

EVALUATION OF LIPOSOMAL BISMUTH-ETHANEDITHIOL-TOBRAMYCIN FOR  
TREATMENT OF CYSTIC FIBROSIS PULMONARY *PSEUDOMONAS AERUGINOSA*  
INFECTION

by

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A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science (MSc) in Chemical Sciences

The School of Graduate Studies  
Laurentian University  
Sudbury, Ontario, Canada

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# THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE

## Laurentian University/Université Laurentienne School of Graduate Studies/École des études supérieures

Title of Thesis Titre de la thèse	EVALUATION OF LIPOSOMAL BISMUTH-ETHANEDITHIOL- TOBRAMYCIN FOR TREATMENT OF CYSTIC FIBROSIS PULMONARY <i>PSEUDOMONAS AERUGINOSA</i> INFECTION		
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Degree Diplôme	Master of Science		
Department/Program Département/Programme	Chemical Sciences	Date of Defence Date de la soutenance	September 20, 2013

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## Abstract

The effectiveness of liposomes incorporating bismuth-ethanedithiol and loaded with tobramycin (LipoBiEDT-TOB) at sub-inhibitory concentrations to inhibit the production of quorum sensing signaling molecules and virulence factors induced by *P. aeruginosa* was evaluated *in vitro*. In addition, we evaluated the efficacy and safety of free and encapsulated tobramycin in liposomal formulations administered intratracheally to rats chronically infected with *P. aeruginosa*. LipoBiEDT-TOB significantly reduced the production of quorum sensing signaling molecules and virulence factor secretion compared to free tobramycin. The LipoBiEDT-TOB formulation significantly reduced the bacterial count in lungs, modulated the IL-8 level in blood and minimized the nephrotoxicity that is associated with aminoglycoside treatment. These results support the hypothesis that aerosolization of liposomal aminoglycosides may enhance the management of chronic lung infections caused by resistant *P. aeruginosa* in patients with cystic fibrosis.

## Keywords

Cystic fibrosis, liposomes, aminoglycosides, bacteria and bismuth.

## Acknowledgements

I would like to thank my research supervisor, Professor Abdelwahab Omri for welcoming me into his lab, introducing me to liposomal delivery systems, providing me with time, and resources as well as his guidance to complete my research work.

I would like to extend my gratitude and thanks to my committee members Professor H  l  ne Joly and Professor Gustavo Arteca for their time, endless encouragement, unfailing support throughout my study and reviewing my thesis. Without their willingness to help, I would be unable to accomplish my academic goals.

I am also indebted to the members of Dr. Omri's group with whom I have interacted during the course of my graduate studies. Particularly, I would like to acknowledge Mai Al-Hajlan, Ahad Hasanin and Solleti Saran Tejaswi for the many valuable discussions that helped me understand my research area better. I also would like to acknowledge the generous financial support provided by the Ministry of Higher Education in the Kingdom of Saudi Arabia.

## Dedication

I would like to dedicate this research to my Father, Abdulaziz Alhariri and Mother, Lila Obeid for their calls, understanding, endless patience and encouragement when it was most needed. I would like also to share the accomplishment of this thesis with my lovely wife Wejdan Radhwan for her support, putting me before herself and standing by my side throughout this entire journey.

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## List of Abbreviations

AACs:	Aminoglycoside acetyltransferases
AcCoA:	Acetyl co-enzyme A
ADP:	Adenosine diphosphate
AHL:	Acyl homoserine lactone
AIDS:	Acquired immunodeficiency syndrome
AMP:	Adenosine monophosphate
ANTs:	Aminoglycoside nucleotidyltransferases
APHs:	Aminoglycoside phosphotransferases
Ara4N:	4-amino-4- <i>deoxy</i> -L-arabinose
ATP:	Adenosine-5-triphosphate
BiEDT:	Bismuth ethanedithiol
BiEDT-TOB:	Bismuth ethanedithiol-tobramycin
cAMP:	Cyclic adenosine monophosphate
CF:	Cystic fibrosis
CFTR:	Cystic fibrosis transmembrane regulator
CFU:	Cell forming unit
CHEMS:	Cholesteryl hemisuccinate
C <sub>4</sub> -HSL:	<i>N</i> -3-oxo-dodecanoylhomoserine lactone
Cl <sup>-</sup> :	Chloride ions
CLSM:	Confocal laser scanning microscopy
COPD:	Chronic obstructive pulmonary disease

CpG ODN:	CpG oligodeoxynucleotide
DCP:	Dicethyl phosphate
ΔF508:	Delta phenylalanine amino acid at Codon 508
DLS:	Dynamic light scattering
DMPC:	Dipalmitoyl glycerophosphocholine
DMPG:	Dimyristoyl phosphatidyl glycerol
DOPE:	Dioleoylglycero-3-phosphoethanolamine
DOTAP:	N-(1-(2,3-dioleolyloxy)propyl)-N,N,N-trimethylammonium methyl sulphate
DPIs:	Dry powder inhalers
DPPC:	Dimyristoyl glycerophosphocholine
DPPG:	Dipalmitoyl phosphatidyl glycerol
DRV:	Dehydration-rehydration vesicles
DSPC:	Distearoyl glycerophosphocholine
EDT:	1, 2-ethanedithiol
EE:	Encapsulation efficiency
ENaC:	Epithelia sodium channel
Fab:	Fragment antigen binding
FEV <sub>1</sub> :	Force expiratory volume in 1 second
GFAAS:	Graphite furnace atomic absorption spectroscopy
GM1:	Asialoganglioside
GNAT:	GCN5-related <i>N</i> - acetyltransferases
HPLC:	High performance liquid chromatography
IL-6:	Interleukin-6

IL-8:	Interleukin-8
LipoBiEDT-TOB:	Liposomal-bismuth ethanedithiol-tobramycin
LUVs:	Large unilamellar vesicles
LPS:	Lipopolysaccharides
LDH:	Lactate dehydrogenase
mAb:	Monoclonal antibody
MBC:	Minimum bactericidal concentration
MSDs	Membrane spanning domains
MIC:	Minimum inhibitory concentration
MLVs:	Multilamellar vesicles
MPS:	Mononuclear phagocytic system
MRSA:	Methicillin resistant <i>Staphylococcus aureus</i>
MTT:	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na <sup>+</sup> :	Sodium ions
NBDs:	Nucleotide-binding domains
NO <sub>2</sub> <sup>-</sup> :	Nitrite
NO <sub>3</sub> <sup>-</sup> :	Nitrate
OD:	Optical density
OLVs:	Oligolamellar vesicles
3O-C <sub>12</sub> -HSL:	<i>N</i> -butanoylhomoserine lactone
PA:	<i>Pseudomonas aeruginosa</i>
PBS:	Phosphate-buffered saline
PEG:	Propylene glycol

PHEA:	Poly(hydroxyethyl-L-asparagine)
PHEG:	Poly(hydroxyethyl-L-glutamine)
PG:	Polyethylene glycol
pMDI:	Pressurized metered dose inhalers
PQS:	<i>Pseudomonas</i> quinolone system
QS:	Quorum sensing
RFB:	Rifabutin
RES:	Reverse-phase evaporation vesicles
REV:	Reticuloendothelial system
RND:	Resistance-nodulation-division
rRNA:	Ribosomal ribonucleic acid
SAXS:	Small-angle X-ray scattering
SEM:	Scanning electron microscopy
SUVs:	Small unilamellar vesicles
TEM:	Transmission electron microscopy
TMD:	Transmembrane domain
TPP:	meso-tetraphenylporphine

# Chapter 1

## 1. Introduction

### 1.1. Cystic Fibrosis

Cystic fibrosis (CF), the most common autosomal recessive disorder, affects 1 in 2000 Caucasians and results in shortening of the life span of the individual (1). [Please note that the references for chapter 1 are listed on page 97.] Cystic fibrosis is caused by a mutation in a single gene on the long arm of chromosome 7 that is responsible for encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein (2). The CFTR is a multi-functional protein which is located on the apical membrane of epithelial cells. A defect in the CFTR function is responsible for CF-related multi-system injuries including the lungs, the endocrine system, the pancreas, the gastrointestinal tract and the reproductive system (3). However, pulmonary disease is the main cause of morbidity and mortality among the CF population due to persistent bacterial infection and inflammation (4).

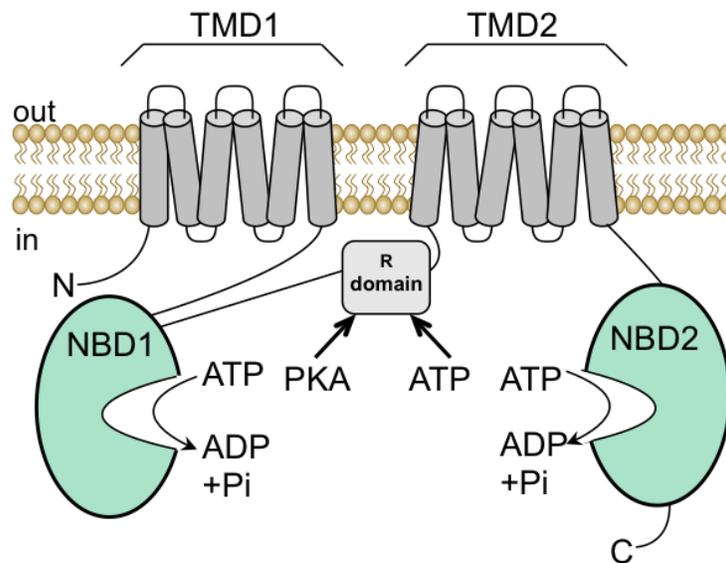
#### 1.1.1. CFTR gene

The CFTR gene is located on the long arm of chromosome 7. It is 25,000 base pairs long, contains 27 exons and encodes the CFTR protein which consists of 1480 amino acids. The severity of symptoms associated with CF and the rate of disease progression in organs depends on the type of mutation (5). The more than 1900 different mutations, that can cause CF with mild symptoms to serious problems, have been sorted into six classes, depending on their effect on CFTR protein production (6). The most common mutation is the loss of a phenylalanine residue at position 508, hence the name  $\Delta F508$ , which causes severe disease in 70% of Caucasian CF

patients (7). This defective protein does not fold normally and consequently, the protein is unable to enter the Golgi apparatus to be expressed on the cell membrane. Retention and subsequent degradation of immature proteins in endoplasmic reticulum decreases the number of chloride channels which are responsible for transporting chloride ions (6).

### 1.1.2. Structure of CFTR

The CFTR protein is part of a larger family of adenosine-5-triphosphate (ATP) binding cassette transporters (8). It contains two ATP-hydrolysis domains, which are also called nucleotide-binding domains (NBDs), and two transmembrane domains (TMDs), each of them consisting of six membrane-spanning alpha helices that form an anion channel (diagram 1). The NBDs are responsible for binding and hydrolysis of ATP which provides the required energy for channel activity. The activation of CFTR depends also on phosphorylation of the cytoplasmic R domain by cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA), and ATP to power a NBD for opening and closing the channel gate (9).



**Diagram 1: Illustration of the CFTR structure.**

### 1.1.3. CFTR function in lungs

The biological function of the CFTR protein can be understood by the observation that the sweat of CF patients contains high levels of electrolytes (10). The CFTR protein functions as a channel, across the membrane of secretory epithelial cells, to transport chloride ions ( $\text{Cl}^-$ ) in and out of the epithelial cells (11). It also down regulates trans-epithelial sodium ( $\text{Na}^+$ ) channels (8). The transport of  $\text{Cl}^-$  and inhibition of  $\text{Na}^+$  flux help to control the movement of water from the epithelial cells to the airways and *vice versa*, which is a necessary process for the production of a thin and freely-flowing mucus that protects the lungs from inhaled particles and pathogens (9).

Mutation of the CFTR gene is the basic pathology of CF due to abnormal CFTR function and subsequent electrochemical alterations across the membrane and dehydrated mucus (12). The absence of the CFTR protein results in blocking the  $\text{Cl}^-$  secretion and increases epithelial sodium channel activity (ENaC), leading to an increased absorption of  $\text{Na}^+$  by the epithelial cells lining the airways (13). In addition, abnormally thick and sticky mucus accumulation prevents cilia to beat normally and thus cleanse an airway (14). Furthermore, the thick and sticky mucus becomes an ideal growth medium in the lungs for several pathogens such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* and *Pseudomonas aeruginosa* (15). Here, we focus on the pathogenesis of *P. aeruginosa* due to the fact that this organism persists in the lungs of CF patients for the rest of their lives causing recurrent infection and inflammation (16). However, a complete discussion of the inflammation is beyond the scope of this thesis and those who are interested in CF inflammation can refer to recent review article by Cohen-Cyberknoh *et al.* (17).

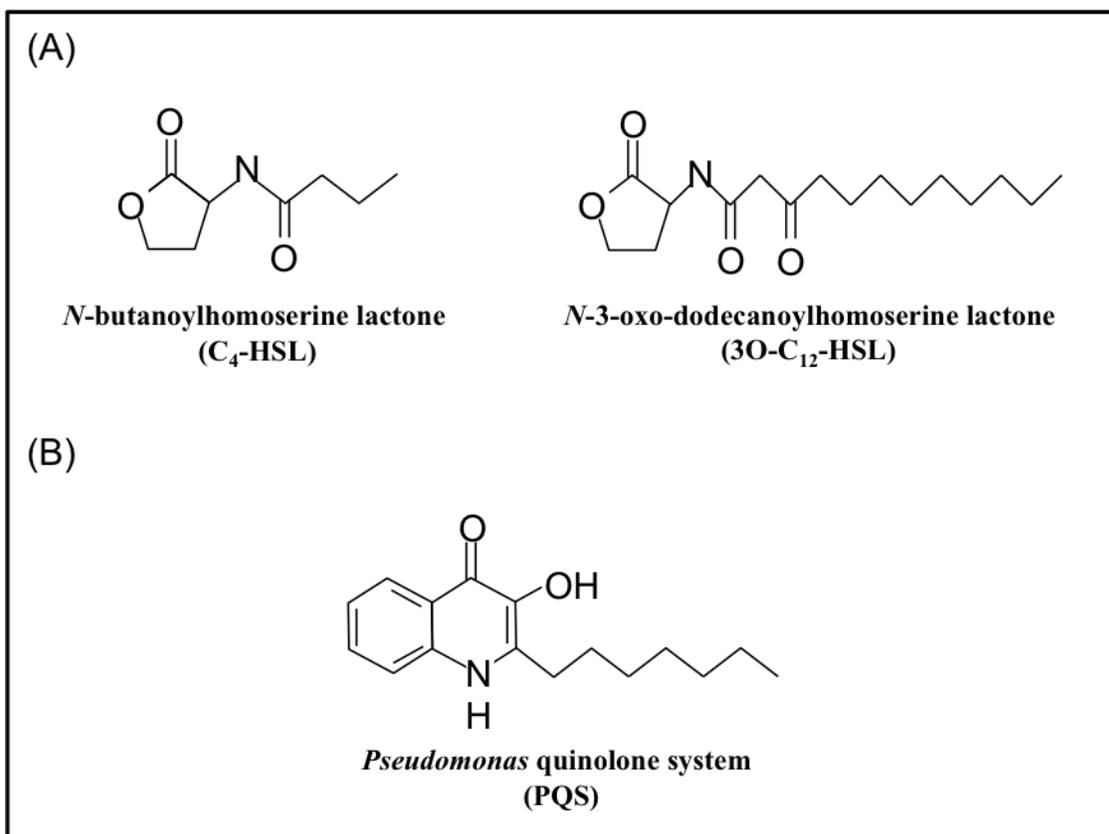
## 1.2. *Pseudomonas aeruginosa*

*P. aeruginosa* is a Gram-negative bacillus found in water, soil, plants and hospital equipment (18). *P. aeruginosa* is categorized as an aerobic bacterium, but has the capability to grow in anaerobic conditions by using nitrogenous oxides such as nitrite ( $\text{NO}_2^-$ ) or nitrate ( $\text{NO}_3^-$ ) as respiratory electron acceptors (19, 20). This opportunistic human pathogen is responsible for a number of serious acute and chronic infections in patients with compromised immunity and mucosal defenses (21). The first step, to establish *P. aeruginosa* chronic lung infection in CF patients, involves the inhalation of *P. aeruginosa* from the surrounding environment. The bacteria basically attach to the upper respiratory tract and then gain access to the lung parenchyma because of a defect in the mucociliary cleansing mechanism in CF individuals (22, 23). *P. aeruginosa* expresses a variety of different surface ligands such as type IV pili which recognize epithelial cells' receptors, in particular asialoganglioside (GM1) (24). *P. aeruginosa* adhesion can also be dependent on flagella that adhere to human respiratory mucin (25, 26). *P. aeruginosa* is also equipped with highly complex cell-to-cell signaling systems, known as quorum sensing, that control gene expression of virulence factors (27).

