, 800 KPa atomizer pressure with a feed rate of 3 mL/min. The outlet temperature was 70 $^{\circ}$C ± 5 $^{\circ}$C. The proliposome powder was collected from the collecting chamber and it was kept in the desiccator until use.

4.2.3 Scanning electron microscopy of proliposome powder

The surface morphology of proliposome particles was examined by scanning electron microscopy (SEM). The sample was sprinkled on double-sided adhesive tape on an aluminum stub and was coated with gold by sputtering technique

with fine coating machine (JFC-1200, JEOL, Tokyo, Japan) for 120 seconds. The sample was observed under SEM (JSM-6301F, JEOL, Tokyo, Japan) at 3 kV.

4.2.4 Content uniformity of INH in proliposome powder

Each INH-proliposome powder (10 mg) was randomly sampled and weighed. The powder was suspended in 10 mL of methanol to dissolve the lipid content. Distilled water was used to adjust to the volume of 25 mL following by sonication to obtain a clear solution. INH content from the clear solution was analyzed by HPLC. The HPLC system (Thermo electron corporation, California, USA) equipped with AS 3000 autosampler, P 1000 pump and UV 2000 detector. The mobile phase was phosphate: acetonitrile (97:3 v/v), at a flow rate of 1 mL/min, and UV detection was 254 nm. The microbondapak C18 column (Phenomenex, USA) (250×4 mm i.d., 5 µm) was used in this study.

4.2.5 Reconstitution of INH proliposome into liposome

INH-proliposome powder (10 mg) was weighed and reconstituted with 1 mL of 0.2 M phosphate buffer solution (PBS) pH 7.4 and incubated for 10 min to form liposome suspension. 100 µL of reconstituted suspension was transferred to volumetric flask of 10 mL. Methanol (2 mL) was added to dissolve the lipid coated on the particles and adjusted to the volume with deionized water and analyzed by HPLC for INH content as total drug loading. The remaining reconstituted suspension (900 µL) was centrifuged at 12000 rpm for 15 minutes in order to separate the large liposome vesicles. The supernatant was filtered through the 100 nm polycarbonate membranes which prevent liposome particles passing through and deposited on the

membrane while the unencapsulated INH was filtered through the membrane. The filtrate was analyzed as unencapsulated INH by HPLC technique as described previously. The percent encapsulation was obtained from the following equation.

$$\text{Percent encapsulation} = \frac{\text{Total drug loading - unencapsulted drug amount}}{\text{Total drug loading}} \times 100$$

4.2.6 X-ray diffraction measurement of INH-proliposome

The employed method was previously described in section 3.2.2.2

4.2.7 Differential scanning calorimetry

DSC 2920 (TA instrument, Newcastle, UK) was used to investigate the interaction of the INH-mannitol-SPC of the proliposome powder produced by spray drying method. The powder sample was placed in an aluminum pan, sealed hermetically, and then assessed by DSC from –50 °C to 200 °C at a rate of 10°C/min. The DSC thermograms were analyzed using the Universal analysis 2000 program version 3.4c.

4.2.8 *In vitro* deposition of INH-proliposome dry powder by Andersen Cascade Impactor (ACI)

The method was already described in section 3.2.2.5.

4.2.9 Cell cultures

Cell culture and maintenance protocol was already described in section 3.2.3.1 for normal human bronchial epithelial cell (NHBE) and small airway epithelial cell (SAEC) and section 3.2.3.2 for alveolar macrophages (AM).

4.2.10 Determination of cytotoxicity of INH-proliposome to the cell in respiratory tract

Viabilities of NHBE, SAEC and AM, after exposed to free INH, INH-proliposome Formulation No. 3 and INH-proliposome Formulation No. 7 in the concentration range of 5-0.15 mg/mL was determined by using MTT assay as described in section 3.2.4.

4.2.11 Determination of alveolar macrophage response to INH- proliposome

4.2.11.1 Production of inflammatory cytokines

The TNF-α and IL-1β produced from alveolar macrophage after challenged with free INH, INH-proliposome Formulation No. 3 and INH-proliposome Formulation No. 7 in the concentration range of 5-0.15 mg/mL were analyzed from the cell culture supernatant immunochemically with commercial ELISA kit as described in section 3.2.5.1.

4.2.11.2 Nitric oxide assay by the Griess reaction

Nitric oxide (NO) released from alveolar macrophage after challenged with free INH, INH-proliposome Formulation No. 3 and INH-proliposome Formulation 7 No. in the concentration range of 5-0.15 mg/mL was detected by the Griess reaction as procedure described in section 3.2.5.2.

4.2.12 Assessments of antimycobacterial activity of free INH and INH-proliposome

4.2.12.1 Culture of *M. bovis* from BCG vaccine

M. bovis from BCG vaccines was cultured according to method in section 3.2.6.1.

4.2.12.2 Determination of minimum inhibitory concentration against *M. bovis*

The MIC of free INH and INH-proliposome (Formulation No. 3) at a drug concentration range of 0.2-1.6 µg/mL was determined as indicated in section 3.2.6.2.

4.2.12.3 Determination of minimum inhibitory concentration against *M. tuberculosis*

Antimycobacterial activity of INH-proliposome (Formulation No. 3) against *M. tuberculosis* H37Ra was determined as described in section 3.2.6.3.

4.2.12.4 Determination of intracellularly antimycobacterial against *M. bovis*

The antimycobacterial effectiveness of INH-proliposome and free INH against intracellular growth of *M. bovis* in AMs was examined as described in section 3.2.6.4. The relative INH concentrations in this section were 5, 10 and 15 µg/mL.

4.2.13 Statistical analysis

Data, when applicable, are presented as mean ± standard deviation (SD) from at least three samples unless indicated. The data were compared using analysis of variance (ANOVA). All statistical comparisons were calculated using the SPSS software version 11.5 (SPSS, Inc., Chicago, IL). A significance level of p-value < 0.05 was considered statistically significant).

4.3 Results and Discussions

4.3.1 Surface and morphology of INH-proliposome particles under SEM

The INH-proliposome in the present study was prepared successfully. The mannitol particles and INH-proliposome particles produced by spray drying technique were observed for their size and morphology under SEM as shown in Figure 4.3–4.6. The smooth surface of spherical particle of mannitol with the size around 3 μm (Figure 4.3) was obtained from spray dried of low concentration mannitol. A large amount of fine particle less than 5 μm in the aerosol cloud be achievable after dispersed this mannitol powder by Diskhaler® (Chew and Chan, 1999). Furthermore, mannitol has been evaluated as a potential drug carrier in dry powder inhaler formulation (Steckel and Bolzen, 2004).

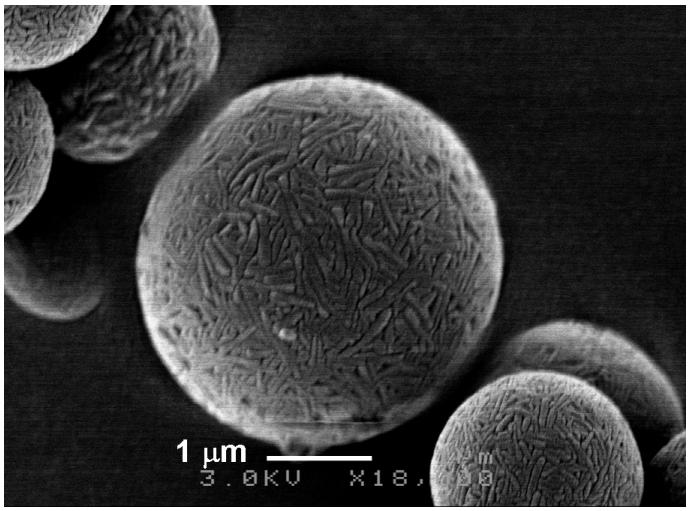


Figure 4.3 The SEM images of spray dried microparticulate mannitol using as a core carrier in proliposome production (magnification 18,000 X, bar =1 μm)

The proliposome formulations were optimized (Table 4.1) to obtain the powder particle suitable for lung delivery. Figure 4.4A and 4.4B displayed a surface and morphology of INH-proliposome obtained from Formulations No. 1 and 2, respectively. The only difference of these 2 formulations was the content of coating material whereas the powder part was kept constant at 1: 9 of INH to mannitol. The total lipid content (SPC and CH) was 0.12 mmole for Formulation No.1 (low lipid) and 0.24 mmole for Formulation No.2 (high lipid).

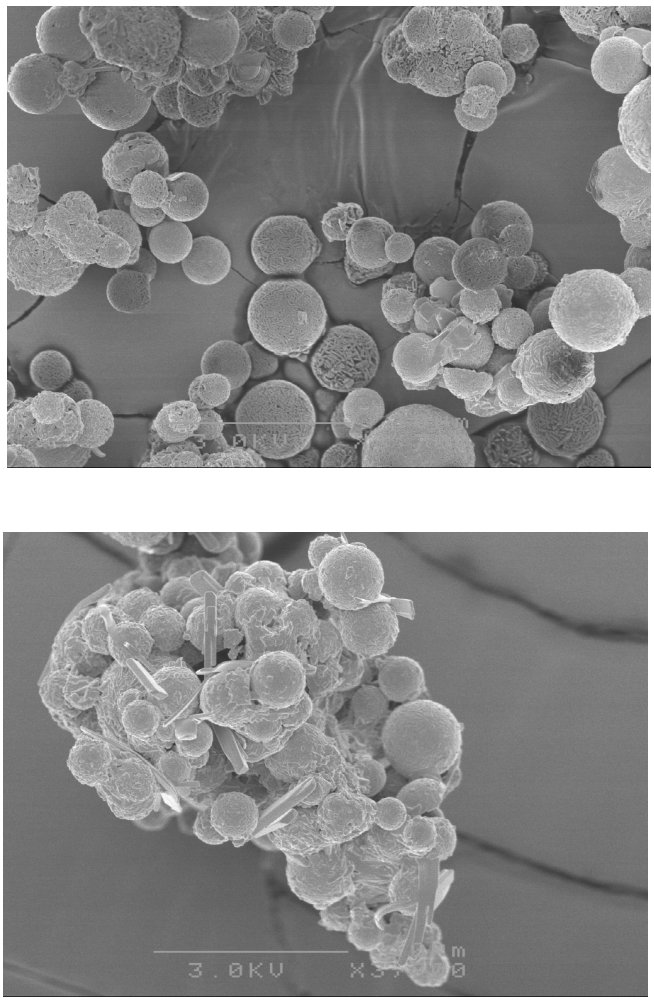


Figure 4.4 The SEM images of INH-proliposome powder of Formulation No. 1 (A) and Formulation No. 2 (B) (magnification 37,000 X, bar = 10 µm)

From pure INH in the Formulation No.3 and successively 20% decrease of INH content (80, 60, 40, and 20% INH) while mannitol was 20% progressively increased from Formulation No.4 toward Formulation No.7 (20, 40, 60, 80% mannitol). The final ratio of INH to mannitol were 4:1, 3:2, 2:3 and 1:4 w/w, for formulation No. 4 to No.7, respectively. The surface and morphology of proliposome obtained from Formulation No. 3 to No.7 are shown in Figure 4.5A to E for 5,000X magnification and Figure 4.6A to E for 10,000X magnification. INH-proliposomes observed from 10,000X magnification SEM images were irregular in shape, some tiny particles (less than 1 μm) adhered on large aggregated particles. Some spherical microparticulate mannitol particles were observed in Formulation No. 6 and 7 when 60% mannitol or higher (80%) was added into the formulation. From an observation, INH itself cannot be spray dried to produce a spherical particle.