### 1.2.1. Quorum sensing

Quorum sensing (QS) is associated with a system of signaling molecules, known as autoinducers, that are produced and released by bacteria in response to bacterial cell density (diagram 2). *P. aeruginosa* does not express its virulence factors until it has adhered to, penetrated and proliferated in the epithelial cells in order to avoid the host immune response and to colonize the lung successfully (28). Ultimately, *P. aeruginosa* releases an excess of virulence factors which contribute to morbidity and mortality in the lungs of CF patients (29). *P.*

*aeruginosa* utilizes two homologues known as *lasI/lasR* and *rhlI/rhlR* which control synthesis of autoinducers and transcription activator proteins (30). *lasI* and *rhlI* synthesize LasI and RhlI proteins respectively. These proteins were subsequently identified as *N*-3-oxo-dodecanoylhomoserine lactone (3O-C<sub>12</sub>-HSL) and *N*-butanoylhomoserine lactone (C<sub>4</sub>-HSL), respectively (31). As the *P. aeruginosa* cell density increases and the level of 3O-C<sub>12</sub>-HSL reaches a threshold concentration, 3O-C<sub>12</sub>-HSL binds to LasR. The 3O-C<sub>12</sub>-HSL/LasR complex then binds to promoters encoding several virulence factors such as lipase, chitinase, protease, and elastase (32-36). The 3O-C<sub>12</sub>-HSL/LasR also regulates the *Pseudomonas* quinolone system (PQS), a signaling molecule that is integral to the QS cascade. This signaling molecule functions as an additional regulatory link between the Las/Rhl QS circuits and regulates production of rhamnolipids, elastase, biofilm and pyocyanin production (37-39). The 3O-C<sub>12</sub>-HSL/LasR can also activate the second transcriptional regulatory *rhlR*. Once *rhlR* is activated, the synthesized protein RhlR binds to C<sub>4</sub>-HSL to activate expression of virulence factors such as alkaline protease, lectins A, lectins B and exoenzyme S (40, 41).



**Diagram 2: Quorum sensing signaling molecules. A) Structure of acyl homoserine lactone; B) *Pseudomonas* quinolone system exploited for cell-to-cell communication by *P. aeruginosa*.**

### 1.2.2. Biofilm and secreted virulence factors

Once *P. aeruginosa* colonizes the tissues successfully, it forms a biofilm and secretes a variety of virulence factors such as elastase, protease, and chitinase which participate in extensive tissue damage.

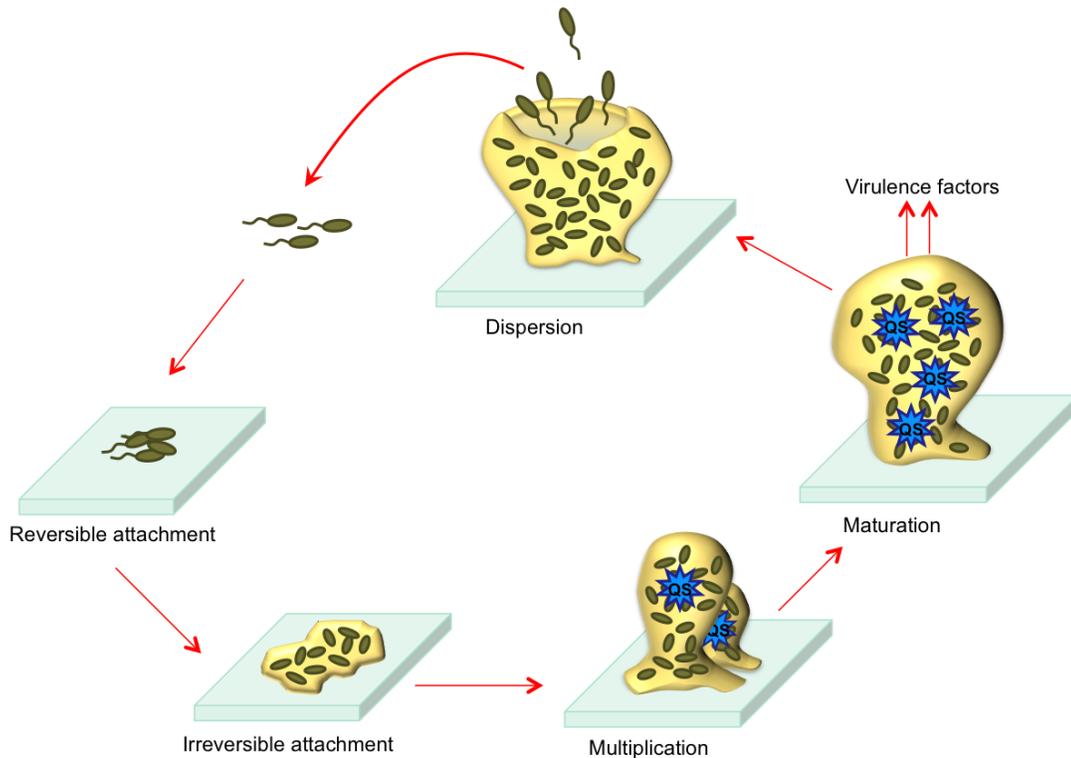
#### 1.2.2.1. Biofilms formation

Biofilms are a structured community of bacterial cells surrounded by a self-produced polymeric matrix that can adhere to biotic and abiotic surfaces (42). It consists of

polysaccharides, DNA and other macromolecular components such as protein and lipid (42, 43). Bacteria develop the biofilm mode of growth in order to survive in a harsh environment and protect themselves from bactericidal challenges such as the host immune response (44, 45). Several steps are involved in developing mature biofilms including reversible attachment, irreversible attachment, multiplication, maturation of the biofilm to a mushroom-like structure (diagram 3).

Reversible attachment is initiated when *P. aeruginosa* binds to the GM1 through the flagella encoded by *fliC* (46). *P. aeruginosa* mutants deficient in flagella showed a significant decrease in the attachment and biofilm formation compared to flagellated strains (47). The irreversible attachment involves the loss of flagellum and expression of type IV pili that bind to GM1 (48). The type IV pili are also responsible for twitching motility and have been reported to have an important role in aggregation and microcolony formation. Bacteria also multiply and upregulate their genes' expression such as *algC*, *algD*, and *algU*, which are responsible for extracellular synthesis (49). The last step in biofilm formation is the maturation of biofilms to mushroom-shaped structure, and it has been reported that QS plays a significant role in biofilm structural changes from a flat unstructured shape to a mushroom-shaped structure via the expression of more than 550 genes including other virulence factors (50). Studies showed that the increase in proteins, at this stage, correlates with a decrease in the oxygen levels at the stalk portion of the biofilm and a decrease in bacterial metabolic activities, whereas the cap portion of the mushroom-shaped biofilms tend to have a higher level of oxygen and bacterial metabolic activities (51, 52). Bacteria then disperse away from the interior portions of cell clusters and proceed to develop new biofilm. The dispersion process was shown to be synchronized with downregulation of gene expression; thereby it might suggest that dispersion and finding new

niche are an active process that the bacteria are programmed to undergo (50). Studies have shown that a biofilm in a hypotoxic environment is advantageous for bacterial survival in the host system because they limit antimicrobial penetration (53, 54).



**Diagram 3: Biofilms formation. Model of the development of a mature *P. aeruginosa* biofilm from planktonic cells.**

#### 1.2.2.2. Elastases

*P. aeruginosa* elastase is encoded by the *lasB* gene and its activity has been reported to play an important role in CF lung infection (55). It has been reported that elastase ruptures the respiratory epithelium via tight-junction destruction, thereby increasing epithelial permeability and facilitating recruitment of neutrophil (56, 57). Elastase also degrades several biological molecules including elastin, laminin, fibrin and collagen as well as surfactant proteins A and D

in the respiratory tract (58-60). Elastase is capable of cleaving the complementary components, interferon- $\gamma$  and immunoglobulins, such as IgG and IgA (61, 62).

### 1.2.2.3. Proteases

Protease is an enzyme encoded by the *lasA* gene. LasA cleaves proteins into short fragments, preferentially at sites glycine-glycine-alanine sequence in the protein (63). LasA enhances the elastolytic activity of LasB elastase as well as human neutrophil elastase, leading to tissue destruction (64). LasA- or LasB-deficient mutants exhibit decreased *invasion* of epithelial cells *in vivo* as well as in cultured epithelial cells (65). Furthermore, protease plays an important role in *P. aeruginosa* pathogenesis through the so-called shedding process, which involves the cleavage of cell surface proteins, followed by the release of ectodomains from the cell surface. Ectodomains convert membrane-anchored growth factor receptors such as IL-6 into diffusible factors, membrane receptors to soluble competitors of their own ligand, and cell adhesion receptors into substances that are no longer capable of mediating interaction with other cells (66, 67). *P. aeruginosa* seems to use the host shedding mechanism to increase its virulence (68). Beside the elastolytic activity of LasA, it also possesses a staphylolytic activity, which rapidly lyses *S. aureus* cells by cleaving the pentaglycine cross-links of bacterial cell wall peptidoglycan (69).

### 1.2.2.4. Chitinases

Chitin is the most abundant polysaccharide polymer found in nature (70). *P. aeruginosa* chitinase is encoded by the *chiC* gene and its chitin-binding protein is encoded by *cbpD* (71). Bacteria produce chitinase in the presence of chitin and repress its production when growing in a rich medium. Degradation of chitin requires two enzymatic steps; the breakdown of chitin into

disaccharide by chitinase followed by hydrolysis of the disaccharide to *N*-acetylglucosamine, which can be used by bacteria as an energy source (72).

### 1.3. Treatment of *P. aeruginosa* infection

A wide range of antibiotics including macrolides,  $\beta$ -lactams, and aminoglycosides have been developed to overcome *P. aeruginosa* infection (73-75). Combination therapy against *P. aeruginosa* has been recommended because of the concern that monotherapy might be associated with elevated levels of antimicrobial resistance (76). Aminoglycosides, however, are the drugs of choice in treatment of pulmonary infections caused by *P. aeruginosa*.

#### 1.3.1. Aminoglycosides

##### 1.3.1.1. Mechanism of action and toxicity

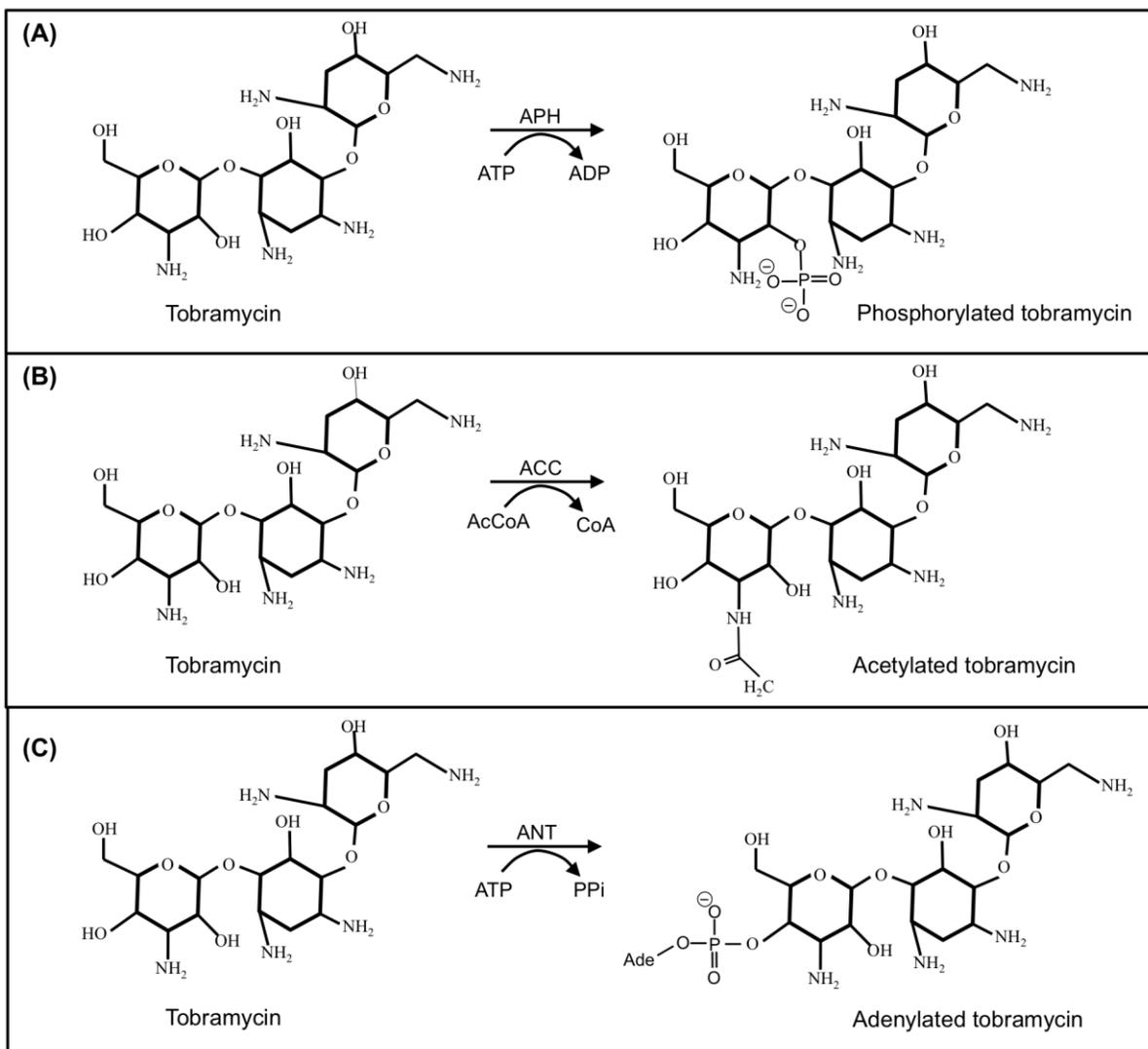
The most useful class of antibiotics for treating *P. aeruginosa* pulmonary infection in CF patients is that of aminoglycosides such as tobramycin (diagram 4) (77). Aminoglycosides contain amino sugars linked to an aminocyclitol ring or hexoses by a glycosidic bond (78). Aminoglycosides, including tobramycin, are water soluble, positively charged, and are poorly absorbed in the gastrointestinal tract, when delivered orally due to efflux of the drug by the P-glycoprotein pump in the brush border of the small intestine (79, 80). Hence aminoglycosides are commonly administered intravenously or intratracheally to maintain high bioavailability (81). Inhalation of nebulized aminoglycosides (primarily tobramycin), in treating *P. aeruginosa* pulmonary infection, has advantages in providing a direct deposition of drug at the site of infection and reducing systemic exposure and toxic effects (82, 83). Aminoglycosides exert their



### 1.3.1.2. Resistance of *P. aeruginosa* to aminoglycosides

*P. aeruginosa* is among the most antibiotic resistant bacterial species that commonly causes infections (91). Several mechanisms have been reported for *P. aeruginosa* resistance to aminoglycoside antibiotics including enzymatic modification of the aminoglycoside, reduction in membrane permeability and up-regulation of the bacterial efflux system.

Inactivation of aminoglycosides by *P. aeruginosa*-resistant strains is due to the enzymatic modification of the amino or hydroxyl groups of these antibiotics (92). Three groups of enzymes that are responsible on the drug modification in the bacteria cytoplasm have been identified (diagram 5); these are aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs). Phosphorylation of aminoglycosides is carried out by aminoglycoside phosphotransferases, utilizing adenosine triphosphate (ATP) as a substrate to phosphorylate the hydroxyl groups at positions 2", 3', 3", 4, 6, 7", and 9 (93-95). Aminoglycoside acetyltransferases belong to the GCN5-related *N*-acetyltransferases (GNAT) superfamily and catalyze the acetylation of the amino groups by utilizing coenzyme A as a substrate (96). Acetylation of aminoglycosides can occur with the amino groups located at positions 1, 2', 3, and 6' (92). Aminoglycoside nucleotidyltransferases mediate inactivation of aminoglycosides by utilizing ATP as a substrate and transferring adenosine monophosphate (AMP) to the hydroxyl group at positions 2", 3", 4', 6, and 9 of the aminoglycosides (97-100).



**Diagram 5: Modification of tobramycin by aminoglycoside-modifying enzymes. (A) phosphorylation of tobramycin by aminoglycoside phosphotransferases; (B) acetylation of tobramycin by aminoglycoside acetyltransferases; and (C) adenylation of tobramycin by aminoglycoside nucleotidyltransferases.**

Bacterial resistance to cationic antimicrobial agents is usually due to outer membrane impermeability resulting from lipopolysaccharide (LPS) modification (101). Poly-cationic antimicrobials competitively displace divalent cations which cross-link anionic LPS to destabilize the bacterial outer membrane, thereby promoting their own entry into the cell by a

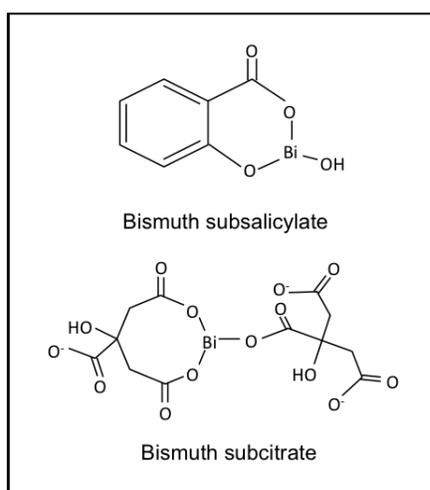
process called self-promoted uptake (102). The interaction relies on the presence of phosphate groups at the lipid A domain (103). Bacteria such as *P. aeruginosa* modify their lipid A structure by adding polar groups such as 4-amino-4-*deoxy*-L-arabinose (Ara4N) (104). Ara4N neutralizes the negative charge of the phosphate residue, thereby decreasing bacterial susceptibility to cationic antimicrobials (105).

Resistance to multiple antimicrobials, including aminoglycoside antibiotics, in *P. aeruginosa* can be also explained by the involvement of the multidrug efflux system of the resistance-nodulation-division (RND) family, MexXY-OprM, which allows bacteria to get rid of the aminoglycoside antibiotics from the bacterial interior (106). The RND family of pumps generally comprises three components which include an inner membrane drug-proton antiporter encoded by *mexY*, an outer membrane channel-forming protein encoded by *oprM*, and a periplasmic membrane fusion protein encoded by *mexX*, which facilitates assembly and joins the other two components into a functional efflux pump (107). Expression of MexXY-OprM in *P. aeruginosa* has been shown to confer resistance to aminoglycosides (108). In order to restore aminoglycoside activity and counter bacterial resistance, combination therapy against *P. aeruginosa* has been recommended because of the concern that monotherapy might be associated with elevated levels of antimicrobial resistance (76). Recently, *in vitro* studies have found that combination bismuth-thiols with tobramycin possess a potent antimicrobial activity in treating Gram-negative bacteria (109).

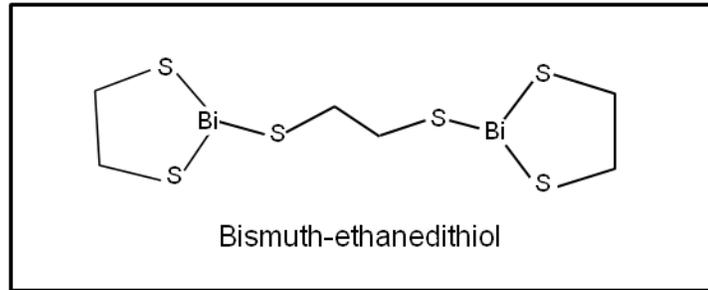
### 1.3.2. Bactericidal activity of bismuth agents

Bismuth agents, such as bismuth subsalicylate and bismuth subcitrate (diagram 6), have antimicrobial activities against a wide variety of gastrointestinal tract infections caused by *Escherichia coli*, *Vibrio cholera*, and *Helicobacter pylori*. Bismuth components disrupt the cell

wall structure and inhibit secretion of virulence factors by *H. pylori* (110, 111). Biofilm production and adherence of *Klebsiella pneumoniae* and *H. pylori* to epithelial cells are also reduced by bismuth agents (112, 113). However, bismuth subsalicylate and bismuth subcitrate alone or combined with antibiotics do not exhibit potent antibacterial activities against Gram-negative bacteria such as *P. aeruginosa* (114, 115). One study reported an improvement in antimicrobial activity against *P. aeruginosa* by chelating bismuth to a lipophilic thiol which increases its solubility (diagram 7) (116). Furthermore, exposing *P. aeruginosa* to bismuth-ethanedithiol at sub-inhibitory concentration showed reduction in alginate and biofilm production, as well as reduced adherence to epithelial cells. However, bismuth-ethanedithiol exhibited an increased toxicity on epithelial cells at concentrations that might be required to eliminate infection (117). Due to the toxicity of bismuth complexes and aminoglycoside antibiotics as well as increasing resistance of *P. aeruginosa* to currently available antibiotics, there is a strong demand for a carrier system such as liposomes which are safe on host cells and effective against bacterial infection.



**Diagram 6: Chemical structures of bismuth subsalicylate and bismuth subcitrate.**



**Diagram 7: Chemical structure of bismuth-ethanedithiol.**

## 1.4. Liposomes

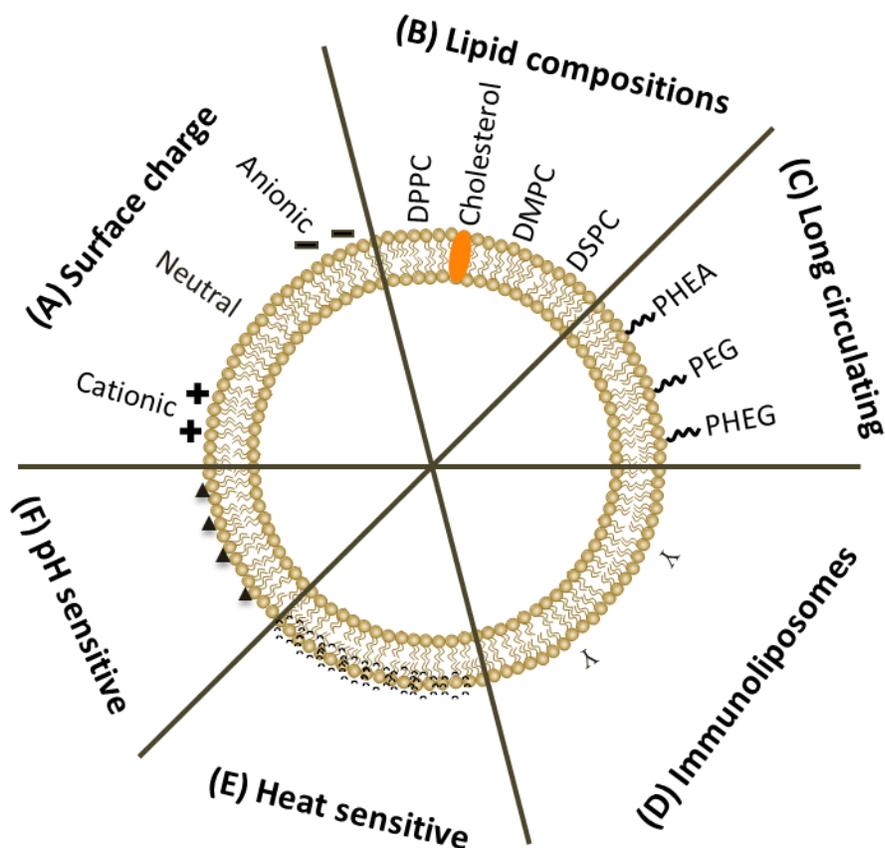
Liposomes are spherical lipid vesicles ranging from nanometers to micrometers in size. They consist of one or more lipid bilayers surrounding an aqueous core. Liposomes are a relatively safe delivery system because they are biocompatible and biodegradable (118). There are several reasons for using liposomes as carriers for biologically active compounds. Currently, liposomes are designed to eliminate or reduce the toxicity of entrapped biologically active agents, to direct active agents to a desired delivery site, to protect the drug from unwanted metabolic breakdown, to improve the pharmacokinetics of the active agents and to increase their accumulation at the target site (119-121). They are usually classified into three categories (Diagram 8): small unilamellar vesicles (SUVs) or oligolamellar vesicles (OLVs), large unilamellar vesicles (LUVs), and multilamellar vesicles (MLVs) depending on their size and the number of bilayers present in the vesicle (122).



**Diagram 8: Classification of liposomes by size. Multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs).**

There are a wide variety of liposomal applications because the physicochemical properties of liposomes can be modified by changing: i) the net surface charge; ii) pH sensitivity; iii) heat sensitivity; or iv) proportions and type of lipids in the formulation (Diagram 9). The ability of liposomes to encapsulate both hydrophilic and lipophilic agents has proven to be a highly valuable characteristic (123). Increased bacterial drug resistance to common therapies and the ability of some species to form drug-impermeable capsules and biofilms impermeable to antibiotics have become a major problem in modern medicine (124). Liposomes can introduce components with poor penetration such as antibiotics into bacterial cells such as those of *P. aeruginosa* (125). Intensive research is now focused on liposome-encapsulated antibiotics in order to enhance their pharmacokinetic properties and bactericidal activities, as well as reducing the drug's adverse effects (123, 126). Due to the complex physicochemical properties of liposomes, there are many factors to consider when preparing a liposomal formulation, e.g., the different lipid compositions that constitute the liposome, as well as its size and surface charge will affect both the encapsulation efficiency (EE) and the release rate of the antibiotics. While

many investigators are making efforts to discover new antibiotics, others are focused on enhancing the efficacy of currently available antibiotics in the form of liposomal formulations (127-129). The present thesis work falls into the latter category.



**Diagram 9: Modification of the physicochemical properties of liposomes. (A) Neutral or charged, (B) lipid compositions, (C) long circulating, (D) immunoliposomes, (E) heat sensitive and (F) pH sensitive. DPPC, dipalmitoyl glycerophosphocholine; DMPC, dimyristoyl glycerophosphocholine; DSPC, distearoyl glycerophosphocholine; PHEA, poly(hydroxyethyl-L-asparagine); PHEG, poly(hydroxyethyl-L-glutamine); and PEG, polyethylene glycol.**

## 1.5. Liposomes as a drug delivery system

Liposomes have been successfully used in the delivery of anti-cancer, antibacterial and anti-fungal drugs *in vitro* and *in vivo*. Developing liposomal formulations holds great interest in biomedical research because they may serve as a sustained drug release system (130), which results in prolonged half-life of the active agents. The extended half-life provided by liposomal formulation may lead to a decrease in frequency and length of drug administration (131, 132). Different methods of preparation and characterization, as well as the stability and interaction of liposomes with cells, are discussed in this section of the thesis.

### 1.5.1. Methods of preparation

There is a wide variety of liposomal methods that used in the preparation of liposomes fall into two main categories, i.e., conventional or mechanical methods. Conventional methods include the dehydration-rehydration vesicle, reverse-phase evaporation, injection, and detergent removal methods which all involve the dissolution of lipids in an organic phase followed by the addition of an aqueous solution. The mechanical methods, on the other hand, involve a mechanical force that results in a homogeneous mixture of liposomes.

#### 1.5.1.1 Conventional methods

##### 1.5.1.1.1 Dehydration-rehydration method

The dehydration-rehydration vesicle (DRV) method is the simplest and most widely used procedure for liposome preparation (127, 129). The process of preparing DRVs involves dissolution lipids in organic solvents such as chloroform or a chloroform/methanol mixture in a round bottom flask, followed by evaporating the organic phase to form a thin lipid film. The last

step in the preparation involves rehydration of the lipid film with an aqueous phase. When the dry lipid films are rehydrated, lipid lamellae are formed, mechanical agitation such as shaking or vortexing are used to detach the lipid film from the flask (133). The DRV technique has been employed by several laboratories. Halwani *et al.* (134) determined that liposomal formulation consisting of distearoyl glycerophosphocholine (DSPC) and cholesterol in a 2:1 molar ratio, encapsulated amikacin with an EE of 52.1 %. Another study investigated the encapsulation of ciprofloxacin in two cationic liposomal formulations that consisted of phosphatidylcholine (PC), cholesterol and N-(1-(2,3-dioleolyloxy)propyl)-N,N,N-trimethylammonium methyl sulphate (DOTAP) in a molar ratio of 3:4:3 or PC, dioleoylglycero-3-phosphoethanolamine (DOPE) and DOTAP in a molar ratio of 3:4:3 by DRV method (135). The EE of liposomal ciprofloxacin was 67 % for PC/DOPE/DOTAP and 74 % for PC/cholesterol/DOTAP.

#### 1.5.1.1.2. Reverse-phase evaporation method

Reverse-phase evaporation is a procedure for the preparation of liposomes with a large internal aqueous space. Preparation of reverse-phase evaporation vesicles (REV) results in large unilamellar and oligolamellar vesicles that are able to entrap large macromolecules with high EE (136). The procedure is based on two steps: adding an aqueous phase to form a phospholipid monolayer surrounded by water, and then adding an excess quantity of organic solvent. The lipid mixture is transferred into a round bottom flask and dissolved in a solvent, followed by removing the solvent under reduced pressure using a rotary evaporation, followed by flush-drying with nitrogen gas (137). The solvents that have been successfully used are diethyl ether, isopropyl ether, halothane and trifluorotrichloroethane. Lipids are then redissolved in the organic phase, followed by adding an aqueous solution of the bioactive agent. The solution is then sonicated to produce inverted micelles. The organic solvent is removed and a viscous, gel-like matrix forms.

As the majority of solvent has been removed, the gel collapses and an aqueous suspension of vesicles forms. The drawbacks to using REV method are the encapsulated drug is in contact with the organic phase exposed to mechanical agitation (133). Ciprofloxacin-loaded liposomes with different compositions and surface charge have been prepared by REV (138). Positively charged liposomes exhibited the highest EE (82.01 %). The maximum amount of ciprofloxacin entrapped was achieved in liposomes prepared from soya PC, cholesterol and stearylamine in a molar ratio of 5:3:1. Nicolosi *et al.* (139) reported the successful encapsulation of vancomycin into liposomes consisting of dioleoyl phosphatidyl ethanolamine, dipalmitoyl phosphatidylcholine and cholesterol hemisuccinate in molar ratio of 4:2:4 using the REV method. The liposomal vancomycin exhibited an EE of 65%.

#### 1.5.1.1.3. Injection method

The ethanol/ether injection method involves dissolving lipids in ethanol or ether, then slowly injecting this lipid solution through a fine needle into the aqueous phase, followed by evaporating the organic solvent. The injection results in the formation of unilamellar liposomes with a high EE (140). When comparing the ethanol injection with the ether injection method, the latter is more advantageous since residual ethanol might be a concern; in contrast to ethanol, ether is immiscible with aqueous solutions and can be removed upon heating the solvent under vacuum (133). Chorachoo *et al.* (141) prepared rhodomyrtone liposomal formulations that consisted of PC and cholesterol with ratio of 4:1 using the ethanol injection method. The encapsulation efficiencies of the liposomal formulations ranged between 51 and 65 %. The highest percentage of rhodomyrtone entrapment was in liposomes of 60 µmol/ml of total lipid concentration. The liposomal rhodomyrtone exhibited higher activity compared with the free formulation against *Staphylococcus aureus*. Another study investigated the encapsulation of

amphotericin B into three liposomal formulations prepared by the ethanol injection method and consisted of soya PC and cholesterol, dimyristoyl phosphatidylcholine and cholesterol, or hydrogenated soya PC and cholesterol in a molar ratio of 7:3 (142). The EE of amphotericin B ranged between 93 and 97 %.

#### 1.5.1.1.4. Detergent removal method

The detergent is used to solubilize the lipids in a micellar solution (143). The detergent protects the hydrophobic part of lipids from interacting with the aqueous solution; consequently micelles are formed instead of liposomal vesicles. After drying the lipid mixture, an aqueous phase, that contains hydrophilic drugs, is added to prepare detergent-lipid micelles. Liposomes are spontaneously formed once the detergent is removed by dialysis, column chromatography or adsorption. One of the drawbacks of liposomes formed by this technique is the use of detergent removal procedures, which are time consuming and might result in removing other hydrophilic components (144). As well, only a few detergents are appropriate for use with this method such as alkylglycosides, sodium cholate and alkyloxypolyethylenes. Using this method, Daemen *et al.* (145) showed that 98 % of the doxorubicin was encapsulated within liposomal vesicles consisting of PC, cholesterol and phosphatidylserine in a molar ratio of 4:5:1.

#### 1.5.1.2. Mechanical methods

A mechanical force is applied to alter the size, lamellarity or homogeneity to produce a liposomal population with a specific size or property. The methods applied most often after liposome preparations are sonication and extrusion. These methods result in size, lamellarity and heterogeneity reduction (144).

### 1.5.1.2.1. Sonication

Sonication is a simple method for liposome size reduction that can be achieved by exposing the MLVs to ultrasonic irradiation. Two sonication techniques can be used: i) probe sonication, and ii) bath sonication. The probe sonicator delivers high energy to the lipid, but has the disadvantage of degradation caused by overheating the lipid suspension (146). The probe sonicator also tends to release titanium particles that need to be removed from lipid suspension (147). The bath sonicator, however, enables one to control the energy that is delivered to the lipids, thereby preventing lipid overheating and enhancing the reduction of liposomal size (148, 149). Bath sonication also is the most widely used technique for large volume preparations (136). Mugabe *et al.* (150) successfully prepared liposomal formulations that loaded different antibiotics including gentamicin, tobramycin, amikacin and erythromycin by the DRV method. After homogenization was performed via sonication, the resulting liposomal formulations had average sizes that ranged from 163 to 260 nm.