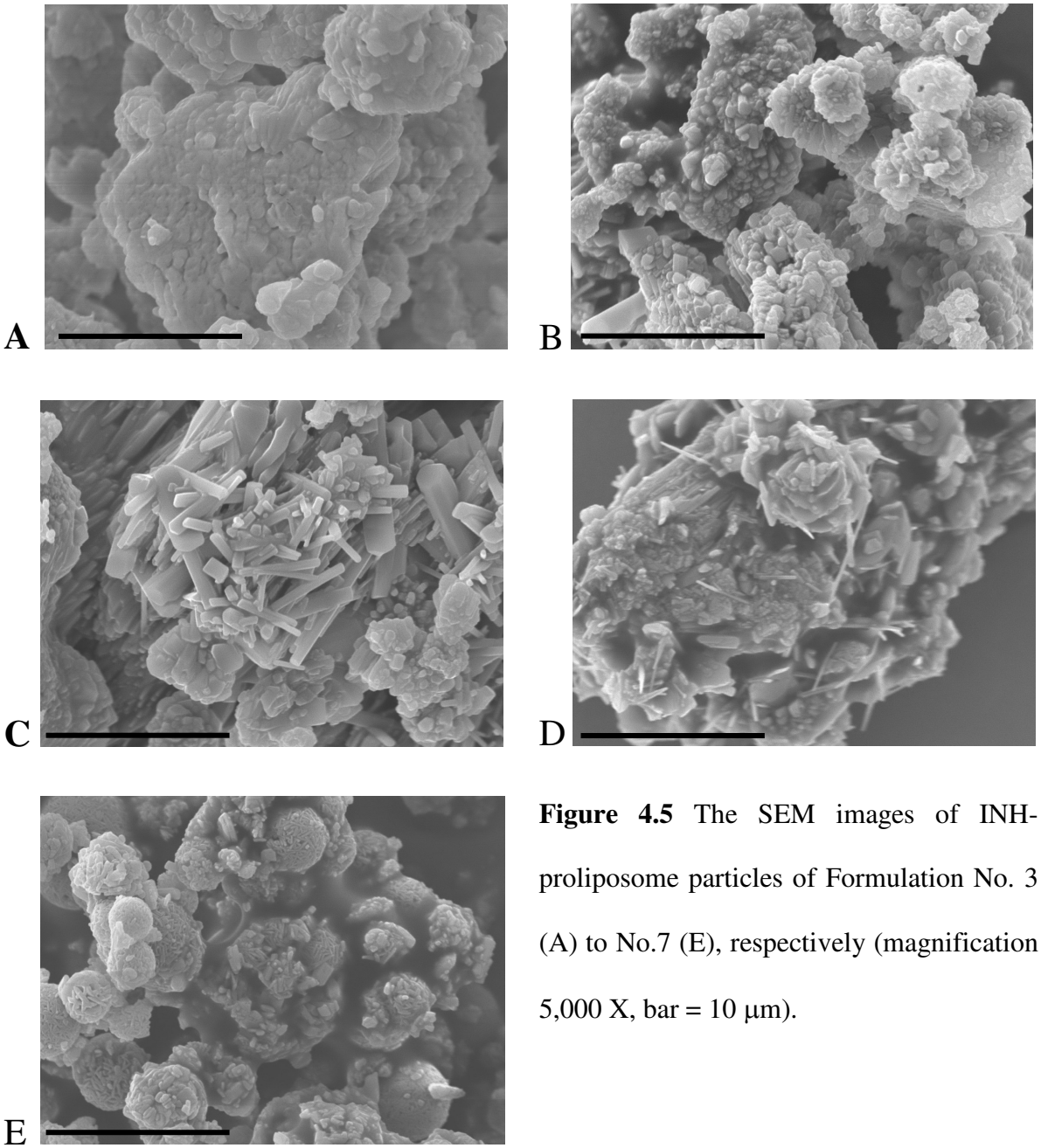
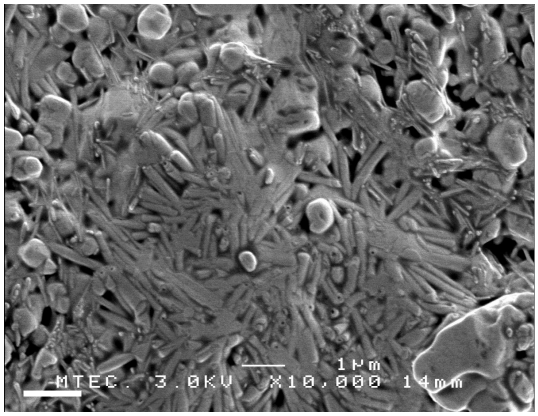
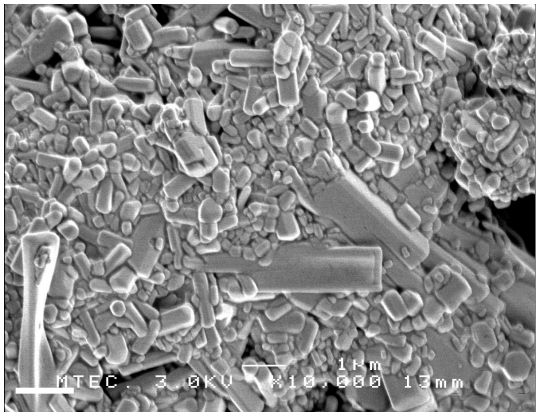
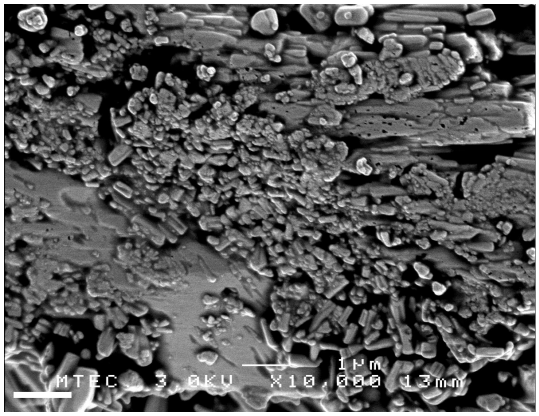
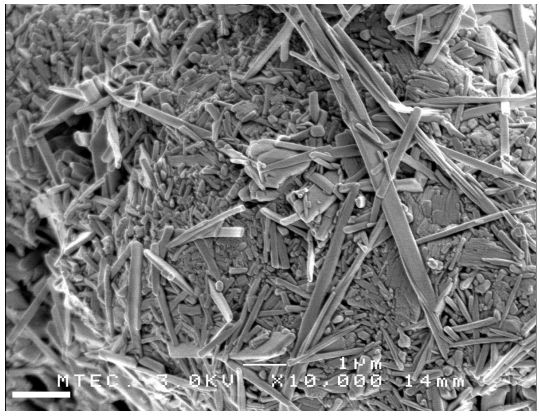


Figure 4.5 The SEM images of INH-proliposome particles of Formulation No. 3 (A) to No.7 (E), respectively (magnification 5,000 X, bar = 10 μm).

A 

B 

C 

D 

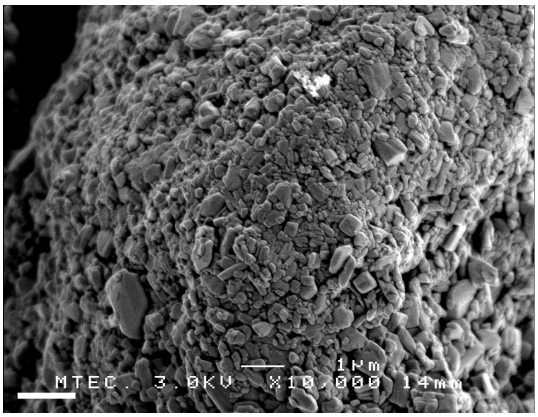
E 

Figure 4.6 The SEM images of INH-proliposome particles of Formulation No. 3 (A) to No.7 (E), respectively (magnification 10,000 X, bar = 1 μm).

4.3.2 Interaction of INH-proliposome powder and its physical properties

The amorphous characteristic of SPC is shown in Figure 4.7 which the intensity peaks due to SPC are at about 2θ of 20°. INH exhibited some degree of crystallinity before being spray dried but after spray dry INH exhibit lower diffraction intensity (Figure 4.8). This indicates that spray dry processes decrease the crystallinity of INH. XRD patterns of various proliposomes are also shown in Figure 4.8, which all exhibited changes of XRD pattern of INH and mannitol. This indicates the interaction of INH and mannitol which mannitol crystalline characteristic was dominated in Formulations No. 6 and No. 7. However, there were no amorphous characteristics of SPC appearing on all proliposome XRD patterns, this may due to the low concentration of SPC as compared to other components. As a result, SPC effects on XRD patterns of proliposome were unable to detect.

From the DSC thermogram (Figure 4.9) the onset, end, peak temperature and enthalpy of spray dried INH are INH-proliposome summarized in Table 4.2. Spray dried INH was in crystalline form with a lower endothermic peak temperature and enthalpy as compared with the starting INH. This result correlated with obtained XRD patterns (Figure 4.8). For proliposome powder; the interactions of INH-lipid-mannitol were depend on the formulation compositions. For example, Formulation No.3 contained INH and lipid, showed a single broad peak of 166.8 °C (7 degrees lower than spray dried INH). This endothermic peak was belonged to INH-lipid interaction. The inclusion of 20% mannitol (Formulation No.4) contributed to two completely separated endothermic peaks. First peak at 143.0 °C was related to INH-mannitol interaction. Second peak at 163 °C (3 degree lower than INH-lipid interaction) is expected to be complex interactions of INH and/or mannitol with lipid.

Increasing of mannitol to 40 and 60% in the spray dried formulation while decreasing INH to 60 and 40% (Formulations No. 5 and No. 6, respectively), two incomplete separated peaks were obtained at 143.0°C and 155.8 °C. This was expected to be from the complex interaction of three components (INH-lipid-mannitol) which a large enthalpy contributed to INH-mannitol-lipid interaction. However, when the INH content was lower to 20%, two completely was again appeared. The small endothermic peak (140 °C) of INH-mannitol interaction was obtained due to some content of INH was lost during spray drying processes (10% of INH was found in the products, see section 4.3.3). This may render to small peak of INH-mannitol interaction. However, the endothermic peak of 163.2 °C exhibited in the similar manner of Formulation No.3.

In overall, the maximum interaction of INH-lipid-mannitol was found with the Formulations No. 5 and No. 6.

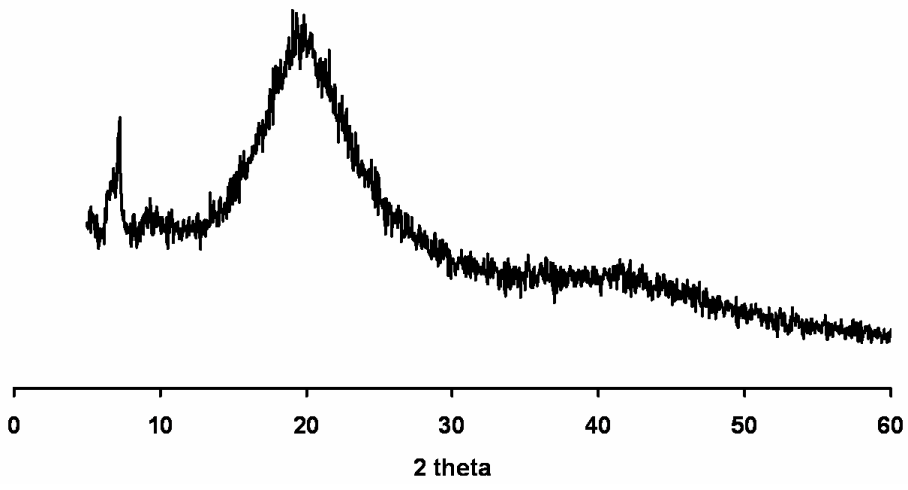


Figure 4.7 The XRD pattern of amorphous characteristic of soybean phosphatidylcholine (SPC)

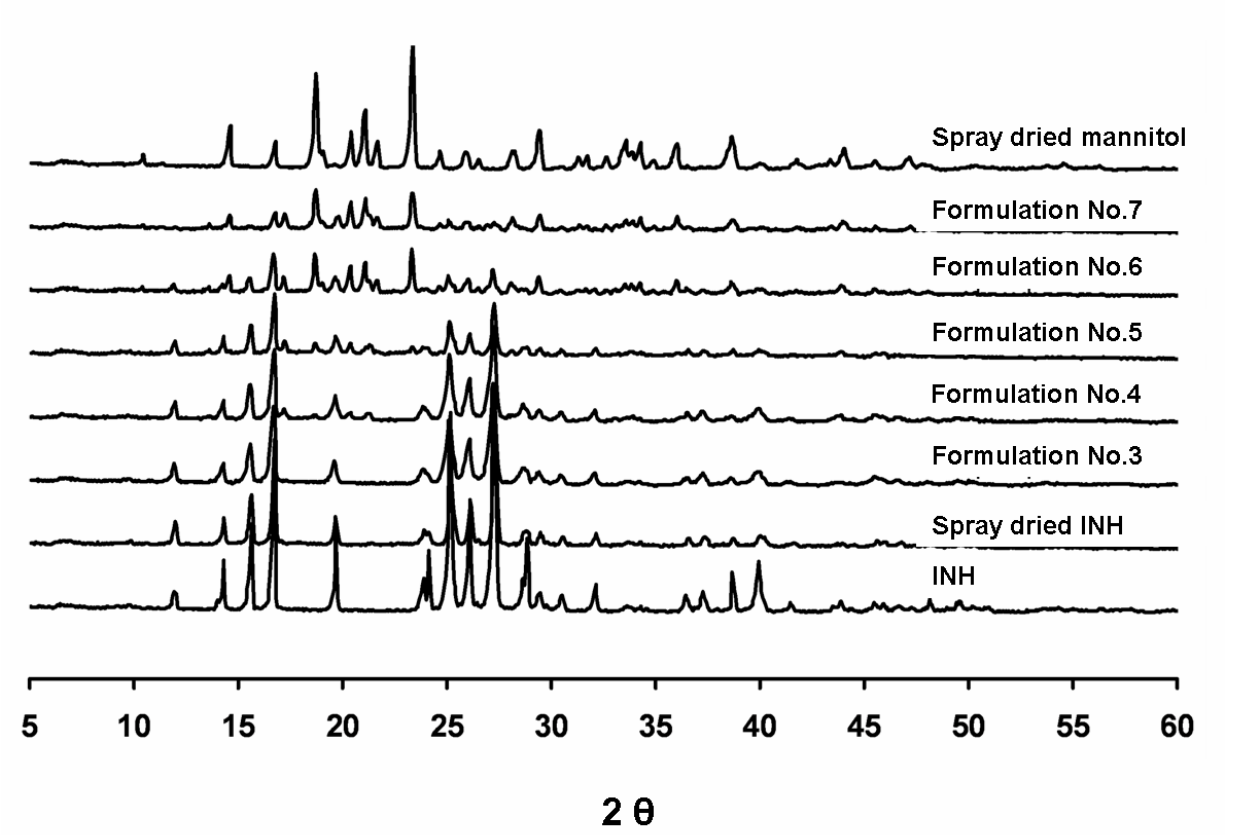


Figure 4.8 The XRD patterns of crystalline characteristics of INH, spray dried INH, spray dried mannitol and INH- proliposome powder Formulation No. 3-7.

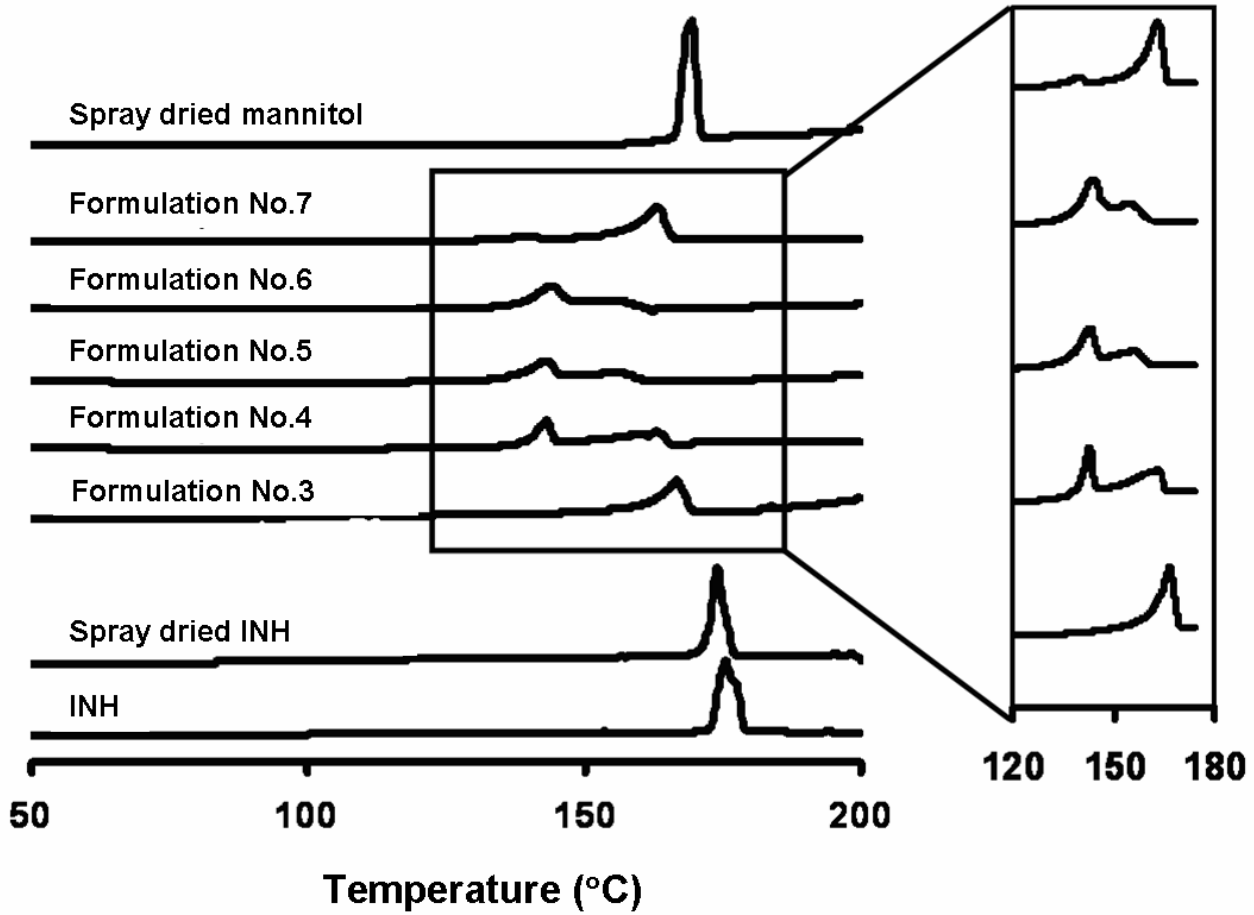


Figure 4.9 DSC thermogram of INH, spray dried INH, spray dried mannitol and INH- proliposome powder Formulation No. 3-7.

Table 4.2 DSC data of INH, spray dried INH, spray dried mannitol and INH-proliposome powder.

Formulation	Peak (°C)	Onset (°C)	End (°C)	Peak area (J/g)
INH	175.7	173.0	178. 8	223.1
Spray dried INH	174.0	172.0	176.4	218.9
INH Proliposome				
Formulation No.3	166.8	161.8	169.3	169.6
Formulation No.4	143.0	139.8	144.8	61.1
	163.0	149.7	165.3	82.3
Formulation No.5	143.0	137.2	160.8	192.6
	155.8			
Formulation No.6	143.5	138.6	161.1	208.7
	154.8			
Formulation No.7	140.0	134.0	142.5	15.6
	163.2	157.4	165.6	171.0
Spray dried mannitol	169.5	166.9	171.0	283.4

4.3.3 INH content uniformity and % encapsulation efficacy (% EE)

After the reconstitution of INH-proliposome, liposome vesicles were obtained in widely size distribution as shown in Figure 4.10. The reconstituted proliposome suspension contained vesicles varying from large size (10 µm) to the small nanometer size which are unable to observe by the light microscope. It is likely that the large size liposome formed under the relatively static condition, like *in situ* forming of liposome from reconstituted with the absence of any shear force condition.

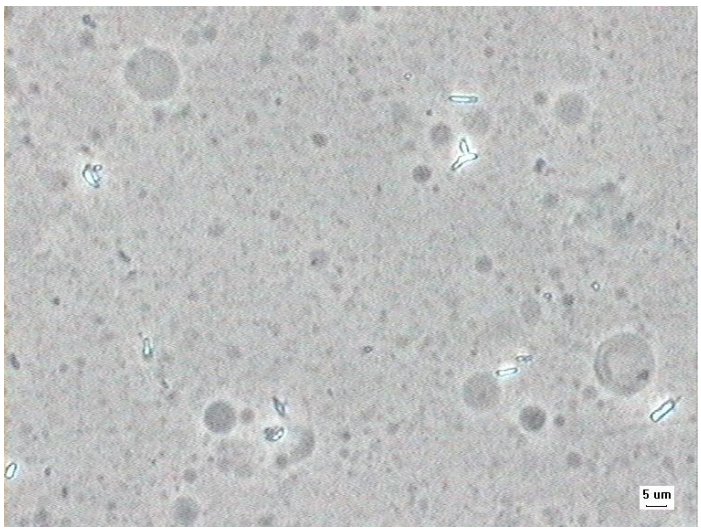


Figure 4.10 Digital image of liposome vesicle produced from reconstituted INH-proliposome powder (magnification 200 x, bar = 5µm)

The INH content and encapsulated INH in the proliposome powder is illustrated in Figure 4.11. The percentage of relative standard deviation (% RSD) of each formulation was used to assess the uniformity of drug content. %RSD less than 6 (USP 30 & NF 25, 2007) suggests that the spray drying processes are able to produce the uniform distribution of active ingredient throughout the proliposome product.

However, the percentage of recovering INH content in the formulation No. 6-7 was less than the INH content in the Formulations No. 3-5, which remained closely to 100%. From the observation, when INH content is lower than mannitol content in the formulation, significant lost of INH is clearly observed as shown in Figure 4.11. When INH was only 20 - 40%, INH may transform to amorphous state in a certain degree. The lower diffraction intensity peak at 2θ region of 23-30 obtained from Formulations No. 6 and 7 comparing to other formulations indicates lower crystallinity of INH. When such formulation was processed in spray dryer, the majority of INH was lost from melting and depositing on drying chamber of the equipment, thus low % recovery was obtained.

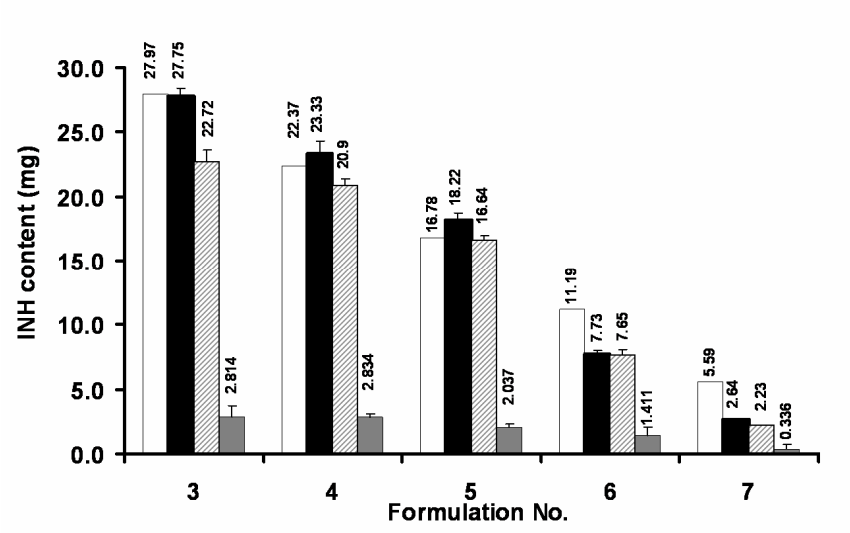


Figure 4.11 The INH theoretical content (□), INH recovering content after spray drying process (■) and after storage of 6 months (▨) and content of encapsulated INH (▩) in proliposome powder (mean ± SD), n ≥10.