### 1.5.1.2.2. Extrusion

In this method, the size is reduced when the liposomes are forced to pass through polycarbonate filters with pore sizes of 1  $\mu\text{m}$  under moderate pressures, followed by several cycles of extrusions through filters of decreasing pore size ranging from 0.6 to 0.1  $\mu\text{m}$  at elevated temperatures (147). There are disadvantages with the extrusion method, including the long period of time required to reduce the size and the high product losses that may occur due to clogging of the extrusion membrane (151). Liposomal polymyxin B of defined size and homogeneity was prepared by sequential extrusion of multilamellar vesicles through a double-stacked polycarbonate membrane. The resulting liposomes had a mean diameter of 172 nm

(152). Another study showed how extrusion also improved the homogeneity and reduced the size of liposome-loaded vancomycin (139). The average size of the liposomal vesicles was 103 nm.

#### 1.5.1.2.3. Microfluidization

Microfluidization is a technique for reducing liposomal size, used in the pharmaceutical industry for large-scale production (153). The method is based on splitting a fluid stream into two parts and passing them through a fine orifice under high pressure (10,000 psi) to guide the flow inside the interaction chamber (154). The high pressure then directs the flow stream through microchannels toward the impingement area. Inside the interaction chamber, cavitation, along with shear and impact, reduces liposome size (153, 154). However, a disadvantage of the microfluidization method is the high pressure required to reduce the size, which may result in partial degradation of the lipids (155). As far as we know, this method has not been widely applied in preparing liposomes for treating infectious diseases. However, Boltic *et al.* (156) have reported the suitability of the microfluidization method to reduce the size of liposome-loaded antibiotics. The mean diameter of the liposomal vesicles after five homogenizing cycles was 380 nm.

#### 1.5.2. Methods of characterization

Liposomes prepared by any of the above-mentioned methods must be characterized. The most important characteristic parameters to be determined for optimizing stability and shelf life of liposomal formulations are particle size, lamellarity, zeta potential, and EE.

### 1.5.2.1. Particle size

Size and size distribution measurements of liposome formulations are important characteristic parameters that indicate the homogeneity of liposomes. Unchanged in the size and size distribution of liposomes can be used as indicators of long-term stability (154).

Dynamic light scattering (DLS), electron microscopy and gel exclusion chromatography are widely used to measure the size of liposomes. The basic principle of DLS is a measurement of the diffusion coefficient as a result of Brownian motion. The diffusion coefficient is then used to calculate the size of the liposome (157). Transmission electron microscopy (TEM) at cryogenic temperature and freeze-fracture TEM are also used to determine polydispersity and size (158). However, light scattering and electron microscopy have their own advantages and drawbacks (154). For instance, light scattering provides the particle size of the entire sample, but it does not determine the morphology of liposomes. Additionally, this technique measures aggregation of two or more liposomal spheres as one vesicle which is larger than the actual size of a single liposomal sphere. Electron microscopy can determine the shape and actual size of particles; however, only a small population can be measured. A simple but powerful method is gel exclusion chromatography, in which an actual hydrodynamic radius can be detected. The main drawback of this method is the presence of positively-charged colloidal particles in columns tend to clog due to the possibility of electrostatic interactions with the medium which may have a negative charge (147).

### 1.5.2.2. Lamellarity

The lamellarity of liposomes can be determined by measuring the phosphorus nuclear magnetic resonance ( $^{31}\text{P}$ -NMR) signal of the phospholipid head groups of liposomes before and

after the addition of manganese ions ( $Mn^{2+}$ ) as a paramagnetic agent. The  $Mn^{2+}$  interacts with the negative charge on phospholipids of the outer liposomal surface. The interaction results in broadening and reduction of the resonance signal. Direct comparison of the peak height of the two signals reveals the ratio of outer to inner phospholipid content (159). Although this method is commonly used, parameters including buffer and  $Mn^{2+}$  concentrations as well as pH may affect the method's accuracy (160). Microscopic techniques including scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and cryo-TEM are also used to determine the lamellarity (161). Not only do these show the accurate lamellarity, but they also give additional information such as shape and size. The size and lamellarity can be provided as well by small-angle X-ray scattering.

### 1.5.2.3. Zeta potential

This potential indicates the overall charge present on the colloidal systems. The zeta potential measurement can also indicate stability of colloidal systems. Positive or negative surface charges on liposomes indicate higher stability due to surface repulsion between similarly-charged particles, hence inhibiting the aggregation of liposomes (162). Measuring the zeta potential is also important for monitoring the fusion and precipitation of liposomes, which relate to liposomal stability (154).

### 1.5.2.4. Encapsulation efficiency (EE)

EE is usually defined as the percent fraction of entrapped drug to that of the initial concentration used in the liposomal preparation (150). EE is indicative of the quantity of drugs entrapped in liposomal formulations. EE can be used to optimize the formulation composition before studying the behavior of these entrapped agents in physical or biological systems. For

water-soluble drugs, encapsulation corresponds to the entrapment within the aqueous core. For lipophilic drugs, on the other hand, it implies entrapment within lipid bilayers. After removal of non-entrapped drugs from the aqueous phase, total lysis of liposome vesicles can be induced by addition of a detergent such as Triton X100 in an effort to measure EE.

High performance liquid chromatography (HPLC) is commonly used for determination of EE. Spectrophotometry, fluorescence spectroscopy, enzyme-based methods, microbiological assays and electrochemical techniques are also used for determining the EE depending on the nature of the entrapped materials (127, 154).

### 1.5.2.3. Stability of liposomes

In the pharmaceutical industry and in drug delivery, the capability of the product formulation to remain stable within a defined period of time is very important. The stability of liposomes is determined by a number of physical and chemical properties.

The physical instability can be indicated by: 1) the increase of liposome size, 2) ratio between lipid and entrapped agent due to fusion, and 3) aggregation of membrane bilayers or leakage of encapsulated materials. Physical stability can be improved by storing the liposomes at low temperatures. Geusens *et al.* (163) studied the stability of cationic liposomes for 28 days at 4°C and 25°C; they found that the particle size was stable at 4°C but increased from 100 nm to 160 nm at 25°C.

Chemical instability can occur by hydrolysis or oxidation of lipids. Hydrolysis removes the hydrophobic chain of ester bonds, thereby leading to lipid destruction and leakage of encapsulated materials. Unsaturated lipids are more likely to be prone to oxidation from reactive oxygen species, thereby affecting liposomal fluidity (164). However, chemical and physical

stability of liposomes can be enhanced by lyophilization process which has been found to be suitable for long-term stability (165).

## 1.6. Interaction of liposomes with cells

Interest in liposomes is based on their biological membrane-like structure consisting of lipids organized in bilayer configuration. The device prevents its content from being released rapidly, and ensures that they are delivered to the desired target because of the liposomes' ability to interact with host cells via lipid exchange, adsorption, endocytosis, or fusion (Diagram 10)

### 1.6.1 Lipid exchange

Lipophilic materials can be transferred from liposomes to the cell membrane by lipid exchange. Lipid exchange is a method where liposomes exchange their own lipids for the lipids of diverse cell membranes (166). Although the exact mechanism is not fully understood, one possibility is the transfer of lipid monomers mediated by lipid exchange proteins existing at the cell surface. It is also possible that the outer monolayer of vesicles and the cell membrane undergo a reversible transient merger. Finally, an enzymatic exchange of acyl chains may take place between the liposomes and plasma membrane lipids of the host cells (167).

### 1.6.2. Adsorption

Adsorption of liposomes to the cell membrane is another mechanism of liposome-cell interaction. Adsorption occurs without merging the liposomes with the cell membrane. Adsorption of liposomes into the cell membrane proceeds when attractive forces (e.g., long-distance electrostatic interactions, van der Waals interactions, hydrophobic insertion and

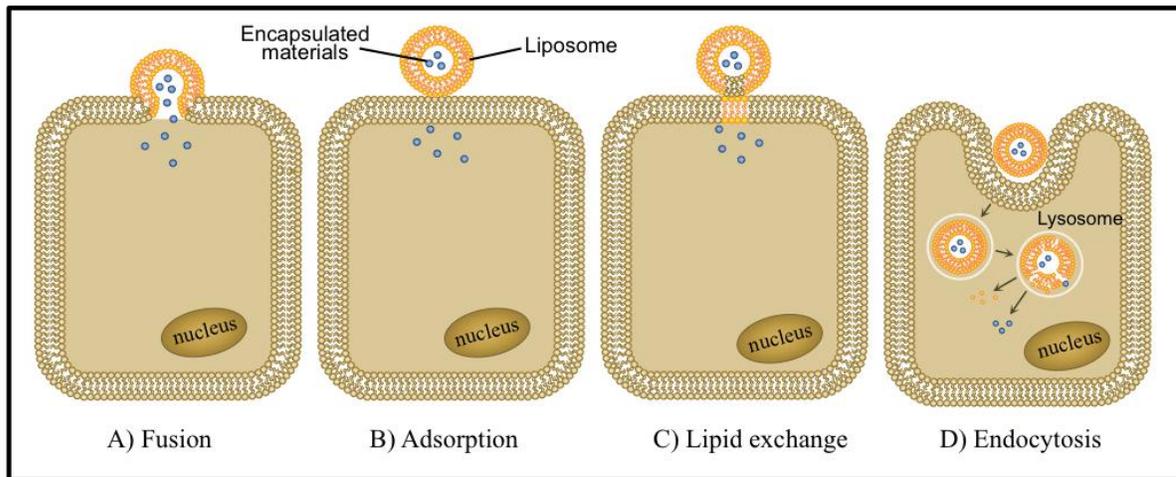
hydrogen bonding) exceed the repulsive forces (e.g., short-distance electrostatic interactions, steric hindrance, hydration and protrusion) (168).

### 1.6.3. Endocytosis

Cells with phagocytic activity engulf liposomes into endosomes. In turn, the endosomes fuse with the lysosomes resulting in the formation of phagosomes. Lysosomal enzymes digest the lipids in the phagosomes and convert them to fatty acids. The liposomes' content is then released intracellularly (169).

### 1.6.4. Fusion

Fusion of biological membranes is a crucial process in the intracellular delivery of lipids. Close contact of liposomes leads to intermixing and diffusion of liposomal lipids with the lipids of the target plasma membrane, thereby allowing entrapped agents in the aqueous compartment to be injected directly into the cytoplasm (127). On the other hand, incorporated agents in the lipid bilayer are delivered into the bilayer membrane of the cell.



**Diagram 10: Schematic representation of possible mechanisms of liposome-cell interaction. (A) fusion, (B) adsorption, (C) lipid exchange and (D) endocytosis.**

## 1.7. Routes of administration

Encapsulated agents in liposomes might be introduced into a biological system by different anatomical routes. Delivering medication to a biological system may proceed via oral, intravenous or pulmonary routes.

### 1.7.1. Oral administration

This route is the preferred means of delivering medication due to the safety, ease of administration, and its widespread acceptance by patients. Oral administration of lipid based encapsulation systems has a direct impact on drug performance *in vivo*. Lipophilic drugs may dissolve in water very poorly, but in gastrointestinal fluids they are often solubilized by bile to a significant extent. Thus, orally-administered liposomes that have a large surface area enable pancreatic lipase to efficiently hydrolyze triglycerides, promoting solubilization of the lipophilic drug in the aqueous environment of the intestinal lumen (170). Gershkovich *et al.* (171) examined the pharmacokinetics of liposome-loaded amphotericin B administered orally and found that the oral liposome formulation has the potential for improving therapeutic treatment and prophylaxis of systemic fungal infections.

### 1.7.2. Intravenous administration

Intravenous administration is the infusion of water-soluble materials directly into a vein. As systemic circulation supplies blood to the whole organism, liposomes are transported from the site of injection directly to the heart via the venous network and then to organs. On administration of liposomes to systemic circulation, however, liposomes are cleared rather quickly from the blood by the mononuclear phagocytic system (MPS) (172). Thus, diverse strategies have been developed to extend the drugs' half-life in circulation. For example,

incorporation of certain glycolipids such as phosphatidyl inositol in the bilayer resulted in shortened clearance time and reduced uptake by the MPS in the spleen and the liver (173, 174). Conjugating a stealth component such as polyethylene glycol (PEG) on the liposome's surface resulted in prolonged circulation (175). The pharmacokinetics of intravenously-administered conventional liposome-loaded gentamicin showed that plasma half-life was prolonged compared with the free drug (176).

### 1.7.3. Pulmonary drug delivery

Liposome-encapsulated active agents can be delivered directly to the lung for local treatment of pulmonary diseases. This route offers greater access of active agents to the target site and allows for the use of effective but lower drug doses with reduced systemic toxicity. Liposomes seem to be a suitable and attractive option for therapeutic agent delivery to the lung, since they can be prepared from components compatible to the lung (177). Liposomal formulations have been delivered to the lung in the form of an aerosol, which can deliver the drug particles by inhalation either through nebulization as droplets or dry powder inhalation.

#### 1.7.3.1 Nebulization

Nebulization is a method of delivering active agents dissolved in liquid in the form of a fine mist inhaled into the lung by using spraying devices. These fall into two categories: ultrasonic and jet flow devices (178). Nebulization depends on whether we deal with a drug that is soluble in solution (e.g., water, saline or cyclosporine in alcohol) or an insoluble drug suspended in liquid (179).

Ultrasonic devices have the advantage of delivering medication in a short time, but they are not widely applicable with macromolecules due to denaturation of recombinant human

deoxyribonuclease (e.g., dornase  $\alpha$ ) by overheating (180). Thus, ultrasonic devices are limited in their therapeutic use (181). Jet flow devices can be applied in order to deliver various types and volumes of drug solutions in higher concentrations. For example, Weers *et al.* (182) investigated the inhalation of liposome-loaded amikacin in terms of pulmonary deposition, clearance and safety. Inhalation of liposome-loaded amikacin was well tolerated up to 120 mg and resulted in the prolonged retention time when a commercial jet nebulizer was used.

### 1.7.3.2. Dry powder inhalation

Dry powder inhalation is an alternative methodology for aerosolization and delivery of medication to the lung in the form of a dry powder. Dry powder inhalers (DPIs) are an alternative technique to pressurized metered dose inhalers (pMDI) for delivering active agents. DPIs provide the advantages of increased efficacy with simplified and shortened time of drug administration (179).

A case in point is budesonide, a corticosteroid that inhibits inflammatory symptoms like edema seen in chronic obstructive pulmonary disease (COPD). Liposome-encapsulated budesonide for DPIs has been found to provide a sustained release for longer periods of time while reducing systemic toxicity (183).

## 1.8. Biodistribution

The use of liposomes in drug delivery can alter the biodistribution and the rate of clearance of the drug by causing it to adopt the pharmacokinetic parameters of the liposomes. After intravenous administration, liposomes are rapidly cleared from the systemic circulation since they are recognized as foreign bodies by the MPS of the reticuloendothelial system (RES),

particularly Kupffer cells in the liver and spleen (173, 174). The increased rate of mononuclear phagocyte uptake is due to opsonization by serum proteins such as albumin, lipoproteins, immunoglobulins and the complement C3a and C5a fragments (184). Numerous studies have focused on investigating the factors responsible for the regulation of this interaction. The uptake rate of mononuclear phagocyte is affected by liposome properties such as size, surface charge, membrane lipid packing, and fluidity.

The circulation kinetics of liposomes with a mean size of 120 nm was shown to exhibit a slower removal rate from the blood compared with those having a mean size of 230 and 360 nm, respectively (185). The use of charged lipids in liposomes has a direct effect on liposome pharmacokinetics as well. Previous studies have shown that the presence of negatively and positively-charged lipids in liposomes resulted in a high uptake rate of liposomes by the RES (186, 187). Furthermore, the interactions of liposomes with serum proteins, including apolipoprotein, are highly dependent on lipid composition. It is generally accepted that the effect of bilayer fluidity and manipulation of lipid composition can have an impact on liposome clearance from circulation. For instance, absence of cholesterol from liposomes resulted in bilayer destabilization by high-density lipoproteins (188), thus quickly eliminating liposome components from systemic circulation. Different strategies have been developed to overcome the rapid liposomal systemic clearance by coating the surface of liposomes with immobile molecules. The common characteristic of these inert molecules is the occurrence of a hydrophilic flexible chain that forms a periliposomal layer (e.g., glycolipid or poly amino acid). This periliposomal layer hinders the binding of blood plasma protein to liposomes, thereby inhibiting the interaction of the MPS with liposomes (189). Methods for extending liposome blood

circulation times are based on grafting them with: PEG, poly(hydroxyethyl-L-asparagine) (PHEA) and poly(hydroxyethyl-L-glutamine) (PHEG) (175, 185).