However, at higher concentration of INH (from 60% or higher in the formulation), the DSC and XRD reveal higher degree of crystallinity of INH (Figure

4.8) as compared to those in Formulations No. 6 and 7 therefore such loss of INH during spray drying may not be occurred. The INH content was significantly decreased in Formulation No. 3-5 after being kept in the desiccator for 6 months (p-value < 0.05) whereas that of the formulations containing lower content of INH (Formulations No. 6-7) was not significantly decreased (p-value > 0.05).

The encapsulation efficacy of INH was 10-18 % of the recovering INH. A about 2 mg INH was encapsulated in Formulation No. 3-5 whereas only 1.41 and 0.3 mg were incorporated in Formulation No. 6-7 (Figure 4.11) due to the low INH content in these two formulations. These indicate the low INH loading in the formulation resulted in the low amount INH encapsulated. However, the method used to evaluate encapsulated INH amount is still questionable. As INH molecules are easily leaked from the liposome membrane during dialysis, this method is considered to be inappropriate. In this study, the membrane filter of 0.10 μm is expected to remove liposome vesicles larger than 100 nm while free INH passes through the membrane pore. Therefore, some small liposome encapsulated INH (< 100 nm) may contain in the filtrate portion which may distort the percent encapsulation of INH. However, the results in this study rather show the minimum encapsulated INH. Alternative method in encapsulation efficacy analysis may be applied such as ultracentrifugation for appropriate force and time. This point may need to be confirmed in the future.

4.3.4 Aerosolised performance of the pro-liposome powder

The aerosolized characteristics of INH proliposome powder are shown in Figure 4.12A for MMAD and 4.12B for % ED and % FPF. Different formulation of INH-proliposome produced by spray dried technique gave the insignificantly different MMAD (7-8 μm, *p*-value =0.165). This was correlated with the low FPF of 11-19 % (*p*-value =0.41) which is not significant different between each formulation. The 85-93 of % ED was obtained with the highest value of 93.50 from Formulation No 3 (*p*-value = 0.023). However, the MMAD should be less than 5 μm which is able to reach to the lower part of respiratory tract. The use of microparticulate mannitol as a core carrier, in this case, does not improve the aerosolized characteristics of the INH-proliposome powder. Since all aerosol parameters (MMAD and FPF) were not a satisfied value, either the composition or operating condition of spray drying must be optimized. The large MMAD perhaps resulted from INH itself and agglomeration of microparticulate mannitol from SPC coating material. Pure INH cannot be spray dried to obtain the perfect spherical of the particle having diameter less than 5 μm based on conditions in this study. SPC was mainly deposited on the surface of the microparticulate mannitol particles which enhance particle agglomeration (Payne *et al.*, 1986). In order to overcome this problem, microporous particles should be used instead of smooth surface microparticulate particle. These types of particle allow lipid to deposit in the porous structure and render less lipid surface contact with other particles resulting in lower agglomeration (Payne *et al.*, 1986). Using of microporous particles, more lipid content may be applicable in order to maximize encapsulation. In addition, microporous particle contain high void space, the particle density is low then enhance the aerosol performance (Chan, 2006).

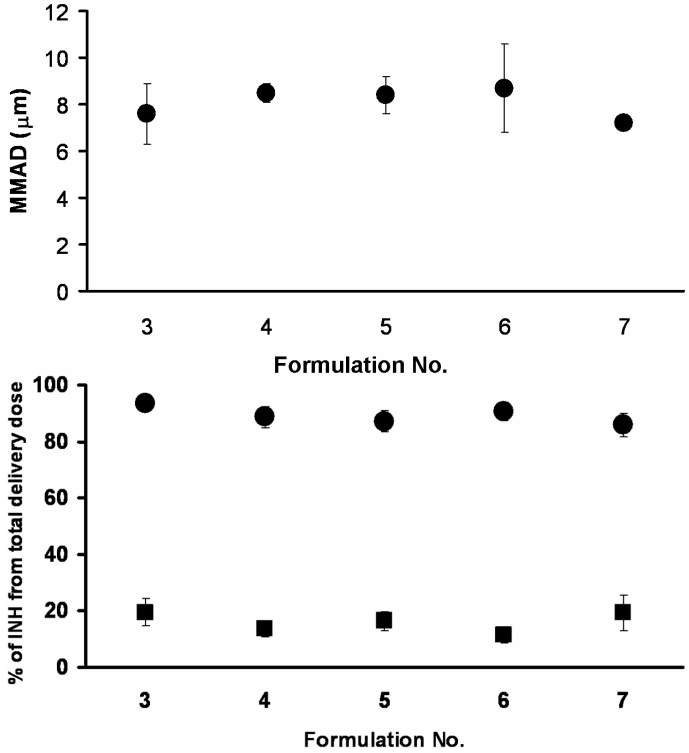


Figure 4.12 Aerosolised characteristics of INH-proliposome powder including (A): MMAD and (B); % FPF (■) and % ED (●)

4.3.5 Effect of INH proliposome on viability of the respiratory tract cell lines

The viabilities of NHBE, SAEC and AMs were estimated after challenging with different concentration of free INH and INH-proliposome (Formulation No. 3). Both free and INH-proliposome formulations are likely to be safe to deliver to the respiratory tract at the concentration less than 2.5 mg/mL (Figure 4.13) which almost 100 % viability of AMs were obtained. Viability of AMs cell was decreased when high concentrations of 2.5 and 5 mg/mL of INH formulation was added, for example only 40% viability was obtained for INH concentration of 5 mg/mL. The % viability of AMs is not significantly different between free INH and

INH-proliposome (*p*- value > 0.05). However, for respiratory epithelial cell line both SAEC and NHBE were likely to be more sensitive to INH induced toxicity since all the concentration of free INH and INH-proliposome Formulation No. 3 reduced the viability of both cells to 70%. The viability of 100% was obtained with the INH-proliposome Formulation No. 7 which contain the lowest amount of INH (10% INH content compared to the Formulation No. 3).

However, this toxic concentration (2.5 mg/mL) which is unlikely to occur in the respiratory tract following inhalation administration is 5,000 times higher than the MIC of INH against the TB bacilli as will be discussed later.

4.3.6 Effect of INH proliposome on production of IL-1β, TNF- α and nitric oxide by AMs

As detailed in section 3.3.8 that liposome may be recognized by AMs to produce the immunological response, for safety reason, level of inflammatory cytokines was examined from cultured supernatant of AMs after they were exposured to the proliposome for 24 h.

As it is expected, AMs did not produce IL-1β, TNF-α and nitric oxide responding to INH-proliposome and free INH, compared to LPS (lipopolysaccharaide) from *E. coli* as shown in Figure 4.14A-C, respectively. The concentration of LPS used to stimulate AMs to produce the immunological response was less than the concentration of INH-proliposome sample for 10,000 times. LPS activated AMs to produce inflammatory cytokines significantly higher than INH-proliposome (*p*-value < 0.05). The results correlated with the section 3.3.8 since the product contains the same ingredients, including SPC, CH and mannitol, except for INH.

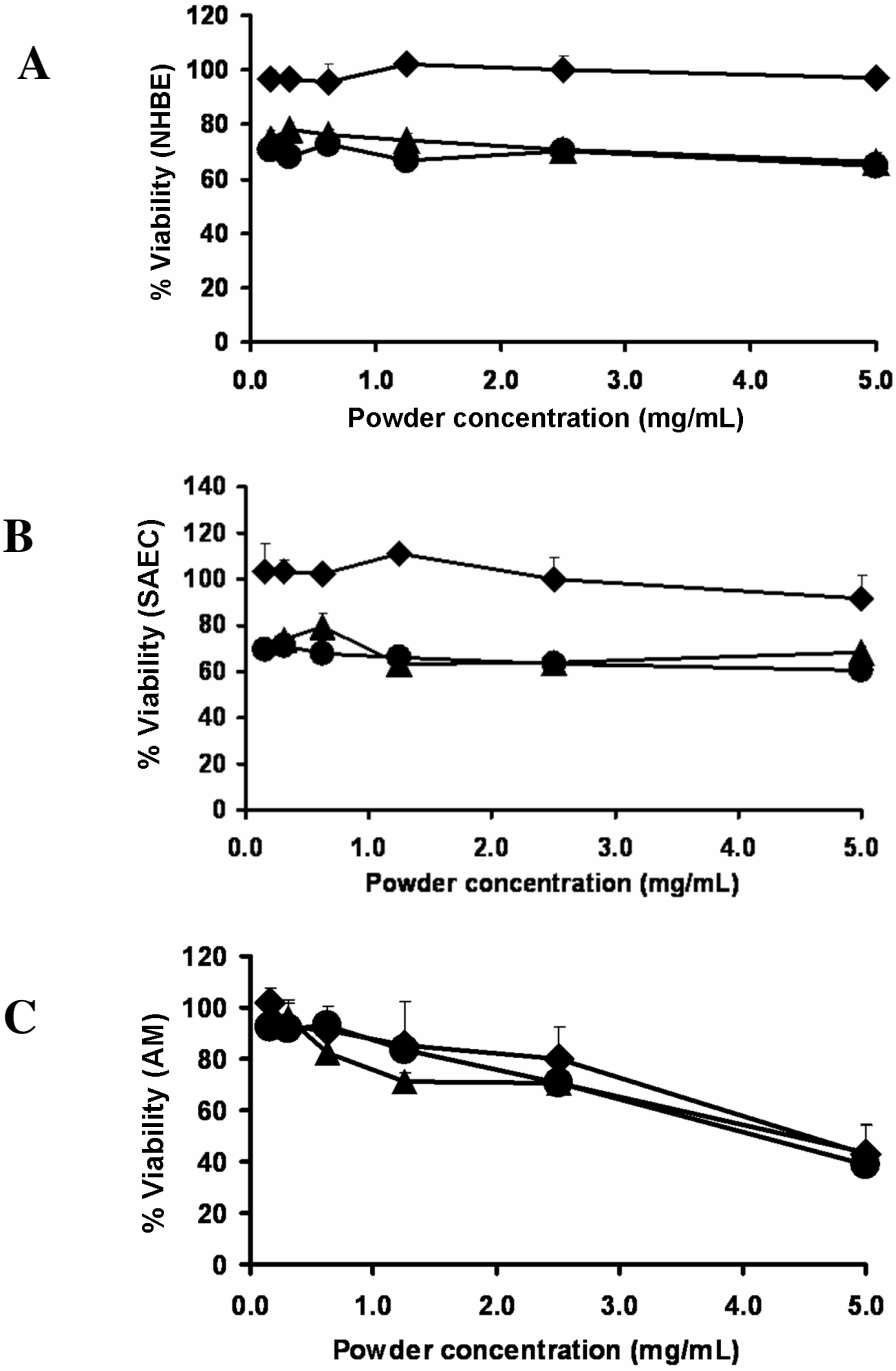


Figure 4.13 Viability of NHBE (A), SAEC (B) and AM (C) cell lines after exposed with different concentrations of free INH (●), INH-proliposome Formulation No. 3 (▲), INH proliposome Formulation No. 7 (◆) (mean ± SD, $n \geq 6$).