## 1.9. Targeting of liposomes

The delivery of liposomes to a specific site involves shuttling them to the target area while reducing their exposure to normal tissues. Liposomes have been employed for accomplishing the delivery of therapeutic agents to a selected site by two mechanisms, known as passive and active targeting.

### 1.9.1. Passive targeting

Passive targeting for liposome delivery uses the natural tendency of certain cells upon injection into the circulatory system. For example, liposomes can be taken up by the RES in a passive manner. This uptake can be very useful in targeting diseases associated with parasites living inside macrophages such as leishmaniasis, candidiasis, and listeria. Once the liposomes are engulfed by the macrophages, the macrophages will degrade the liposomes resulting in the release of encapsulated drug within the macrophage (190). Therefore, the drug will reach the target site directly. Similarly, liposomes with a relatively small diameter can extravasate and accumulate in tissues characterized by leaky vasculature, such as solid tumors (191). This accumulation, which occurs due to retention of liposomes at sites of enhanced vascular permeability, will result in the creation of a high local drug concentration (192).

## 1.9.2. Active targeting

Active targeting of liposomes involves controlling and directing the movement of liposomal vesicles with specific ligands coupled into the liposomal structure to target-specific tissues or cells. These so-called immunoliposomes improve the therapeutic availability of encapsulated drugs and minimize the adverse effects to non-target cells within pathological tissues. Examples of active targeting liposomal structure include concanavalin A-modified liposomes, mannose-modified liposomes, monoclonal antibody (mAb)-modified liposomes, folate-modified liposomes, and transferrin-modified liposomes.

### 1.9.2.1. (Concanavalin A)-modified liposomes

Lectins are glycoproteins or protein receptors that recognize sugar molecules, what makes them capable of binding to glycosylated molecules on cell membranes (193). The immobilization of carbohydrate ligands onto liposomal surfaces have led to the development of targeted liposomal delivery systems based on a carbohydrate–lectin interaction (194). Sudheesh *et al.* (195) reported that a concanavalin A-anchored liposomal amphotericin B exhibited higher activity on inhibiting the *Candida albicans* growth within a biofilm community, compared with conventional liposomes loaded with amphotericin B.

### 1.9.2.2. Mannose-modified liposomes

Another approach to target liposomes in infectious diseases consists of grafting mannose, instead of lectin, on liposomal surfaces. Mannose has the ability to recognize mannose receptors including mannose-binding lectins, which are highly expressed in cells of the immune system such as macrophages and dendritic cells (196). In addition, mannose binding proteins are capable of binding to a wide variety of cellular components including lipopolysaccharides,

lipoarabinomannan and lipophosphoglycan (197). Chono *et al.* (198) reported a higher efficacy for mannosylated liposomal ciprofloxacin against bacteria compared with unmodified liposomal ciprofloxacin *in vitro*. Same study showed that targeting ciprofloxacin to alveolar macrophages in a rat model of pulmonary infection was significantly higher in mannosylated liposomal ciprofloxacin than conventional liposome-loaded ciprofloxacin.

### 1.9.2.3. (Monoclonal antibody)-modified liposomes

Antibodies are frequently used as ligands because they can be applied against a variety of antigens and often exhibit high affinity and selectivity for their antigen. A tumor-specific mAb 2C5 is capable of recognizing a wide variety of tumor cells. This antibody was used to modify the surface of liposome loaded meso-tetraphenylporphine (TPP) for tumor photo-dynamic therapy *in vivo*. The modification of liposome-entrapped TPP by mAb 2C5 resulted in enhancing the efficacy of TPP and increasing the accumulation of the drug in tumor cells (121). However, it has been reported that using whole antibodies may trigger complement-mediated cytotoxicity and antibody-dependent cellular toxicity (199). To prevent these effects, Fab fragment (a region on the antibody that binds to antigens) can be used instead of using the whole antibody. Modifying liposomes with antibody fragments resulted in prolonging the circulation time and enhancing the accumulation of liposomes in solid tumors (200).

### 1.9.2.4. Folate-modified liposomes

Since folate receptors are often overexpressed in tumor cells, targeting these cells with folate-modified liposomes has been successfully applied (201). Indeed, intravenously administered folate-targeted liposome-loaded doxorubicin in mice bearing KB tumors was investigated (202). Surface modification of liposome-loaded doxorubicin with folate increased the accumulation of

drug in tumor tissues and antitumor efficacy of the doxorubicin while prolonging the circulation time.

#### 1.9.2.5. Transferrin-modified liposomes

In addition to antibody- and folate-targeting liposomes for tumor cells, modifying the liposomal surface with transferrin has also attracted attention. Transferrin receptors are overexpressed on the surface of a variety of tumor cells and can be internalized after binding of transferrin to cell receptors (203). Therefore, the coupling of transferrin into the liposome structure offers longevity and the ability for drug delivery into the tumor. Transferrin-PEG-liposome-loaded oxaliplatin injected intravenously into colon tumor-bearing mice was investigated and found that surface modification of liposomal formulations exhibited a long circulation time, low uptake rate by the RES *in vivo*, an enhanced accumulation in tumor cells for over 72 h after administration and suppressed tumor growth (204).

### 1.10. Toxicity of liposomes

Liposomes are usually considered as biocompatible, biodegradable, and relatively non-toxic because they are composed of lipids from natural sources that reduce the toxicity of entrapped bioactive agents. Previous studies showed that liposomes prepared from naturally occurring compounds are not toxic to culture cells *in vitro* (205, 206). However, when toxicity is associated with empty liposomes, it has not resulted from the lipids; rather, toxicity is coupled with the liposomal net charge and presence of volatile organic solvents (154, 206).

A previous study has reported that charged liposomes show highly cytotoxic effects whereas neutral liposomes are not toxic (206). Preparation of liposomes using positively charged lipids

exhibited a highly toxic effect to epithelial lung cells, whereas negatively charged liposomes were relatively not toxic (206). Another study investigated the effect of charged liposomes on the buccal cells and found that positively charged liposomes were toxic; in contrast, negatively charged liposomes exhibited relatively low toxicity (207). The cytotoxicity of charged liposomes has not been completely elucidated. However, it has been proposed that the cytotoxicity results from the intermixing of cationic lipids in liposomes with the anionic lipids of cell organelles such as cardiolipin in the mitochondrial membrane (208). Another study investigated cationic liposome toxicity in the lungs and showed that positively charged liposomes can induce the production of toxic reactive oxygen intermediates (209). Although cationic liposomes are toxic, they play an important role as vaccine delivery systems (210). The majority of liposomal preparation methods require the use of organic solvents or detergents to solubilize the lipids. Residues of these solvents might not be removed completely from the final liposome solution, thereby causing a high potential for cytotoxicity via a number of suggested mechanisms. These mechanisms could include enzyme inhibition, protein denaturation, and cell membrane modification as well as extraction of outer cellular components such as lipids, cholesterol and proteins (211-213). Classical approaches can be applied to ensure solvent removal such as dialysis, evaporation under vacuum and gel filtration in an effort to reduce the cytotoxicity of liposomal formulations.

## 1.11. Application of liposomes

### 1.11.1. *In vitro* studies

Although polymyxin B can control a variety of bacterial infections including those by *P. aeruginosa*, its toxic side effects, including ototoxicity, nephrotoxicity and neuromuscular blockade are barriers to its use (214). However, it has been shown that the application of liposomal formulations could attenuate the side effects of drugs (215). Furthermore, *in vitro* studies on *Bordetella bronchiseptica*, *P. aeruginosa*, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter lwoffii* and *Acinetobacter baumannii* strains showed that encapsulation of polymyxin B in liposomes improved antimicrobial activity and reduced bacterial population in the presence of polyanions and sputum. This can illustrate the general observation that liposomes protect polycationic antibiotics from inactivation by polyanionic components present in sputum (129, 152).

Liposomal formulations consisting of dicetyl phosphate (DCP) or dimyristoyl phosphatidyl glycerol (DMPG) loaded with vancomycin were evaluated *in vitro* against methicillin-resistant *Staphylococcus aureus* (MRSA) (216). Both liposomal formulations showed an improvement in the antimicrobial activity of vancomycin. Minimum inhibitory concentrations (MIC) of the liposomal formulations against MRSA strains were two- to fourfold lower than that of free vancomycin. Likewise, the minimum bactericidal concentrations (MBC) of both liposomal vancomycin formulations were fourfold less as well. Incorporation of vancomycin or ciprofloxacin in cationic, anionic and neutral liposomes improved the efficacy of encapsulated drugs against *S. aureus*. Cationic liposome, however, showed more potent activity than neutral and anionic formulations (217). Another study demonstrated that a cationic liposome-loaded

ciprofloxacin showed two to four times higher antibacterial activity compared with free drug against Gram-negative bacteria including *P. aeruginosa*, *E. coli* and *K. pneumoniae* (135). The enhanced activity of cationic formulation might be explained by the interaction of the negatively charged bacterial cell membrane with the positively-charged liposome surface (218). This interaction led to increased accumulation of encapsulated drugs in the periplasm, allowing a large number of antimicrobial molecules to diffuse in the cytoplasm (135).

Aminoglycosides, including amikacin, netilmicin and tobramycin, loaded into cationic liposomes composed of lecithin, stearylamine and cholesterol or anionic liposomes consisting of lecithin, DCP and cholesterol, were investigated *in vitro* (125). Although the liposomal formulations exhibited a consistent release profile in human pooled sera; however, there was no significant difference in antibacterial activity between encapsulated and free drugs. Absence of enhanced activity of liposomes-encapsulated amikacin, netilmicin and tobramycin was explained in another study by the slow release of the drugs from liposomes, which prevented a sufficient amount of antibiotic to act directly on bacteria (219). However, other studies reported that liposomal aminoglycosides fuse with the outer membrane of *P. aeruginosa* and *Burkholderia cenocepacia* leading to the delivery of a high dosage of aminoglycosides into bacterial cells as confirmed by TEM, immunocytochemistry, lipid mixing assays and flow cytometry (127, 134). In other words, liposomes suppress the drug resistance of infectious organisms by offering a protection for antimicrobial agents from being effluxed (214).

Encapsulation of amikacin, tobramycin and gentamicin in liposomes consisting of dipalmitoyl glycerophosphocholine (DPPC) and cholesterol (in a molar ratio of 2:1) exhibited more potent anti-pseudomonal activity than the free drug (127). The liposome-encapsulated aminoglycoside formulations also improved killing time and prolonged antimicrobial activity. Furthermore, a 64-

fold reduction of the MIC with amikacin (512 mg/l for free drug vs 8 mg/l for liposomal amikacin), a 128-fold reduction with tobramycin (1,024 mg/l for free tobramycin vs 8 mg/l for liposomal tobramycin) and a 16-fold reduction with gentamicin (256 mg/l for free drug vs 8 mg/l for liposomes) were observed (127). Similar results for liposome-loaded gentamicin in the MIC reduction were noted for liposomes consisting of dimyristoyl glycerol phosphocholine (DMPC) and cholesterol (in a molar ratio of 2:1) (220). Furthermore, co-encapsulation of bismuth-ethanedithiol (BiEDT) with tobramycin in liposomes showed a synergistic effect against *P. aeruginosa*, by enhancing its penetration into sputum and inhibiting bacterial growth within the biofilm structure. Moreover, the formulation reduced toxic side effects of BiEDT on lung epithelial cells as indicated by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays (123, 128). We reported that liposomal BiEDT-encapsulated tobramycin reduces *P. aeruginosa* quorum sensing signaling molecules, as well as production of lipase, chitinase, and protease *in vitro*, via a fusion mechanism (120).

The efficacy of liposomal formulations of different surface-charged encapsulated clarithromycin was investigated against *P. aeruginosa* clinical isolates from CF patients. Liposomal formulations improved the MIC and MBC against clinical isolates compared with free formulation. Although using neutral liposomes were more effective than free formulations in reducing bacterial growth, positively and negatively-charged liposomes showed more elimination of the bacterial growth within the biofilm and were more effective in reducing virulence factors and bacterial motility (206).

Liposomal meropenem was also tested against sensitive and resistant strains of *P. aeruginosa* *in vitro* and showed an improvement in bactericidal activity of the encapsulated drug as the MIC results were two to four-times lower than the MICs of free meropenem for sensitive strains

(221). Benzyl penicillin-loaded liposomes inhibited the growth of a penicillin-sensitive strain of *S. aureus* at lower concentration and shorter exposure time than the free drug (222).

Application of liposomes is an effective therapeutic delivery system in fungal infections. Polyene antibiotics such as amphotericin B, are utilized in the treatment of candidiasis and aspergillosis systemic infections. The mechanism of action of amphotericin B involves binding to ergosterol, a major sterol molecule in fungal membranes, resulting in changes to membrane permeability leading to metabolic disruption, osmotic imbalance, and as a consequence, cell death (223). The conventional amphotericin B with deoxycholate as a surfactant is associated with significant toxicities including infusion-related reactions, nephrotoxicity, and hypokalemia (224). However, liposome-loaded amphotericin B appears to have less nephrotoxicity than the conventional formulation and amphotericin B lipid complex (225, 226).

### 1.11.2. *In vivo* studies

Liposomes can alter the distribution of the encapsulated drug molecules in the body, which might extremely improve the efficacy of the treatment. Much interest has been focused on the application of liposomes as drug carriers using *in vivo* studies for treatment of several bacterial and parasitic infections (120, 214, 216, 227).

Encapsulation of aminoglycoside antibiotics have been shown to have a superior effect on treating bacterial infections. A previous study showed that encapsulation of amikacin into liposomes administered intravenously in *Mycobacterium avium* mice infection model resulted in an increase of drug concentration in liver and spleen for up to 28 days (228). Furthermore, a once monthly of liposomal formulation treatments increased the survival time of infected mice up to 7 months, compared with 4 months in the control group. Although liposomal amikacin reduced the bacteria load in the lungs (compared with the control) after a single injection of liposomal

formulation in a month, it did not eliminate bacterial growth due to low drug concentration measured in the lungs.

A previous study showed that labeled liposomes administered intravenously in mice were found in high concentration in the liver and spleen, whereas lower amounts of liposomes reached the lungs (119). However, the bactericidal effect of long circulating liposomal amikacin given intravenously to mice showed two to six times higher antibacterial activity in the lungs compared with free drug (229). Liposomal amikacin showed two to six times higher antibacterial activity in spleen and lungs compared with free drug or combination of free amikacin and streptomycin when they were administered three-times a week. Administration of long circulating sterically stabilized liposomal amikacin to mice infected with *M. avium* showed an elimination of bacteria from infected organs, a decreased treatment period, and prevented relapse of bacterial infection (230). A similar finding is reported with the use of liposomal amikacin in a chronic *P. aeruginosa* lung infection in rats (231). This formulation also resulted in prolonged amikacin residence time in the airway thus allowing longer dosing intervals. Liposomal amikacin loaded into a PARI LC STAR nebulizer was investigated in a healthy male volunteer (182). The study demonstrated that a high percentage (70% of total liposomal formulation delivered to the body) was deposited in the lung and cleared slowly. The treatment was well tolerated with no significant clinical changes, including vital signs and lung function parameters. A randomized placebo-controlled Phase II study conducted in Europe demonstrated that administration of liposomal-loaded amikacin at 280 and 560 mg once-daily for 28 days improved force expiratory volume in 1 second (FEV<sub>1</sub>) versus placebo (232). The liposomal amikacin exhibited a prolonged release of the antibiotic with no observed toxicity. A continuous open-label study of liposomal amikacin administered at 560 mg once-daily for 6 cycles, each cycle consisting of 28 days on,

followed by 56 days off treatment, showed a sustained improvement in lung function (233). There was also a significant reduction in non-mucoid and mucoid strains of *P. aeruginosa* cell counts, even during the 56 days off treatment. There was no significant change in the MICs over the study and Phase III trial was initiated.

Results of investigations into the pharmacokinetic and the bactericidal effect of liposomal tobramycin are promising as well. Intratracheal administration of liposomal tobramycin in animal models of chronic pulmonary infection caused by mucoid *P. aeruginosa* improved the pharmacokinetic of tobramycin and was more effective in eliminating the bacteria (132, 234). We reported a significant improvement in efficacy of co-encapsulated BiEDT and tobramycin administered intratracheally in rats with chronic pulmonary infection caused by a resistant clinical strain of *P. aeruginosa* (120). In addition to improving bactericidal activity, the liposomal formulation showed a sustained concentration of tobramycin at the infection site, a reduced nephrotoxicity, minimal systemic absorption and a modulation of IL-8 levels in the lungs and sera.