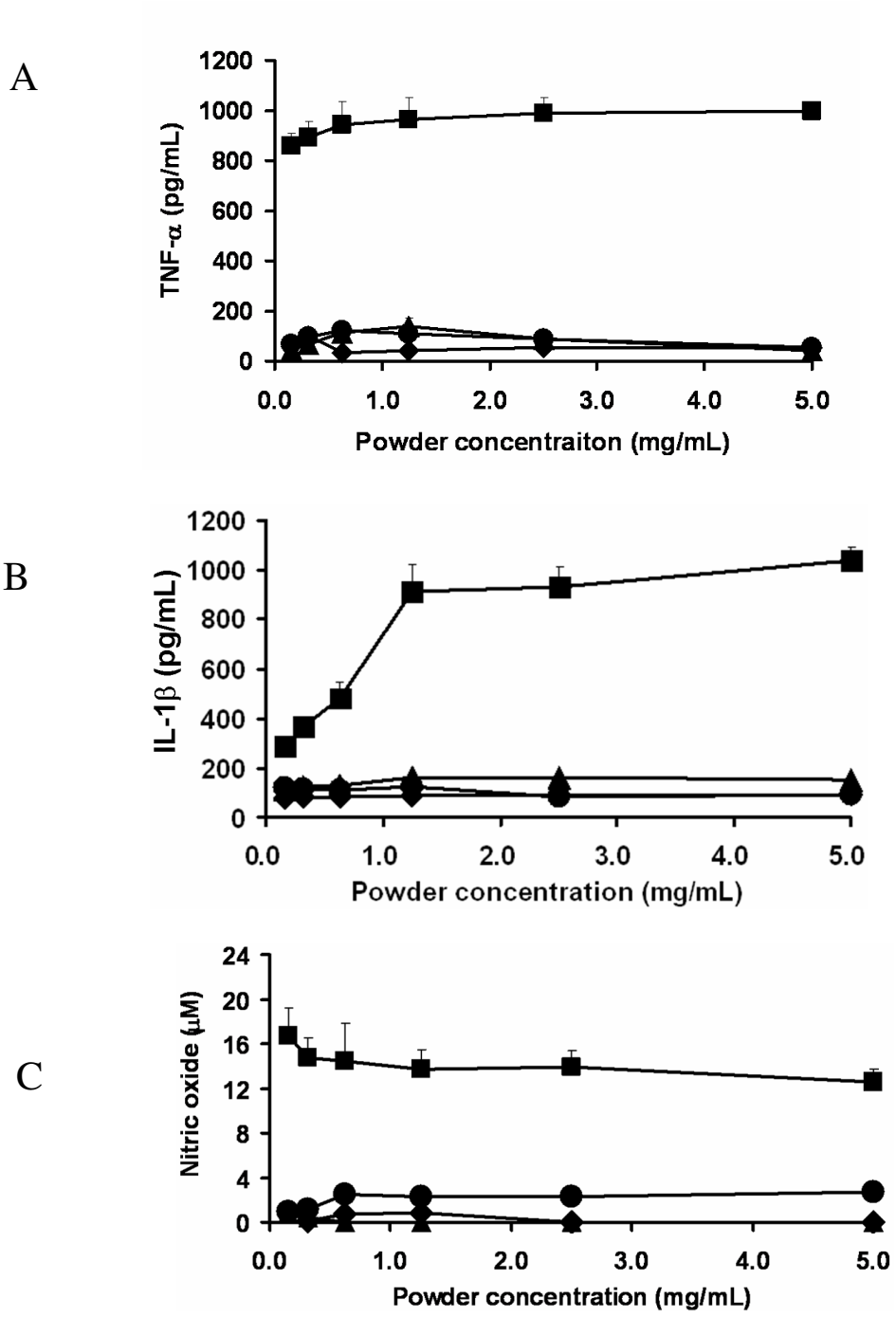


Figure 4.14 The level of inflammatory cytokine (A: IL-1β, B: TNF-α) and C: nitric oxide produced from AMs after exposure with free INH (●), INH-proliposome Formulation No. 3 (▲), INH-proliposome Formulation No 7 (◆) and LPS from *E. coli* (■) for 24 h (mean ± SD, n ≥ 6).

4.3.7 Activity of INH and INH-proliposome against *Mycobacterium bovis*

The remaining colony forming unit of *M. bovis* after incubation with samples of INH and INH-proliposome at various times and concentrations are shown in Figure 4.15. The free INH and INH-proliposome showed similar activity against *M. bovis*. At first day, the drug cannot reduce the number of *M. bovis* at all concentrations. However, the CFU was completely cleared with the INH concentration higher than 0.4 μg/mL at day 3 (both free INH and INH-proliposome). In addition, at day 7, there were no viable cells when *M. bovis* was challenged with all concentration of INH and INH-proliposome. From the results, INH and INH-proliposome was able to kill the bacilli after 3rd day of incubation and the most effective was found in the day 7. The MIC of INH and INH-proliposome for 3rd day was 0.4 μg/mL while the MIC at the day 7 was less than 0.2 μg/mL.

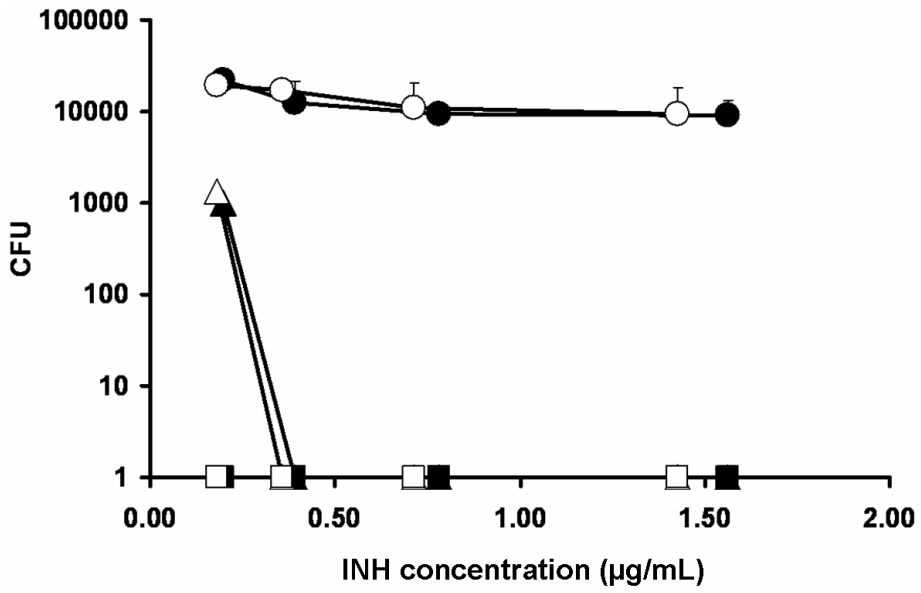


Figure 4.15 The reduction of *M. bovis* CFU after incubated free INH (dark symbol) and INH-proliposome (Formulation No. 3, blank symbol) at day 1 (●), day 3 (▲) and day 7 (■) (Mean ± SD, n ≥ 3).

4.3.8 Activity of INH and INH proliposome against *M. tuberculosis*

The same MIC values of free INH and INH-proliposome against *M. tuberculosis* were obtained at the concentration of 0.024 μg/mL. This indicated that INH and INH-proliposome is able to kill the bacilli at the low concentration which the MIC was lower than that reported by Mohamed *et al*. (2004) for 2 times and Chanwong *et al*. (2004) 16.7 times. However, this may due to the different method and condition using to determine the MIC. However, the most important point is free INH and INH-proliposome exhibit the efficacy against the TB bacilli in the same manner.

4.3.9 Activity of INH and INH-proliposome on intracellular growth of *M. bovis*

The activity of free INH and INH-proliposome against *M. bovis* infected alveolar macrophage intracellularly was examined for various incubation times and concentrations. Based on the result of the previous section, MIC of INH against *M. bovis* at day 3 was 0.4 μg/mL. The concentrations of 12.5, 25 and 37.5 times of the MICs were used to treat AMs infected *M. bovis*. Based on such calculation, the final concentration of INH (or equivalent in case of proliposome formulation) would be 5, 10 and 15 μg/mL in the cell culture media.

As results shown in Figure 4.16, at the highest concentration of 15 μg/mL, the CFU was completely cleared by both INH and INH-proliposome at day 3 and day 7. There were no significantly different in the forms of INH (free or INH proliposome) at these high concentrations. At 10 μg/mL, the bacilli were completely killed by day 7 and there was no significant difference from the formulation type. The formulation effects exhibited at the concentration of 5 μg/mL, which free INH was not

effective to kill the bacilli at either day 3 or day 7 whereas INH-proliposome showed the antimycobacterial against intracellular *M. bovis* after 7 days of incubation. This may be due to the hydrophilicity properties of INH which is difficult to penetrate to the AMs. However, when INH was incorporated into liposome, it was able to enhance permeation to obtain higher concentration intracellulary. Hence, high activity against *M. bovis* in AMs was obtained.

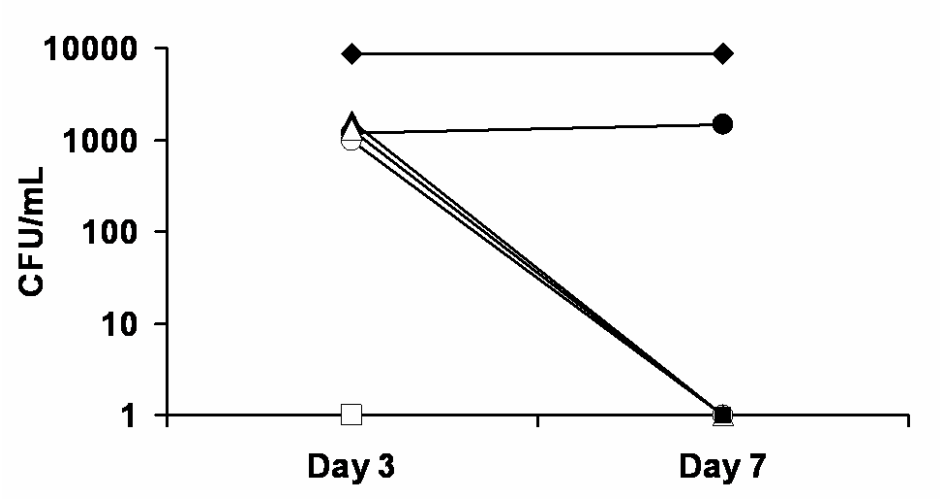


Figure 4.16 Activity of free INH (dark symbol) and INH-proliposome (blank symbol) against intracellular growth of *M. bovis* at concentrations of 5 µg/mL (●), 10 µg/mL (▲), 15 µg/mL (■) and control.

4.4 Conclusions

INH-proliposome is able to produce by the using of spray drying technique. The ratios of INH to mannitol of almost 1:1 (4:6 or 6:4 weight ratio) maximize the interaction of three components of INH-lipid-mannitol. The obtained particles showed high MMAD of 7-8 µm. With the same ingredients, a slight

modification of spray drying condition is required in order to obtain the microporous carrier particle. This may be useful to develop a proliposome powder as dry powder aerosol. INH and INH-proliposome are proved to be safe for respiratory associated cells since they did not induce the toxicity to the cells and did not activate AMs to produce the inflammatory cytokines and nitric oxide. The most important finding is INH-proliposome exhibited the better antimycobacterial activity against *M. bovis* infected AMs than free INH.

CHAPTER 5

CONCLUSIONS

Dry powder of liposomes and proliposomes were successfully prepared by freeze drying and spray drying method, respectively. Proliposome is still needed to further improve the aerosolized characteristics to decrease its MMAD. Both liposome-containing rifampicin and proliposome-containing isoniazid were evidently to be non-toxic to respiratory cell lines. The cytotoxicity examinations of respiratory associated cells exhibited high viability level upon they were exposed to liposomes or proliposomes containing antituberculosis drugs. In addition, alveolar macrophage cell lines were not stimulated to produce inflammatory cytokines upon challenging. Furthermore, it was clearly demonstrated that liposome containing antituberculosis targeted to macrophage could considerably increase the antitubercular activity of the agent compared to free drug. These are supporting evidences of the potential to develop antituberculosis encapsulated liposome dry powder as an alternative tuberculosis treatment and further progressing to clinical trial in phase I.

For future direction, the important points to be considered before transitioning to clinical trial phase I, are as following:

1) There are mixtures of rapidly growing organisms which are exponentially killed, as occurred in log phase cultures, together with organisms that

are metabolically less active and therefore killed less quickly as in stationary phase cultures in a tuberculosis lesion. For oral administration, if all lesional bacilli were rapidly growing, it would only take place about 14 days to sterilize lesions. Unfortunately, effective sterilization takes about 6 months because of the presence of persisting metabolically inactive bacilli (Mitchison, 1997). It is important to concern with the stationary phase tubercle bacilli when the duration of treatment of inhalable liposome containing antituberculosis being designed.

2) The combination of antituberculosis drug may be needed which the potent sterilizing drugs are rifampicin and pyrazinamide while isoniazid acts as the early bactericidal to obtain the sterile lesion (Mitchison, 1997). This may be needed combine activity even the drug are inhalation formulation.

3) The drug molecules should have high potency for the desired biological effect since an effect will be needed from a small fraction of the inhaled dose which may overcome by increasing the drug dose (free and encapsulated) (McCalden, 1990). The 67% of total rifampicin content in one dose of dry powder going to lower respiratory tract equivalent to 33.5 µg. Whereas the MIC of liposome dry powder against *M. tuberculosis* was 0.01 µg/mL. This indicates that 3,350 times rifampicin content higher than MIC in 1 mL. For proliposome, MIC of 0.02 µg/mL was obtained whereas 20% of total isoniazid (Pure INH proliposome formulation) content in one dose of dry powder going to lower respiratory tract equivalent to 5.5 mg. Thus, proliposome INH may potent enough to produce 230,000 times higher drug amount compared with MIC in 1 mL.

4) Another crucial factor is the treatment schedule, as it is a well-known fact that the development of resistance may occur when low dose of antimicrobial agent is administered over a long dosing interval. Some study indicated that the reduction in the level of infection was more dependent on the administration of dose than on the number of dose administered per week (Khuller *et al*, 2004).

5) Only pulmonary tuberculosis is expected to be treated effectively by inhalation route. Whereas other organs tuberculosis occurring from disseminated infection of tubercle bacilli via blood stream may need conjugated oral antituberculosis treatment. Lower oral dose of antituberculosis may be required in this case to minimize drug side effects.

Optimization of liposome formulation as well as regimens is needed to provide a therapeutic advantage of liposomal encapsulation of antituberculosis as an alternative tuberculosis treatment.

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APPENDICES

A. ELISA Test kit for rat IL-1β

Introduction

The interleukin 1 (IL-1) family of proteins consists of IL-1α, IL-1β and the IL-1 receptor antagonist. IL-1β and IL-1α bind to the same cell surface receptors and share biological functions. With the exception of skin keratinocytes, some epithelial cells, and certain cells of the central nervous system, IL-1 is not produced by unstimulated cells of healthy individuals. However, in response to stimuli such as those produced by inflammatory agents, infections, or microbial endotoxins, a dramatic increase in the production of IL-1 by macrophages and various other cell types is seen.

Instructions

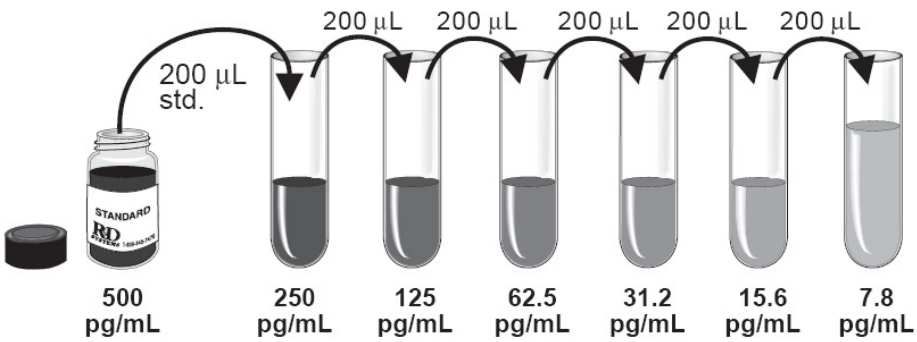
Reagent preparation *(Bring all reagents to room temperature before use).*

1. Rat IL-1β kit control: Reconstitute the kit control with 1.0 mL deionized or distilled water.
2. Rat IL-1β Conjugate: To prepare enough conjugate for 1 plate. Add 0.5 mL conjugate concentrate to 11.0 mL conjugate diluent. *Use sterile container & protect from light.*
3. Wash Buffer: To prepare enough wash buffer for 1 plate; add 25 mL wash buffer concentrate into deionized or distilled water to prepare 625 mL of wash buffer.

4. Substrate solution: Color reagents A and B should be mixed together in equal volumes within 15 minutes of use and protect from light. 100 µL of mixture is required per well.

5. Rat IL-1β Standard: Reconstitute the rat IL-1β standard with 2.0 mL of calibrator diluent RD5Y. This reconstitution produces a stock solution of 2000pg/mL). Allow the standard to sit for 5 min. with gentle mixing prior to making dilutions.

Use polypropylene tube. Pipette 200 µL of calibrator diluent RD5Y in each tube. Use stock solution to produce a dilution series (below).The rat IL-1β standard serves as the high standard (500 pg/mL). Calibrator diluent RD5Y serves as the zero standard (0 pg/mL).



Assay Procedure *(Bring all reagents to room temperature before use. Samples, standards and controls be assayed in duplicate)*

1. Prepare reagents, standard curve dilution, controls and samples as directed in the previous section.

2. Add 50 µL of assay diluent RD1-21 to the center of each well.

3. Add 50 µL of standards, controls or samples to the center of each well. Cover the plate with the adhesive trip. Mix by gently tapping the plate frame for 1 minute. Incubate for 2 h at room temperature.

4. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with wash buffer (400 µL) and completely remove of liquid at each step. After the last wash, remove any remaining wash buffer by aspiration/inverting the plate and blotting it against clean paper towel.

5. Add 100 µL of rat IL-1β conjugate to each well. Cover with a new adhesive strip. Incubate for 2 h at room temperature.

6. Repeat the aspiration/wash as in step 4.

7. Add 100 µL of substrate solution to each well *(prepare before use 15 minutes)* Incubate for 30 minutes at room temperature and protect from light.

8. Add 100 µL of stop solution to each well. Gently tap the plate to ensure through mixing.

9. Determine the optical density of each well within 30 min (at 450 nm or 570 nm)

B. ELISA kit Test (Rat TNF-α)

Introduction

Tumor necrosis factor alpha (TNF-α), also known as cachectin; and tumor necrosis factor beta (TNF-β), also known as lymphotoxin, are two closely related proteins (approximately 34% amino acid sequence identity) that bind to the same cell surface

receptors and show many common biological functions. TNF-α and TNF-β play critical roles in normal host resistance to infection and to the growth of malignant tumors, serving as immunostimulants and as mediators of the inflammatory response. Over-production of TNFs, however, has been implicated as playing a role in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders. TNF-α is produced by activated macrophages and other cell types including T and B cells, NK cells, LAK cells, atrocities, endothelial cells, smooth muscle cells and some tumor cells.

Instructions

Reagent preparation *(Bring all reagents to room temperature before use).*

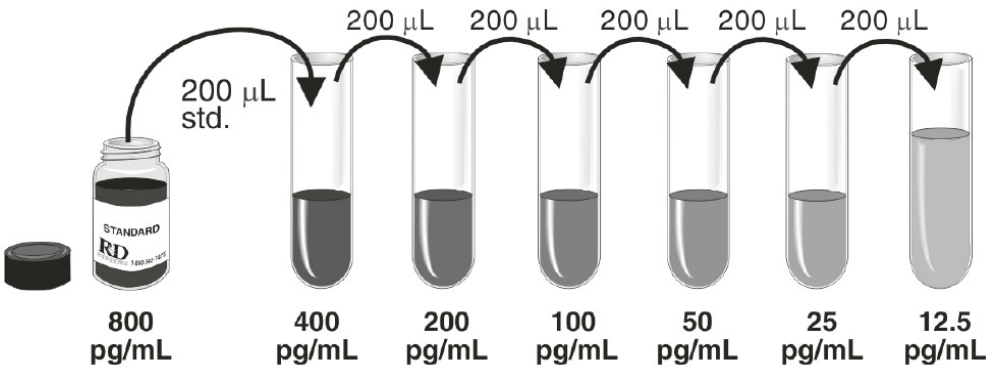
1. Rat TNF-α kit Control: Reconstitute the kit control with 1.0 mL deionized or distilled water. Assayed the control undiluted.

2. Wash Buffer: To prepare enough wash buffer for 1 plate; add 25 mL wash buffer concentrate into deionized or distilled water to prepare 625 mL of wash buffer.

3. Substrate solution: Color reagents A and B should be mixed together in equal volumes within 15 minutes of use and protect from light. 100 μL of mixture is required per well.

4. Rat TNF-α Standard: Reconstitute the rat TNF-α standard with 2.0 mL of calibrator diluent RD5-17. This reconstitution produces a stock solution of 800 pg/mL). Allow the standard to sit for 5 min. with gentle mixing prior to making dilutions.

Use polypropylene tube. Pipette 200 µL of calibrator diluent RD5-17 into each tube. Use stock solution to produce a dilution series (below).The undiluted rat TNF-α standard serves as the high standard (800 pg/mL). Calibrator diluent RD5-17 serves as the zero standard (0 pg/mL).



Assay Procedure *(Bring all reagents to room temperature before use. Samples, standards and controls be assayed in duplicate)*

1. Prepare reagents, standard curve dilution, controls and samples as directed in the previous.
2. Add 50 µL of assay diluent RD1-41 to the center of each well.
3. Add 50 µL of standards, controls or samples to the center of each well. Cover the plate with the adhesive trip. Mix by gently tapping the plate frame for 1 minute. Incubate for 2 h at room temperature.
4. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with wash buffer (400 µL) and completely remove of liquid at each step. After the last wash,

remove any remaining wash buffer by aspiration/inverting the plate and blotting it against clean paper towel.

5. Add 100 µL of rat TNF-α conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hrs at room temperature.

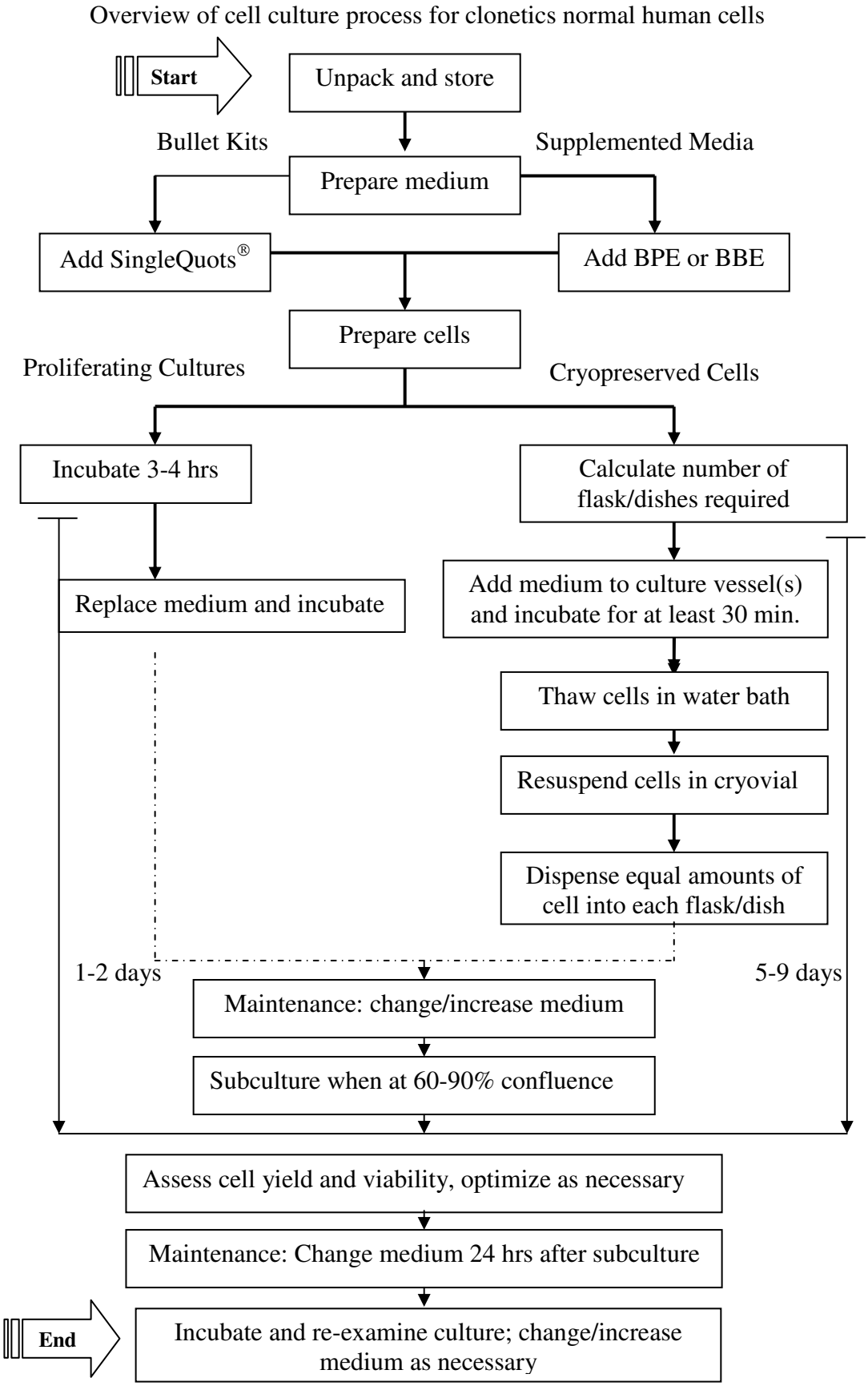
6. Repeat the aspiration/wash as in step 4.