CpG oligodeoxynucleotide (CpG ODN) is a synthetic immunomodulator that increases nitric oxide production by macrophages *in vivo* (235). Encapsulated CpG ODN in cationic liposomes consisting of DOTAP exhibited the highest protective effect compared with the free form against *Burkholderia pseudomallei* in mice (236). The intracellular bacterial counts in mice macrophages treated with CpG ODN-DOTAP were reduced by 92% compared to the control, whereas the group treated with CpG ODN alone exhibited a 45% reduction in intracellular bacterial counts. The reduction in levels of intracellular bacteria resulted from increased production of nitric oxide by macrophages. In addition, the liposomes were able to localize in the liver and spleen, where

many infectious organisms reside, rendering liposomes useful for targeting antibiotics to these organs.

Efficacy of liposomal formulations against challenging intracellular mycobacterial infections has also been tested. Encapsulation of rifabutin (RFB) in liposomes exhibited an improvement in bactericidal activity more than free RFB against *M. avium* (237). Likewise, liposomal formulation administered intravenously in *Mycobacterium tuberculosis* infected mice decreased injuries in the liver, spleen and particularly in the lungs. The liposomal formulation also reduced *M. tuberculosis* in the lung significantly compared with free drug. This study concluded that liposomes with high transition temperature were more efficient in enhancing the delivery of RFB. Administration of the drug delivered by lung-specific liposomes of RFB enhanced RFB's anti-tuberculosis effects in mice and significantly reduced its hepatotoxicity (238). When RFB-loaded tuftsin-bearing liposomes were delivered twice weekly for 2 weeks, the load of lung bacilli was effectively lowered at least 2,000 times more than treatment with the free drug *in vivo* (239).

MRSA is one of the challenging infections that worry public health (240). Conventional liposomal vancomycin administered intraperitoneally reduced bacterial infection in murine systemic infection caused by MRSA in kidney and spleen by 100 to 1000 times compared with the control (216). Various pieces of evidence supported the idea that the improved efficacy of liposomal formulation might be due to fusion of liposomes to the bacterial cell wall (127).

Other research has focused on PEGylated surface-modified liposomes-loaded vancomycin over conventional liposomes in treating MRSA infections of the lungs in murine (241). The liposomal formulations showed a sustained vancomycin concentration in plasma for the 48 days of the study, whereas free vancomycin concentration was not detected after 2 h of injection. In

addition, both liposomal formulations significantly improved the biodistribution of vancomycin compared with free agent in liver and spleen; however, surface modification of liposomes significantly increased deposition of vancomycin in the lung compared with conventional liposomes. It is believed that surface modification of the liposomes protected the liposomes from being cleared rapidly by liver and spleen and prolonged the circulation time; therefore, it might be an efficacious treatment approach against MRSA in pneumonia.

The persistence of infection in osteomyelitis due to contamination of bone tissues can be attributed to the rapid removal of antibiotics by the RES of Kupffer cells of the liver, bone marrow and macrophages of the spleen. Liposomal antibiotics may improve efficacy of antimicrobial drugs in treating infections in hard tissues. Intravenous administration of cationic liposomes-encapsulated vancomycin or ciprofloxacin was more effective in reducing infection in rabbit bone caused by *S. aureus* (217). Administration of liposomal formulations for 7 - 14 days resulted in 1.5- or 2- fold reduction in severity of the disease, respectively. The latter time point cured infections in 30% - 50% of the animal population. The combination therapy of liposomal ciprofloxacin and liposomal vancomycin for 14 days, however, cured all infected rabbits with reduced nephrotoxicity. The successful therapy of combining vancomycin and ciprofloxacin in liposomal form might be explained by the ability of liposomes to prolong circulation time of encapsulated drugs and avoid the uptake by macrophages.

A previous study investigated the pharmacokinetic, the biodistribution and the efficacy of single doses of liposomal ciprofloxacin administered intravenously at concentrations ranging between 0.2 and 8 mg/kg in murine model of systemic *Salmonella dublin* infection (242). Liposomal formulation increased mice survival compared with free ciprofloxacin; a single dose of liposomal ciprofloxacin at 1/16 of the highest concentration resulted in survival 60% of

infected mice, whereas free drug at same concentration did not prevent mortality. Administration of a single dose of liposomal formulation at 1/4 of the highest concentration increased murine survival up to 83%, whereas administration of free agent at same concentration for 5 days exhibited 33% murine survival. The liposomal formulation exhibited a sustained drug release and prolonged the persistence of agents for 48 h in liver and spleen, whereas there was no ciprofloxacin detected in liver and spleen at 8 h. The study also showed that liposomal formulation was more effective in reducing bacteria community in liver, spleen, lymph nodes, Peyer's patches and stool. The effectiveness of liposomal ciprofloxacin was dose dependent. Chono *et al.* (243) investigated the pharmacokinetics and pharmacodynamics of pulmonary-administered liposomal ciprofloxacin in rats with lipopolysaccharides-induced pneumonia. Administration of liposomal ciprofloxacin enhanced the distribution of antimicrobial agents in epithelial lining fluid in the lungs compared with free agent and resulted in a sustained release of encapsulated agent throughout the study. Furthermore, the liposomal formulation increased the delivery of ciprofloxacin to alveolar macrophages and reduced systemic toxicity. Pharmacokinetic and pharmacodynamics parameters showed a markedly increased efficacy for liposomal formulation, suggesting that liposomal ciprofloxacin formulation could be a useful treatment to target intracellular and extracellular infection associated with pneumonia.

The infected macrophages have been shown to overexpress mannose receptors that bind and internalize mannose-terminated glycoproteins (244). These receptors can effectively be targeted with appropriate delivery systems. Mannose-grafted liposomes with entrapped amphotericin B are more effective in reducing *Leishmania donovani* than free and unmodified liposome-entrapped amphotericin B. The improved reductive effect can be attributed to enhanced uptake of mannose-coupled liposomes by the mannosyl-fucosyl receptor present on the

macrophage surface. Thus, macrophage-rich organs such as the liver and spleen exhibited an increased accumulation of the drug for prolonged periods of time in *L. donovani*-infected golden hamsters (245). Chagas disease caused by *Trypanosoma cruzi* leading to cardiac failure is one of the worst parasitic diseases in terms of mortality and morbidity. Liposome-encapsulated amphotericin B was investigated in mice infected with *T. cruzi* and found to reduce infections in heart, liver, spleen, skeletal muscle and adipose tissues. Administration of liposomes-loaded amphotericin B in early stages of infection resulted in reduced *T. cruzi* loads in the spleen and liver, allowing the survival of the infected animals (246). A randomized comparative study evaluated the safety and efficacy of intravenously-administered liposomal amphotericin B compared with free formulation in treatment of febrile neutropenia patients (247). Although there was no statistically significant difference in duration of treatment, time to resolve fever and absolute neutrophil count between treatment groups, adverse effects of treatment such as vomiting, nausea, fever and headache were higher with conventional amphotericin B compared with the liposomal formulation. Furthermore, administration of liposomal formulation at 1 mg/kg/day was equally efficacious and well-tolerated at 3 mg/kg/day.

## 1.12 Thesis objective

The aim of this research thesis was to investigate the efficacy of co-encapsulation of bismuth-ethanedithiol and tobramycin into liposomal vesicles at sub-inhibitory concentration against *P. aeruginosa* to reduce quorum sensing signaling molecules and virulence factors, *in vitro*. In addition, the bactericidal efficacy of this liposomal formulation in an animal model of chronic pulmonary infection with *P. aeruginosa* was investigated.

## Chapter 2

# Efficacy of Liposomal Bismuth-Ethanedithiol Loaded Tobramycin after Intratracheal Administration in Rats with Pulmonary *Pseudomonas* *aeruginosa* Infection

**Running Title:** Efficacy of Liposomal BiEDT-Tobramycin.

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Published in Antimicrobial Agents and Chemotherapy; January 2013, 57(1):569-578

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## Abstract

We sought to investigate alterations in the quorum-sensing signal molecule, *N*-acyl homoserine lactone, secretion and in the release of *Pseudomonas aeruginosa* virulence factors as well as *in vivo* antimicrobial activity of bismuth-ethanedithiol incorporated into a liposome-loaded tobramycin formulation (LipoBiEDT-TOB) administered to rats chronically infected with *P. aeruginosa*. The quorum-sensing signal molecule, *N*-acyl homoserine lactone, was monitored by using a biosensor organism. *P. aeruginosa* virulence factors were assessed spectrophotometrically. An agar beads model of chronic *Pseudomonas* lung infection in rats was used to evaluate the efficacy of the liposomal formulation in the reduction of bacterial count. The levels of active tobramycin in the lungs and the kidneys were evaluated by microbiological assay. LipoBiEDT-TOB was effective in disrupting both quorum-sensing signal molecules, *N*-3-oxo-dodecanoylhomoserine lactone and *N*-butanoylhomoserine lactone, as well as significantly ( $P < 0.05$ ) reducing lipase, chitinase and protease production. At 24 h, after 3 treatments, the CFU counts in lungs treated with LipoBiEDT-TOB were of  $3 \log_{10}$  CFU/lungs comparatively to 7.4 and  $4.7 \log_{10}$  CFU/lungs, in untreated and in lungs treated with free antibiotic, respectively. The antibiotic concentration after the last dose of LipoBiEDT-TOB was  $25.1 \mu\text{g/lung}$ , while no tobramycin was detected in the kidneys. As for the free antibiotic, we found  $6.5 \mu\text{g/kidneys}$ , but could not detect any tobramycin in the lungs. Taken together, LipoBiEDT-TOB reduced the production of quorum sensing molecules and virulence factors and could highly improve the management of chronic pulmonary infection in cystic fibrosis patients.

Keywords: cystic fibrosis, aminoglycoside, cytokines, virulence factors, quorum sensing.

## 2.1. Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutation in a cystic fibrosis transmembrane regulator (CFTR) gene that affects organs and systems including the lungs, the pancreas, the endocrine system and the gastrointestinal tract (1). Pulmonary injury is the most challenging medical problem and is responsible for the majority of morbidity and mortality in the CF population (2). There are more than 1500 mutations in CFTR genes with different degrees of disease severity. The mutation in CFTR gene caused by deletion of phenylalanine at position 508, known as  $\Delta F508$ , is more common and causes severe disease due to non-functional chloride ion channels (3). Normal lung epithelial cells keep the epithelial lining fluid of the airways hydrated to ensure appropriate mucociliary clearance of allergens or microbes from the airways (4). Hydration of the mucosal surface of epithelial cells is linked osmotically to sodium transport and chloride secretion. The mutations in CFTR lead to dysfunctional or compromised chloride ion channels as well as hyper-absorption of sodium through sodium channels (ENaC). The resultant thick sticky mucous (5, 6) provides a suitable growth environment for bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* (7-9). Recurrent *P. aeruginosa*- induced pulmonary infection and inflammation is more common and is associated with reduced lung function and disease exacerbation (10, 11).

*P. aeruginosa* is a Gram-negative opportunist human pathogen found in various environments such as fresh water, plants, sinks, hand soaps, and hospitals (12, 13). *P. aeruginosa* cells interact with specific host cell receptors through appendices such as type IV pili, which recognize the over-expressed asialoganglioside (GM1) in CF epithelial cells; and its monotrichous flagellum binds specifically to secreted respiratory mucins (14-16). *P. aeruginosa* utilizes mucus as a

shield against the host immune system and regulates its cell density, virulence factor production, and biofilm formation through quorum sensing (QS) signaling (17-20). The pathogen carries two homologues that control the QS system: the *lasI/lasR* and *rhlI/rhlR* (21). The autoinducer proteins are responsible for synthesizing specific signal molecules. LasI and RhlI synthesize *N*-3-oxo-dodecanoylhomoserine lactone (3O-C<sub>12</sub>-HSL) and *N*-butanoylhomoserine lactone (C<sub>4</sub>-HSL), respectively; whereas, LasR and RhlR function as transcriptional activator proteins (22-24). Bacteria release 3O-C<sub>12</sub>-HSL at certain cell density into the external environment, where it binds to LasR forming a complex that binds promoters to induce a wide variety of virulence factors, including lipase, chitinase, and proteases (25-29). Activation of airway epithelial cells signaling pathways, in response to *P. aeruginosa* pulmonary infection, results in gene expression and secretion of several cytokines and chemokines including IL-8, a potent chemoattractant of neutrophil (30). While neutrophils eradicate bacteria, their toxic products, such as elastase and reactive oxygen radicals, in the airway, damage the lung tissue as well (31).

Aggressive chemotherapy, through various routes, has been utilized to decrease the persistence of *P. aeruginosa* in lungs (32, 33). Administration of aminoglycosides such as tobramycin, along with  $\beta$ -lactams, is usually prescribed against *P. aeruginosa* to reduce infection (34, 35). Tobramycin at sub-inhibitory concentration reduces production of *P. aeruginosa* virulence factors at translation level by inhibiting the release of C<sub>4</sub>-HSL and 3O-C<sub>12</sub>-HSL levels (36, 37). However, since a high dosage and prolonged use of tobramycin are required to eradicate bacteria, a high risk of ototoxicity and nephrotoxicity exists (34). Furthermore, the presence of mucus, overexpression of multidrug efflux pumps, and bacterial transition to biofilm form result in a poor prognosis (38-41).

Bismuth subsalicylate and bismuth subcitrate have been used for years to treat gastrointestinal disorders associated with *Helicobacter pylori* (42). A combination of bismuth and thiol agents increases the bismuth solubility, lipophilicity, and its anti-microbial activity against Gram-positive and Gram-negative bacteria (43). Huang and Stewart have shown that bismuth dimercaprol was able to reduce biofilm formation by *P. aeruginosa* (44). Bismuth-ethanedithiol (BiEDT) along with tobramycin have a synergistic effect against *P. aeruginosa* and *Burkholderia cepacia in vitro* (45, 46). The cytotoxic effects of bismuth, however, limit its utility. BiEDT, at concentrations of 10 and 20  $\mu\text{M}$ , render human lung cells in culture nonviable (47). Microcarriers such as liposomes are used to overcome toxicities of the drugs, to sustain the release of drugs at the target site, and to prolong their residence time (48, 49).

Liposomes are small lipid vesicles with a size ranging from nanometers to micrometers. They are generally a safe delivery system since liposomes are biocompatible and biodegradable. They consist of phospholipid bilayers with an aqueous core. Hydrophilic drugs can be encapsulated in the aqueous core, whereas lipophilic drugs can be incorporated into the bilayers. Recently, more research has focused on utilizing liposomes to deliver therapeutic molecules to target sites including the lungs (50). Liposomes are preferred for antibiotic delivery because they provide a sustained release of the drugs and reduce side effects, as well as increasing the bioavailability of insoluble hydrophobic drugs (50, 51). Previous studies in our laboratory have shown that coencapsulation of BiEDT with tobramycin in liposomes resulted in elimination of the BiEDT toxic effect on human lung cells while increasing its antibacterial efficacies against *P. aeruginosa* and *B. cepacia* (52, 53).

The current study was performed to test whether liposomal BiEDT loaded tobramycin (LipoBiEDT-TOB) at sub-inhibitory concentrations is able to reduce production of virulence

factors and QS signal molecules by *P. aeruginosa in vitro* and to enhance the antimicrobial efficacy as well as to examine anti-inflammatory effect of LipoBiEDT-TOB on the animal model of chronic pulmonary infection with the aforementioned bacteria.

## 2.2. Materials and methods

### 2.2.1. Chemicals and media

1, 2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Northern Lipids, (Vancouver, BC, Canada). Cholesterol, bismuth nitrate [Bi (NO<sub>3</sub>)<sub>3</sub>·5H<sub>2</sub>O], 1, 2-ethanedithiol (EDT), propylene glycol (PG), heavy mineral oil, xylazine, saline, chitin azure, β-D-galacopyranoside and Triton X-100 were obtained from Sigma–Aldrich (Oakville, ON, Canada). Sodium hydroxide (NaOH), sodium deoxycholic acid (C<sub>24</sub>H<sub>39</sub>O<sub>4</sub>Na), tobramycin, chloroform and methanol were purchased from Fisher Scientific (Ottawa, ON, Canada). Tryptic soy agar, Tryptic soy broth, Luria-Bertani (LB) broth, Luria-Bertani agar and Mueller-Hinton agar were purchased from Becton Dickinson Microbiology Systems (Oakville, ON, Canada). Ketamine was obtained from Animal Health Inc.(Cambridge, ON, Canada).

### 2.2.2. Bacterial strains

PA-489122 strains of *P. aeruginosa* were used throughout the experiment and had been isolated from CF patients at Sudbury Regional Hospital (Sudbury, Ontario, Canada). *Staphylococcus aureus* ATCC 29213 was used as an indicator of tobramycin activity, as recommended by the Clinical and Laboratory Standards Institute (CLSI). All strains were stored in Mueller-Hinton broth at -80°C supplemented with 10% glycerol. All strains were grown for 18 h in ABt medium (27 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 30 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 20 mM KH<sub>2</sub>PO<sub>4</sub>, 47 mM

NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 0.01 mM FeCl<sub>2</sub>, 0.5% w/v glucose, 0.5% w/v casamino acids and 0.00025% w/v of thiamine) broth prior to the MIC, QS, and virulence factors experiments. *Agrobacterium tumefaciens* strain A136 (pCF218)(pCF372) (Ti<sup>-</sup>) was used as the biosensor for the detection of AHL and cultured in Luria-Bertani (LB) broth at 30°C.

### 2.2.3. LipoBiEDT-TOB preparation

A dehydration-rehydration method was used to prepare liposomal bismuth tobramycin. To prepare BiEDT, Bi (NO<sub>3</sub>)<sub>3</sub> and 600 mM of NaOH were dissolved first in 25 mL of methanol. One mL of EDT was then added to the mixture. To prepare the liposome vesicles, DSPC and cholesterol (2:1) molar ratio were transferred into a round flask and dissolved in 19 mL of chloroform: methanol (2:1) molar ratio. One mL of ethanedithiol bismuth was then added to the round flask. The organic solvents were removed by using rotary vapor (Buchi Rotavapor R205, Buchi vacuum controller V-800; Brinkman, Toronto, Ontario, Canada) under vacuum at 55°C, which resulted in a thin layer of lipid. The lipid film was rehydrated by adding 12 mL of PBS with hand shaking for 5-7 min in a water bath at 55°C until it became a suspension. The suspension lipids were then sonicated at an amplitude of 50% (Sonic Dismembrator Model 500, Fisher Scientific, Ottawa, ON, Canada) for 10 min (40 sec ON and 5 sec OFF). Tobramycin (8 mg/mL) and PG were added to the sonicated suspension. The solution was then sonicated again for 10 min (40 sec ON and 5 sec OFF). The sonicated liposomes were transferred to 15 mL tubes and frozen for 15 min at -70°C and then freeze-dried overnight (Freeze Dry System model 77540, Labconco Corporation, Kansas City, MO, USA). The powdered liposomes were stored at 0°C. To rehydrate the powder formulation, sterile distilled water was added in the volume of 10% of the volume before lyophilisation, vortexed, and then the mixture was incubated for 30 min at 45°C; then PBS was added to restore the original volume. The solution was centrifuged

(Beckman L8-M Ultracentrifuge) for 20 min, at 100,000 ×g and 4°C and the supernatant was removed. This step was repeated by PBS as described previously (52). The size of liposomes was determined by a Submicron Particle Sizer, Model 270 (Nicomp, Santa Barbara, CA, USA).

#### 2.2.4. Tobramycin encapsulation efficiency (EE) within LipoBiEDT-TOB formulation

The concentrations of tobramycin incorporated into LipoBiEDT were measured by an agar diffusion assay using laboratory strains of *S. aureus* (ATCC 29213) as an indicator organism for tobramycin. We used an overnight culture of the organisms in cation-adjusted Mueller-Hinton broth (CAMH) to prepare a bacterial solution equivalent to 0.5 McFarland ( $1.5 \times 10^8$  bacterial/mL). The bacterial suspension in warm (45°C) Mueller-Hinton agar was then poured into a sterile glass plate (460 mm×360 mm) and left to solidify at room temperature. Wells of 5 mm diameter were made with a well puncher. Standard curves of diluted tobramycin as well as samples of LipoBiEDT-TOB were prepared. Triplicate samples (25 µL) were transferred into the agar plate holes. The plate was covered and incubated for 18 h at 37°C. We then measured the inhibition zones and the averages of triplicate measures were used in data analysis. The standard curve was utilized to calculate concentrations of the entrapped tobramycin that were released from the liposomes by 0.2% (vol/vol) Triton X-100 (with PBS). The sensitivity of the assay was 0.75 µg/ml. The quantifiable limit for tobramycin was 0.75 µg/ml. At concentrations from 0.75 to 12.5 µg/ml, the coefficients of variation (ratio of standard deviation to the mean in percentage) ranged between 1.2 and 2.9%. Over the same concentrations, the intraday coefficients of variation ranged between 2.2 and 3.5%. For 10 samples of spiked tobramycin, the standard curve linearity extended over the range 0.75 to 12.5 µg/ml gave a correlation coefficient greater than

0.999. Concentration measurements were the means of at least three independent experiments, with each experiment measured in triplicate.

#### 2.2.4.1. Encapsulation efficiency

The drug encapsulation efficiency (expressed as a percentage) was calculated by dividing the concentration of LipoBiEDT-TOB (determined by the microbiological assay as described in section 2.2.4.) by the concentration of free tobramycin used in the original preparation of these liposomes.

#### 2.2.5. Determination of the minimum inhibitory concentrations (MICs)

The micro-broth dilution method was used to determine the MICs for tobramycin. Briefly, the reference strain *S. aureus* or clinical isolates of *P. aeruginosa* PA-489122 were exposed to different dilutions of LipoBiEDT-TOB or a combination of tobramycin with BiEDT. The contribution of bismuth-ethanedithiol to the MICs was assessed by exposing the aforementioned bacterial strains to different concentrations of BiEDT-TOB and LipoBiEDT-TOB, with a starting concentration of 128 mg/L for tobramycin as well as 128  $\mu$ M for BiEDT in the LipoBiEDT-TOB and free BiEDT-TOB as reported previously (123). Drug-free bacterial cultures and an ABt broth medium alone were used as positive and negative controls respectively.

#### 2.2.6. Quantification of bismuth in liposomal formulations

The bismuth content within the LipoBiEDT-TOB formulation was measured by graphite furnace atomic absorption spectroscopy (GFAAS) as described previously, with some modifications (52). To simplify, samples were lyophilized, weighed, and then transferred into

Teflon digestion vessels. A total of 1 mL H<sub>2</sub>O<sub>2</sub> (30%, w/w) and 4 mL HNO<sub>3</sub> was added and the samples were digested overnight at 25°C. Samples were then subjected to hot-plate digestion in a glycerol bath at 135–140°C for 3 h and left overnight. Next, the volumes were adjusted to 25 mL with double distilled water. 1.25 mL from each digested sample was then subjected to 20-fold dilution with 2% HNO<sub>3</sub>. Samples were then analyzed by GFAAS (AAAnalyst 600, Perkin Elmer Precisely, Woodbridge, ON, Canada).

### 2.2.7. Evaluation of QS and virulence factor production and activity

PA-489122 was grown in ABt medium for 18 h at 37°C; then the bacteria solution was adjusted to follow the 0.5 McFarland standard (optical density at 600 nm [OD<sub>600</sub>], 0.13) in a 100 ml flask and incubated for 1 h at 37°C for experiments involving QS signal molecules, lipase, chitinase, and protease. When the bacterial concentration doubled to OD<sub>600</sub>=0.26, the solutions were exposed to an equal volume of free or liposomal BiEDT-TOB (1/16-1/2 MICs). Untreated *P. aeruginosa* PA-489122 served as control. After 24 h, bacterial cultures were measured and centrifuged, at 16000 ×g for 15 min at 4°C and filter sterilized (0.22 μm). To test that there were no killing effect of the antibiotic on bacteria at concentrations below the MICs, free or liposomal BiEDT-TOB (1/4 and 1/2 MICs) was introduced to a PA-489122 culture that had been adjusted to 0.5 McFarland standard in 100 ml flasks and incubated at 37°C with agitation (250 rpm). The growth was monitored (OD<sub>600</sub>) for 8 h.

### 2.2.8. Bioassay for AHL production

Supernatant samples were screened for AHL production as described previously with some modification (54). *A. tumefaciens* strain A136 (pCF218)(pCF372) (Ti<sup>-</sup>) cells equal to density of 10<sup>6</sup> CFU/ml with  $\beta$ -D-galactopyranoside (20 mg/ml in dimethylformamide) and LB agar at 45°C were poured into Petri dishes. Wells of 5 mm diameter were made with a well puncher and aliquots (80  $\mu$ l) from control or treated supernatant samples were transferred to the wells. The Petri dishes were incubated for 48 h at 30°C. AHL production levels were confirmed by blue pigmentation around the wells.

### 2.2.9. $\beta$ -Galactosidase activity assay

The level of AHL production from *P. aeruginosa* exposed to free or LipoBiEDT-TOB at sub-inhibitory concentrations was examined by measuring the ability of *P. aeruginosa* AHL signaling molecules released in the supernatants to activate the production of  $\beta$ -galactosidase in the reporter strain *A. tumefaciens* (A136) as described previously (55). Briefly, bioassay tubes containing 4 mL of reporter strain and 1 mL of supernatant were incubated at 30°C in water bath for 5 h with rotation at 100 r.p.m. Next, bacterial cell density was measured at (OD<sub>600</sub>) before centrifugation. The supernatants were removed, and the pellets were suspended in an equal volume of Z buffer (0.06 M Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 0.04 M NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.01 M KCl, 0.001 M MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05 M  $\beta$ -mercaptoethanol, PH 7.0). The cells were then permeabilized by a solution of 200  $\mu$ L of chloroform and 100  $\mu$ L of 0.1 % sodium dodecyl sulphate prior to the addition of 0.4 mL of *O*-nitrophenol- $\beta$ -D-galactopyranoside (4 mg/mL in PBS). After the development of yellow color, 1 mL of 1M Na<sub>2</sub>CO<sub>3</sub> was added to stop the reaction. Optical density of the reaction samples was measured at 420 and 550 nm. Miller units of  $\beta$ -Galactosidase

were calculated as  $(1000 \times A_{420\text{nm}}) - (1.75 \times A_{550\text{nm}}) / (\text{time} \times \text{volume} \times A_{600\text{nm}})$  as described previously (56).

### 2.2.10. Virulence factor assays

Lipase activity was evaluated using Tween 20 as substrate. Briefly, the reaction mixture consisted of 0.6 mL of 10% Tween 20 in Tris buffer, 0.1 mL of 1 M  $\text{CaCl}_2$ , 0.6 mL of filtered supernatant, and 1.6 mL of double distilled water. Samples were incubated at 37°C for 24 h with agitation (200 rpm). In the presence of lipase, Tween 20 is broken down to a fatty acid and alcohol. The fatty acid binds calcium to form a precipitate that was measured at ( $\text{OD}_{400}$ ). For chitinase, 1 mL of filtered supernatant was mixed with 1 mL of PBS and 5 mg of insoluble chitin azure. The reaction mixture was incubated at 37°C for 24 h with agitation (200 rpm). The cleaving of chitin azure in the presence of chitinase results in the release of a blue-coloured dye that can be measured spectrophotometrically at ( $\text{OD}_{290}$ ). The lipase and chitinase experiments were repeated three times with three replicates and the results were normalized by dividing the optical density by cell density ( $\text{OD}_{600}$ ). For the protease assay, 100  $\mu\text{L}$  of filtered supernatants were transferred into the wells of an ABt medium containing 2 % agar and 2 % skim milk. Plates were incubated for 48 h at 37°C. Zones of clearance due to the proteolytic activity of protease could be easily perceived and were measured in (millimeters) by using digital callipers. The experiments were repeated three times with three replicates.

### 2.2.11. Preparation of agar beads

Agar beads were prepared as described previously with some modifications (57). The *P. aeruginosa* PA-489122 strain was grown overnight at 37°C in a tryptic soy broth. The bacteria were then embedded into agar beads by mixing 2% v/v of the aforementioned strain with tryptic

soy agar and mineral oil (1:3 volume ratio) at 45°C. The mixture was then vortexed vigorously and cooled down by placing crushed ice around the vessel while stirring continuously for 5 min. Next, the mineral oil was removed by centrifugation at slow speed for 5 min, 500×g at 4°C. Agar beads were washed once with 0.5% sodium deoxycholic acid, once with 0.25% sodium deoxycholic acid, and three times in PBS for 20 min, 1,000×g at 4°C. The number of bacteria was determined after homogenizing the bacteria-impregnated bead suspension. The bacterial count was ascertained by working 10-fold serial dilutions in phosphate-buffered saline (PBS) on Mueller-Hinton agar plates as described previously (58).

### 2.2.12. Experimental infection and LipoBiEDT-TOB treatment

Fifteen Sprague-Dawley rats weighing 201-225 g (Charles River, Saint Constant, Quebec, Canada) were used in this study. The animals were housed (Nalgene® cages) in groups of three for 1 week before any experiment was undertaken and allowed free access to food and water. Animals were kept at room temperature and were exposed to alternate cycles of 12 hours of light and darkness. Animals used in this study were treated and cared for in accord with the guidelines recommended by the Canadian Council on Animal Care and the Association for Assessment and Accreditation of Laboratory Animal Care. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

To mimic the chronic respiratory tract infection caused by *P. aeruginosa*, PA-489122 was incorporated into agar beads. Animals were anesthetized with a mixture of 70 mg/kg of ketamine hydrochloride and 7 mg/kg of xylazine by intraperitoneal injection before infection and placed in the supine position, and the upper jaw was attached to the operating table with a rubber band brought over the incisor teeth. Using a laryngoscope, the tongue was moved aside and the mouth was opened. The larynx was identified and distinguished by its opening and closing as the rat

breathed. A catheter was inserted between the vocal cords and pushed gently forward into the trachea. The catheter's insertion was confirmed by the formation of water condensation on a cold mirror with each breath of the rat. The rats were then inoculated with 50  $\mu$ L of agar beads containing  $10^6$  CFU of *P. aeruginosa* at the bifurcation of the trachea with a 1mL tuberculin syringe followed by a bolus of air to ensure complete delivery.

Four days after the inoculation with agar beads, the rats were anaesthetized using the same procedure as above to be treated with antibiotics. The rats were sorted to three groups and each group was administered either saline, BiEDT-TOB or LipoBiEDT-TOB for three days. Infected animals received a dose (same method as described for infection) of either 300 mg/L of LipoBiEDT-TOB per kg or 300 mg/L of BiEDT-TOB per kg. The concentration of tobramycin in free or LipoBiEDT-TOB was 300 mg/L and BiEDT in liposomal as well as the combination with tobramycin was 300  $\mu$ M. Saline (90  $\mu$ L) was administered to the infected control animals. Twenty four hours after the last treatment, the animals were euthanized by using CO<sub>2</sub>. The kidneys and the lungs were removed aseptically and homogenized in cold sterile PBS (33% [wt/vol]) for 40 s with a Polytron Homogenizer. The homogenizer was rinsed, immersed in 95% ethanol, flamed, and then cooled down with cold saline between samples. Lung bacterial counts were performed after homogenizing the lungs. Serial 10-fold dilutions of the homogenates in cold PBS were made, and 0.1 mL of each dilution was pipetted and spread on Mueller-Hinton agar. The experiment was done in triplicate and the bacterial counts for each animal were done in triplicate. CFU were counted after 24 h incubations at 37°C and counts were expressed in log<sub>10</sub> CFU per pair of lungs. To measure the quantity of active tobramycin in tissues, the tissue samples were concentrated as follows: 1mL samples of homogenized lungs or kidneys were lyophilized (Freeze Dry System model 77540, Labconco Corporation, Kansas City, MO, USA)

and rehydrated with 100  $\mu$ L of sterile PBS. The presence of active tobramycin was detected by an agar diffusion assay as described above in 2.4.

### 2.2.13. IL-8 assay

Supernatants from sera and the lung homogenates samples were used to quantify secreted IL-8 protein. A 96-well plate was pre-coated with IL-8 capture antibody (primary antibody) overnight. A wash buffer, consisting of 1x PBS and Debecos buffered salts, was used between each step to rinse excess reagents from the treatment plate according to the manufacturer's protocol (BioLegend, San Diego, CA). Next, the protein blocking agent was added to each well of the 96-well plate. The blocking agent was allowed to sit in the wells for one hour while on a shaker (200 rpm) at room temperature. The assay diluent was removed from plate with wash buffer. A standard curve was made with 1:2 serial dilution of known IL-8 antigen. Supernatants were spun in a microcentrifuge for 10 min, at  $106 \times g$  and  $22^{\circ}C$ . The supernatants were added to the well for two hours while shaking. The plates were washed before adding the detection antibody for one hour on a shaker. Detection antibody was washed and Avidin-HRP was added to sit in well for thirty minutes while shaking in the dark. The Avidin-HRP was washed with wash buffer and tetramethylbenzidine substrate Solution C was added for 15 min in the dark without shaking. A 2N  $H_2SO_4$  solution was added to the mixture to stop the reaction. The plate was read with the Beckman Coulter AD 340 microplate reader (Beckman, Brea, CA). Data were normalized and IL-8 concentrations reported in pg/mL.

## 2.2.14. Data analysis

The data presented as mean  $\pm$  S.E.M. of three independent experiments. Comparisons of groups were made by one-way analysis of variance (ANOVA) using InStat 3 from GraphPad (GraphPad Software Inc., Version 5.0) followed by a post t-test. Probability values of  $*P<0.05$ ,  $**P<0.01$  and  $***P<0.001$  are reported as statistically significant.