7. Add 100 µL of substrate solution to each well *(prepare before use 15 minutes)*. Incubate for 30 minutes at room temperature and protect from light.

8. Add 100 µL stop solution to each well. Gently tap the plate to ensure through mixing.

9. Determine the optical density of each well with within 30 minutes (at 450 nm or 570 nm).

C. Clonetics™ Human and Animal Cell Systems



Storage requirements

Cell

Remove cryopreserved cells from dry ice and place immediately into liquid nitrogen. If no dry ice is left in the package, thaw cells, immediately place them into culture vessels.

Medium

Store clonetics cell culture medium in a 4 °C refrigerator. When using medium, take the amount you need under sterile conditions, and return bottle to the refrigerator. Always bring medium to room temperature before use.

Supplement and reagents

Store all growth supplements and reagents at -20 °C. Within 3 days before subculture store all growth supplements, HEPES buffered saline solution and trypsin neutralizing solution at 4°C. Trypsin/EDTA solution has a limited shelf life at 4 °C. If upon arrival, trypsin/EDTA is thawed, immediately aliquot and refreeze at -20 °C. If trypsin/EDTA is frozen, store at -20 °C.

Cell culture proceeding

1. To set up cultures, calculate the number of vessels needed about at 2,500 cells/cm^2. Add appropriate amount of medium to the vessels (1 mL/5 cm^2) and allow the culture vessels to warm and equilibrate in a 37°C, 5% CO_2, humidified incubator for at least 30 minutes.

2. Remove the cryovial containing cells from storage container. Wipe cryovial with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve the internal pressure, and then retighten. Do not open the cryovial completely.

3. Holding the cryovial, dip the bottom ¾ of the cryovial in a 37°C water bath, and swirl gently for 1-2 minutes until contents are thawed. Watch your cryovial closely; remove it when the last silver of ice melts. Do not submerge it completely. Thawing the cells longer than 3 minutes results in less optimal results.

4. Remove the cryovial immediately, wipe it dry, and transfer to a sterile field where the equilibrate vessels should be waiting, ready to seed. Rinse cryovial with 70% alcohol, and then wipe to remove excess.

5. Note the color of the thaw cryovial. Ideally, the color of the thawed cryovial should be pink. If the color is not pink, still seed the cells, note the color and mention this fact to your technical service specialist if seeding is not successful.

6. Remove the cap, being careful not to touch the interior threads with your fingers.

7. Using a micropipette set to 800 μL with a 1,000 μL tip, put the tip into the cryovial and resuspend the cells. Use a gentles, slow and steady up and down pipetting motion no more than 5 times. Do not resuspend quickly, and keep the tip near the bottom of the vial to avoid making bubble.

8. Dispense an equal amount of cells into the flask set up earlier. If four of T-75 flasks were prepared, set micropipette to 250 μL and dispense, if eight of T-25 flask were prepared, set micropipette to 125 μL and dispense, etc.

9. Replace the cap or cover, and gently rock the vessels to evenly distribute the cells. Loosen caps if necessary to permit gas exchange.

10. Return the culture vessels to 37°C, 5% CO_2 incubator. Lay them flat on the shelf, providing the largest surface for cells to attach. The cells will anchor to the bottom surface of the flask.

11. Change the growth medium the day after seeding (to remove residue DMSO and unattached cells), the every other day thereafter. Examine cells daily.

12. Successfully recovered cultures will exhibit the following:

    a. Cells with clear non-granular cytoplasm.

    b. Numerous mitotic figure after day 2.

Cell counting using a hemacytometer

Proper use of a hemacytometer is critical for obtaining and accurate count of cells.

Procedure

1. Prepare a cell suspension in growth medium.

2. Prepare a hemacytometer for use.

    a. Carefully clean all surfaces of the hemacytometer and coverslip.

b. Take care to ensure that all surfaces are completely dry using non-linting tissue.

c. Enter the coverslip on the hemacytometer.

3. Pipet approximately 9 µl (this volume will vary with the brand of hemacytometer) of the cell suspension into one of the two counting chambers.

a. Use a clean pipette tip.

b. Be sure that the suspension is thoroughly, but gently, mixed before drawing the sample.

c. Fill the chambers slowly and steadily.

d. Avoid injection bubble into the chambers.

e. Do not overfill or under fill the chamber.

4. Count the cells.

a. Allow the cell suspension to settle for at least 10 seconds.

b. Count all cells in each of the four 1 mm^3 corner squares.

c. Do count the cells touching the top or left borders.

d. Do not count the cells touching the bottom or right borders.

5. Determine the cell count.

a. Calculate the total cells counted in the four corner squares.

b. If the total cell count is less than 100, or if more than 10% of the cells counted appear to be clustered, carefully remix the original cell suspension and repeat steps 2 through 4 above.

c. If the total cell count is greater than 400, dilute the suspension so the count will be 100-400 cells. Then repeat steps 2 through 4 above.

6. Calculate the cell count using the equation:

$$\text{cells/mL} = (n) \times 10^4$$

(n = the average cell count per square of four corner squares counted)

Example: If the calculated average (n) of the cells in the four 1 mm corner squares of the hemacytometer is 30:

$$\text{cells/mL} = (n) \times 10^4$$
$$= 30 \times 10{,}000$$
$$= 300{,}000 \text{ cells/mL}$$

7. Determine the total number of cells in the total suspension volume.

a. Determine the total volume of the cell suspension.

b. Multiply the volume of the cell suspension by the cells/mL value calculated above.

Example: If the initial suspension volume is 2 mL:

cells/mL x total volume = 300,000 cells/mL x 2mL = 600,000 cells

Assessment of cell viability with trypan blue

Trypan blue is a dye that enables easy identification of dead cells. Dead cells take up the dye and appear blue with uneven cell membranes. By contrast, living cells repel the dye and appear retractile and colorless. Evaluate on bright field and not phase.

Procedure

1. Prepare the hemacytometer for use.

   Carefully clean all surfaces of the hemacytometer and coverslip.

2. Stain the cells

   a. Transfer 50 μL of 0.4% Trypan blue into a clean tube.

   b. Add 50 μL of the prepared cell suspension into the tube containing the stain.

   c. Mix the solution thoroughly, but gently. Take care to avoid making excessive bubbles.

   d. Allow the mixture to sit for 2-3 minutes after mixing.

Note: Do not left the cells sit in the dye for more than 5 minutes because both the living and dead cells will begin to take-up the dye after 5 minutes.

3. Pipet approximately 9μl of the Trypan blue/cell suspension mixture (this volume will vary with brand of hemacytometer) into one of the two counting chambers.

   a. Use a clean pipette tip.

   b. Be sure that the suspension is thoroughly, but gently, mixed before drawing the sample.

   c. Fill the chambers slowly and steadily.

   d. Avoid injection bubble into the chambers.

   e. Do not overfill or under fill the chamber.

4. Determine cell viability

a. Allow the cell suspension to settle in the chambers for at least 10 seconds.

b. Count all the stained cells in each of the four corner squares of hemacytometer.

c. Separately count all of the unstained cells in the same squares.

%Cell Viability = (number of unstained cells/Total cells counted) x 100%

Example: If a total of 300 cells (stained + unstained) are counted and 200 are identified as living cells (unstained), then the viability is calculated as:

% Cell Viability = (200 live cells/ 300 total cells) x 100%

= 67%

Subculture Preparation

Note: The following instructions are for subculturing a 25 cm^2 flask. Adjust all volumes accordingly for other size flasks.

Preparation for subculturing the first flask

1. Subculture the cell when they are 60-90 % confluent and contain at least five mitotic figures per field at 100X.
2. For each 25 cm^2 of cells to be subcultured, allow 2 mL of Trypsin/EDTA to thaw and come to room temperature.
3. For each 25 cm^2 of cells to subcultured, allow 5 mL of HEPES buffered saline solution (HEPES-BSS) to come to room temperature.
4. For each 25 cm^2 of cells to subcultured, allow 4 mL of Trypsin neutralizing solution (TNS) to come to room temperature.
5. Prepare new culture vessels.
    a. Prepare five to ten T-75 flasks. The number of flasks needed depends upon confluence and total yield. Larger flasks may be used to save plasticware and time spent on subsequent subcultures. Smaller flasks reduce the risk of losing a substantial part of your culture.
    b. As before, label each flask with the passage number, strain number, cell type, and date.
    c. In a sterile field, carefully open the bottle and transfer growth medium to new culture vessels by adding 1 mL growth medium for every 5 cm^2 surface area of the flask.

d. Example: 15 mL growth medium for a 75 cm^2 flask.

6. If not using vented caps, loosen caps of flask.

7. Place the new culture vessels into 37°C humidified incubator with 5% CO_2 and equilibrate the flask at least 30 minutes.

8. Subculture one flask at a time. All flasks following the first flask will be subcultured following an optimization of this protocol, based on calculated cell count and cell viability.

Subculturing cells

1. Aspirate the medium from one culture cell.

2. Rinse the cells with 2-3 mL of room temperature HEPES-BSS. Do not forget this step. The medium contains proteins that neutralize the trypsin, making it ineffective.

3. Aspirate the HEPES-BEE from the flask.

4. Repeat this rinse to completely remove serum and protein from the cell monolayer

5. Cover the cells with 2 mL of Trypsin/EDTA solution.

6. Rock the flask to make sure all cells come into contact with the trypsin.

7. Tighten the cap and begin monitoring the flask under the microscope.

8. Continue to examine the cell layer microscopically.

9. Allow the trypsinization to continue until ≈ 90% of the cells are rounded up.

Note: Rounded up cells are spherical, have smooth edges and are refractile or shiny. If the cells still have protruding nubs, they need more time to trypsin. This entire process takes about 5-6 minutes.

10. At this point, rap the flask against the palm of your hand to release the majority of cells from the culture surface. If only a few cells detach, wait and rap again. If cells still do not detach, wait and rap every 30 seconds thereafter.

Note: do not try to get all cells to detach by rapping them severely. This action may damage the cells.

11. After cells are release, neutralized the trypsin in the flask with 2 mL of room temperature TNS.

12. Quickly transfer the detached cells to a sterile 15 mL centrifuge tube.

13. Rinse the flask with a final 2 mL HEPES-BSS to collect residue cells, and add this rinse to the centrifuge tube.

14. Examine the harvested flask under the microscope to make sure the harvest was successful by looking at the number of cells left behind. This should be less than 5%

15. Centrifuge the harvested cells at 220 g for 5 minutes to pellet the cells.

a. Aspirate most of the supernatant, except for 100-200 μl.

b. Flick the centrifuge tube with your finger to loosen the pellet.

16. Dilute the cells in 4-5 mL of growth medium and note the total volume of the diluted cell suspension.

Note: To obtain the best results from your cells, assess cell yield and avibility with Trypan blue.

17. Count the cell with a hemacytometer or cell counter and calculate the total number of cells. Make a note of your cell yield for later use.

Note: The cells suspension shoul contain between 250,000-1,000,000 cells/mL for greatest accuracy.

18. Prepare flask by labeling each flask with the passage number, cell type and date.

19. Carefully open the medium bottle and transfer growth medium to new culture vessels by adding 1 mL growth medium for every 5 cm^2 surface area of the flask (1 mL /5 cm^2).

20. After mixing the diluted cells with 5 mL pipet to ensure a uniform suspension, dispense the volume of suspension calculated into the prepared subculture flasks.

21. After dispensing the cells, gently rock flask to promote even distributuion.

22. If not using vented caps, loosen caps of flasks. Place the new culture vessels into 37°C humidified incubator with 5% CO_2.

Maintenance after subculturing

1. After 24 hrs, examine the cells under the microscope. At least 30% of the cell should have attached to the culture flask. Some cells will be loosely adherent, but most will have spread out on the culture

flask surface. At this stage, most cells will be single or in small colonies.

2. Change the culture medium to remove residual trypsin and non-attached cells.

3. Incubate for an additional 24 hrs, and re-examine the culture.

   a. At this stage, the vessels should have several mitotic figures indicating that the cells have resumed active growth.

   b. If few mitotic figures are observed, contact technical service for assistance.

4. Change the medium again 48 hrs after the day 1 feeding, and every 48 hrs thereafter. Examine the culture daily.

5. Passage again when cells reach. This stage should be taken from 4-10 days.

Instructions for cryopreservation

Cryopresered media: 80% Clonetic (SAGM, BEGM) growth medium, 10% FBS, 10% DMSO

1. Sterile filter freezing media using any 0.2 μm filter.

2. Harvest cells and centrifuge to collect cells into a pellet.

3. Resuspend cells in cold freezing solution at 500,000-2,000,000 cells/mL. Work quickly once exposed to DMSO, cells become very fragile.

4. Pipet aliquot (1 mL each) into freezing vials or ampoules and seal.

5. Insulate aliquots with a Styrofoam or propanol freezing canister.

6. Store cells at -70°C overnight.

7. Within 12-24 h, place in liquid nitrogen for long-term storage (-200 °C). Cells will be compromised by storage in -70 °C.

I. Clonetics™ small airway epithelial cell systems (SAEC)

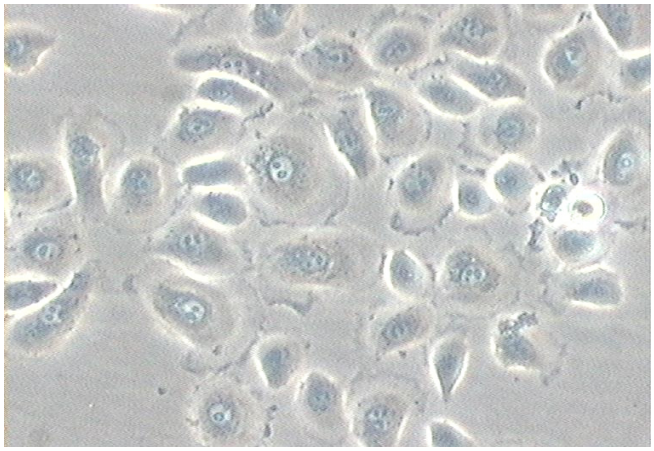
Clonetics SAEC systems contain normal human small airway epithelial cells from the distal airspace and optimized media for their growth. Each system can quickly generate SAEC cultures for experimental applications in asthma, inhalation toxicology and pulmonary inflammatory response.

Cell system components

- Cryopreserved small airway epithelial cell
- Small airway epithelial cell medium BulletKit® 500 mL: Clonetics SAGM BulletKit® contains one 500 mL bottle of small airway epithelial cell basal medium (SABM) and following growth supplements: Hydrocortisone 0.5 mL, Triiodothyronine 0.5 mL, insulin 0.5 mL, Transferrin 0.5 mL, GA-1000 0.5 mL, Retinoic acid 0.5 mL, hEGF 0.5 mL, BPE 2 mL, Epinephrine 0.5 mL , and BSA-FAF 5.0 mL.
- ReagentPack™, containing: Trypsin 0.025%/EDTA 0.01% 100 mL, Trypsin neutralizing solution (TNS) 100 mL and HEPES buffered saline solution (HEPES-BSS) 100 mL.

Characterization of cells

Routine characterization of SAEC includes positive staining for cytokeratin 19 and morphological observation throughout serial passage.



Performance

Recommended seeding density for subculture	2,500 cells/cm^2
Typical time from subculture to confluent monolayer	5-9 days
Additional population doublings guaranteed using clonetics system	15

II Clonetics™ bronchial/tracheal epithelial cell systems (NHBE)

Clonetics™ bronchial/tracheal epithelial cell systems contain normal bronchial/tracheal epithelial cell (NHBE) and optimized media for their growth. Each system can quickly generate NHBE cultures for experimental applications in cancer research, respiratory disease, cellular function and differentiation.

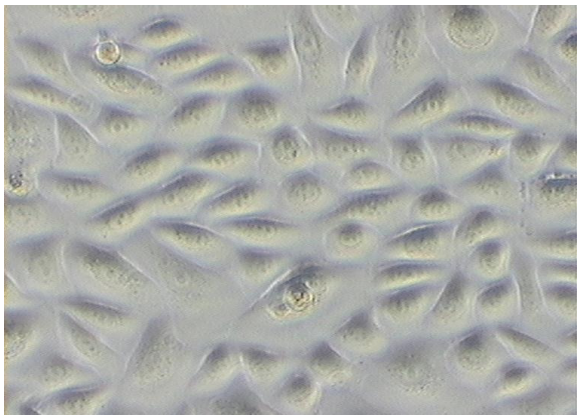
Cell system components

- Cryopreserved bronchial/tracheal epithelial cell

- Bronchial epithelial cell medium BulletKit® 500 mL, Clonetics BEGM BulletKit® contains one 500 mL bottle of bronchial epithelial cell basal medium (BEBM) and following growth supplements: Hydrocortisone 0.5 mL, Triiodothyronine 0.5 mL, insulin 0.5 mL, Transferrin o.5 mL, GA-1000 0.5 mL, Retinoic acid 0.5 mL, hEGF 0.5 mL, BPE 2 mL and Epinephrine 0.5 mL.
- ReagentPack™, containing: Trypsin 0.025%/EDTA 0.01% 100 mL, Trypsin neutralizing solution (TNS) 100 mL and HEPES buffered saline solution (HEPES-BSS) 100 mL.

Characterization of cells

Routine characterization of NHBE cells morphological observation throughout serial passage.



Performance

Recommended seeding density for subculture	3,500 cells/cm^2
Typical time from subculture to confluent monolayer	6-9 days
Additional population doublings guaranteed using clonetics system	15

D. Alveolar Macrophage cell line (NR 8383, ATCC 2192)

NR8383 (normal rat, August 3, 1983) was established from normal rat alveolar macrophage cells obtained by lung lavage. The cells were cultured in the presence of gerbil lung cell conditioned medium for approximately 8 to 9 months. Subsequently the requirement for exogenous growth factors was lost. NR8383 cells were cloned and subcloned from single cells by limiting dilution, and then subcloned from soft agar three times. The cells exhibit characteristics of macrophage cells: Phagocytosis of zymosan and Pseudomonas aeruginosa, nonspecific esterase activity, Fc receptors, oxidative burst, IL-1, TNF beta and IL-6 secretion, and replicative response to exogenous growth factors. The cells respond to appropriate microbial, particulate or soluble stimuli with phagocytosis and killing. NR8383 cells respond to bleomycin by secreting latent transforming growth factor (TGF beta). Stimulation with bleomycin also increases TGF beta mRNA expression. These cells are sensitive to endotoxin. LPS levels of 1-10 ng/mL inhibit replication by 50%. LPS inhibition is nontoxic and reversible even after levels up to 0.001 mg/mL for extended periods. The NR8383 cell line provides a homogenous source of highly responsive alveolar macarophages which can be used in vitro to study macrophage related activities.

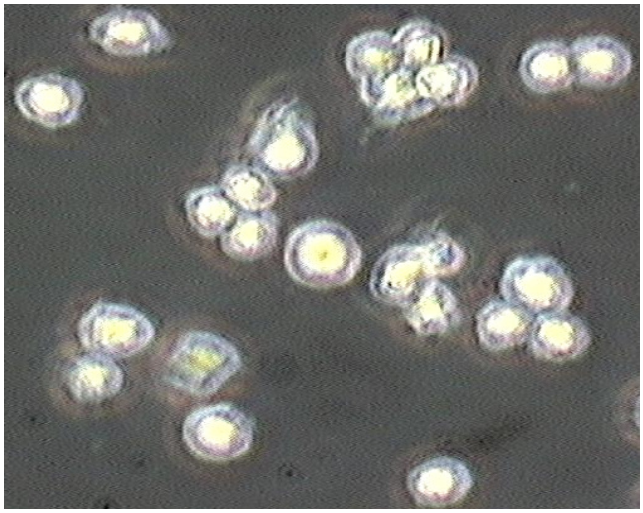
Propagation: ATCC complete growth medium**:** Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 85%; heat inactivated fetal bovine serum, 15% incubated at 37°C.

Subculturing: Cultures can be maintained by transferring floating cells to additional flasks. Adherent cells may be harvested by scraping. Upon reseeding, about one half of the cells will re-attach. Cultures are most successful when set up at a floating cell concentration of 1-4 x 10^5 viable cells.

Medium Renewal: 2-3 times weekly

Preservation: Freeze medium; Complete growth medium, 95%; DMSO, 5%

Storage temperature: liquid nitrogen vapor phase



VITAE

Name Miss Narumon Changsan

Student ID 4653002

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Pharmacy	Prince of Songkla University	2001

Scholarship Award during Enrollment

Royal Golgen Jubilee Ph.D Program, Grant No. PHD/0119/2546, Thailand Research Fund, 2003-2007.

List of Publications and Proceedings

1. Narumon Changsan and Teerapol Srichana. 2005. Rifampicin encapsulated liposomes as dry powder inhaler. Proceeding of Pharma Indochina IV: Pharmacy in coorperation for development and integration. 301-311.
2. Narumon Changsan and Teerapol Srichana. 2007. Factor influencing the properties of rifampicin liposome and application for dry powder inhaler. Proceeding of the 2nd IEEE International conference on Nano/Micro engineered and molecular systems. 1104-1109 (available online IEEE explore and Scopus).
3. Narumon Changsan, Athip Nilkaeo, Pethchawan Pungrassamee, and Teerapol Srichana. 2007. Bioactivity of rifampicin encapsulated liposome

delivery to the respiratory tract. Proceeding of German-Thai Symposium on Nanoscience and Nanotechnology. 275-280.

4. Narumon Changsan, Hak-Kim Chan and Teerapol Srichana. 2007. Isoniazid proliposome for dry powder aerosols and the immunological response of alveolar macrophage. Proceeding of the Fifth Indochina Conference on Pharmaceutical Sciences: Pharmacy for sustainable development. pp 1-8.
5. Narumon Changsan, Frances Separovic and Teerapol Srichana. 2008. The ^{2}H and ^{31}P solid state nuclear magnetic resonance used to determine the location of rifampicin and cholesterol on the liposome lipid bilayer. Proceeding on 2008 2nd IEEE International Nanoelectronics Conferences. pp. 699-703.
6. Narumon Changsan, Frances Separovic, Hak-Kim Chan and Teerapol Srichana. xxxx. Physico-chemical Characterization and Stability of Rifampicin Liposome Dry Powder Formulations for Inhalation. J. Pharm Sci pp. xxx-xxx. (article inpress)