## 2.3. Results

### 2.3.1. LipoBiEDT-TOB characterization

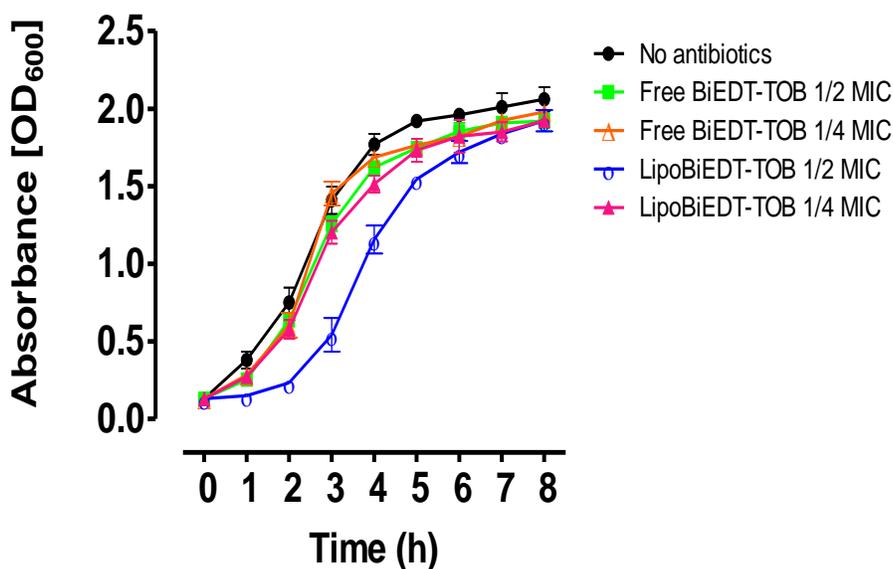
The average size of the LipoBiEDT-TOB formulation was  $907.3 \pm 40.1$  nm and the encapsulated tobramycin in the LipoBiEDT formulation was  $1.0 \pm 0.2$  mg/mL. The percentage of tobramycin that was encapsulated into liposomes  $14.40 \pm 0.001\%$ . Atomic absorption analysis showed that the concentration of bismuth incorporated into LipoBiEDT-TOB formulation was  $1.0 \pm 0.3$  mM.

### 2.3.2. Antimicrobial activity of free and LipoBiEDT-TOB

The MICs of LipoBiEDT-TOB formulation against the *P. aeruginosa* strain used in this study was 16-fold lower than tobramycin alone or fourfold lower than tobramycin in combination with BiEDT. For example, the MIC of tobramycin alone was 16 mg/L, whereas BiEDT-TOB was 4 mg/L for tobramycin and 4  $\mu$ M for BiEDT in free BiEDT-TOB compared to 1 mg/L for tobramycin and 1  $\mu$ M for BiEDT in LipoBiEDT-TOB.

### 2.3.3. Effect of sub-inhibitory concentrations of free or LipoBiEDT-TOB

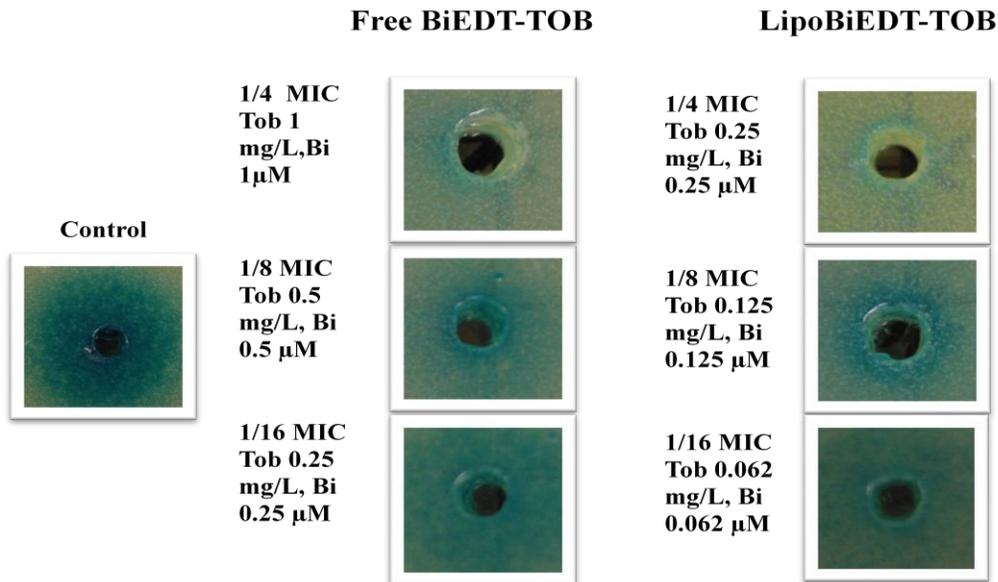
The effect of concentrations (1/4-1/2 MICs) of free BiEDT or LipoBiEDT-TOB on bacterial growth are shown in (Fig. 1). The rate of growth of cells was inhibited when treated with 1/2 of the MIC of the liposomal formulation, therefore this formulation at that concentration is not considered as sub-inhibitory for further investigation. Thus, all the experiments that involve QS and virulence factors were done using concentrations of 1/16-1/4 of the MICs of free or LipoBiEDT-TOB.



**Figure 1: The effect of sub-inhibitory concentrations of BiEDT-TOB on the growth of PA-489122. Growth curve of *P. aeruginosa* without antibiotics (filled circles), in the presence of sub-inhibitory concentrations of 1/2 MIC of LipoBiEDT-TOB [0.5 mg/L of TOB][0.5  $\mu$ M of BiEDT] (open circle), 1/4 MIC of LipoBiEDT-TOB [0.25 mg/L of TOB][0.25  $\mu$ M of BiEDT] (filled triangle), 1/2 MIC of free BiEDT-TOB [2 mg/L of TOB][2  $\mu$ M of BiEDT] (filled squares), or 1/4 MIC of free BiEDT-TOB [1 mg/L of TOB][1  $\mu$ M of BiEDT] (open triangle).**

### 2.3.4. QS molecule reduction

*P. aeruginosa* PA-489122 was grown in an ABt medium for 24 h at 37°C with or without free or liposomal BiEDT-TOB at 1/16-1/4 MICs. Both formulas have reduced AHL production up to 1/16 MIC compared to control, but did not prevent the production completely (Fig. 2). However, LipoBiEDT-TOB reduced AHL production at sub-inhibitory concentrations that were 4 times lower than for free BiEDT-TOB. At 1/4 MICs of free BiEDT-TOB, the production of the blue pigment ring around the edge was darker and more clear than 1/4 MIC of LipoBiEDT-TOB.



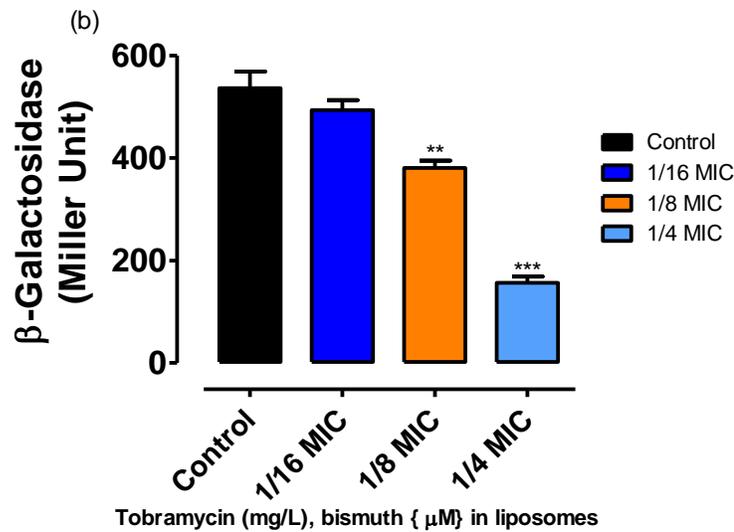
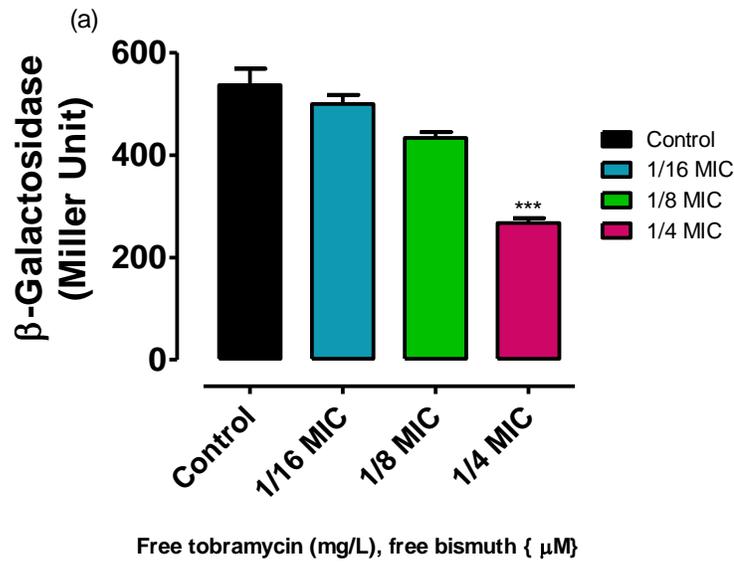
**Figure 2: Effect of sub-inhibitory concentrations of free or LipoBiEDT-TOB (1/16-1/4 MICs) on QS. LB agar containing *Agrobacterium tumefaciens* and  $\beta$ -D-galactopyranoside was poured in Petri dishes. Holes were made in the agar by a vacuum device and 80  $\mu$ l from control or treated supernatant samples were transferred to the wells. The plates were incubated for 48 h at 30°C.**

### 2.3.5. AHL quantification

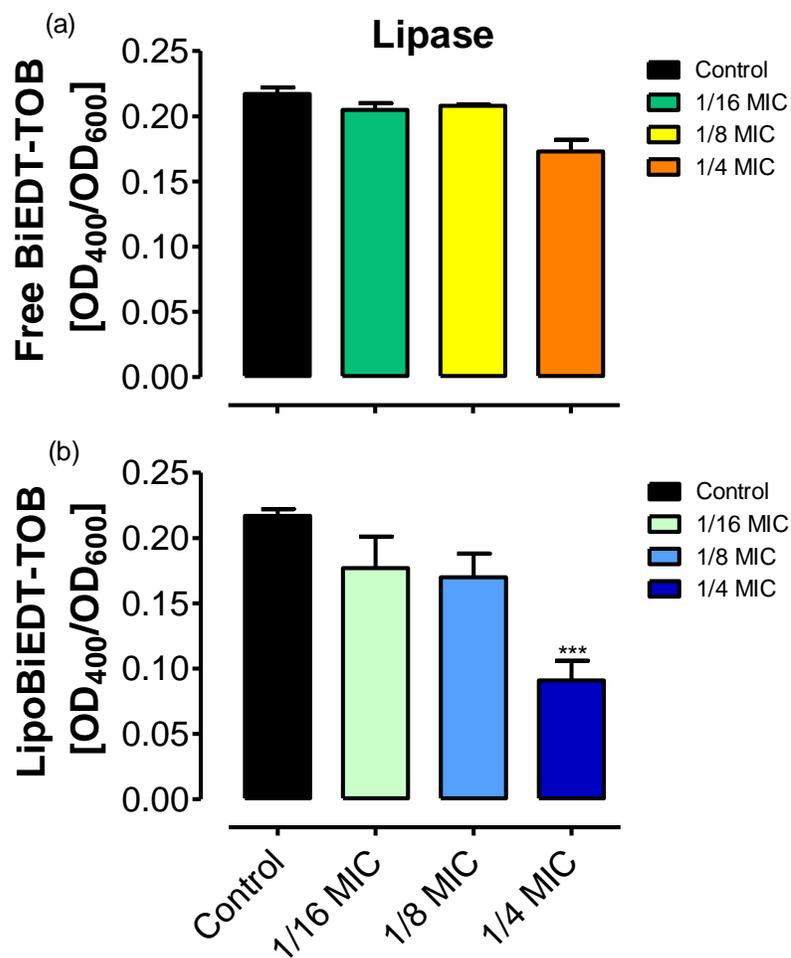
The levels of  $\beta$ -galactosidase activity, in response to AHL, indicated a decrease in the levels of AHL signaling molecules released from *P. aeruginosa* cells exposed to LipoBiEDT-TOB (Fig. 3a and b). For instance, free BiEDT-TOB at 1/8 MIC did not reduce the level of AHL significantly, whereas LipoBiEDT-TOB reduced the level of AHL, at 1/8 MIC significantly ( $P<0.01$ ) compared to the control. LipoBiEDT-TOB was significantly more active in reducing AHL production than free BiEDT-TOB at 1/4 MIC ( $P<0.001$ ).

### 2.3.6. Reduction of virulence factors by BiEDT-TOB

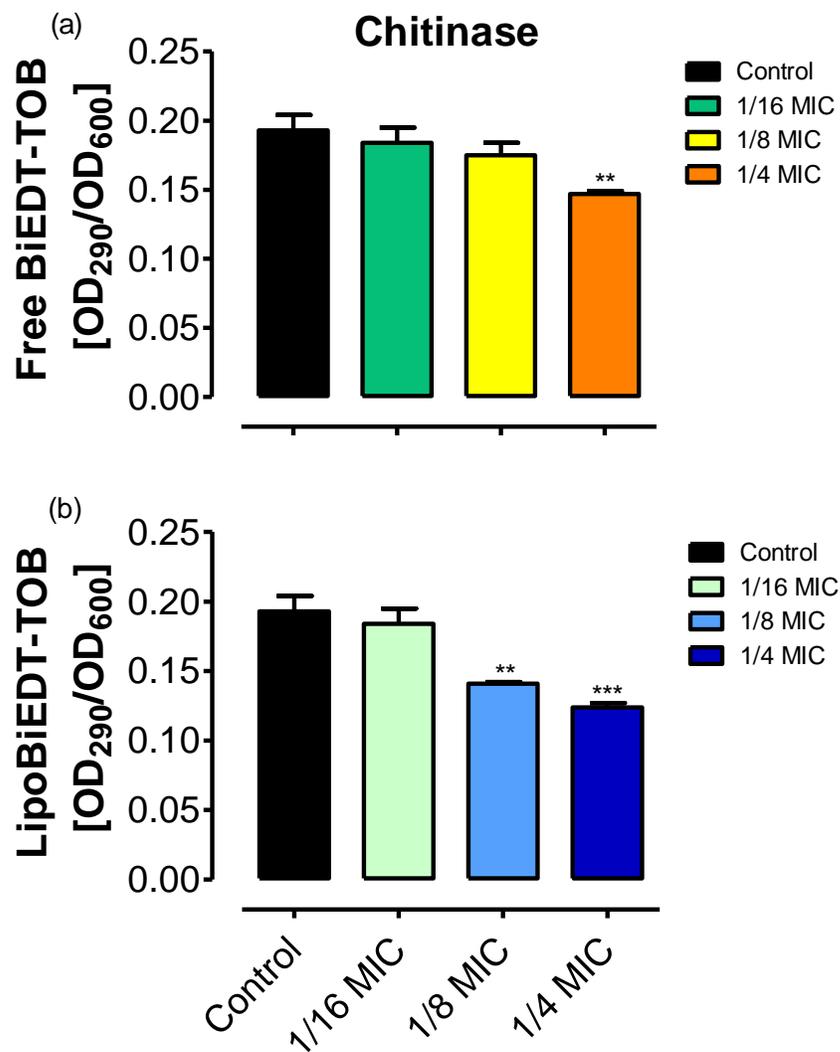
We compared the effects of free and liposomal BiEDT-TOB at 1/16-1/4 MICs on production of the virulence factors, lipase, chitinase, and protease by PA-489122. For the lipase assay, free BiEDT-TOB at 1/4 of the MICs did not reduce the production of lipase significantly compared to the control (Fig. 4a). LipoBiEDT-TOB at 1/4 MIC attenuated lipase production significantly compared to the control ( $P<0.001$ ) (Fig. 4b). Chitinase production in the supernatants was evaluated by quantifying the breakdown of chitin azure. As shown in (Fig. 5a and b), the liposomal formulation was able to reduce chitinase at a concentration 8 times lower than with free BiEDT-TOB (1/8 vs. 1/4) and more effectively ( $P<0.01$ ) than the free formulations. The activity of extracellular protease LasA in filtered sterilized supernatants was measured in agar plates containing 2% skim milk. Free BiEDT-TOB reduced the protease level at 1/4 of the MICs ( $P<0.01$ ) compared to the control, whereas LipoBiEDT-TOB attenuated activity significantly at 1/4 MIC ( $P<0.001$ ). Furthermore, protease activity was able to be reduced by LipoBiEDT-TOB at a concentration eightfold lower than the free formulation (1/8 vs. 1/4) ( $P<0.001$ ) as indicated in (Fig. 6a and b).



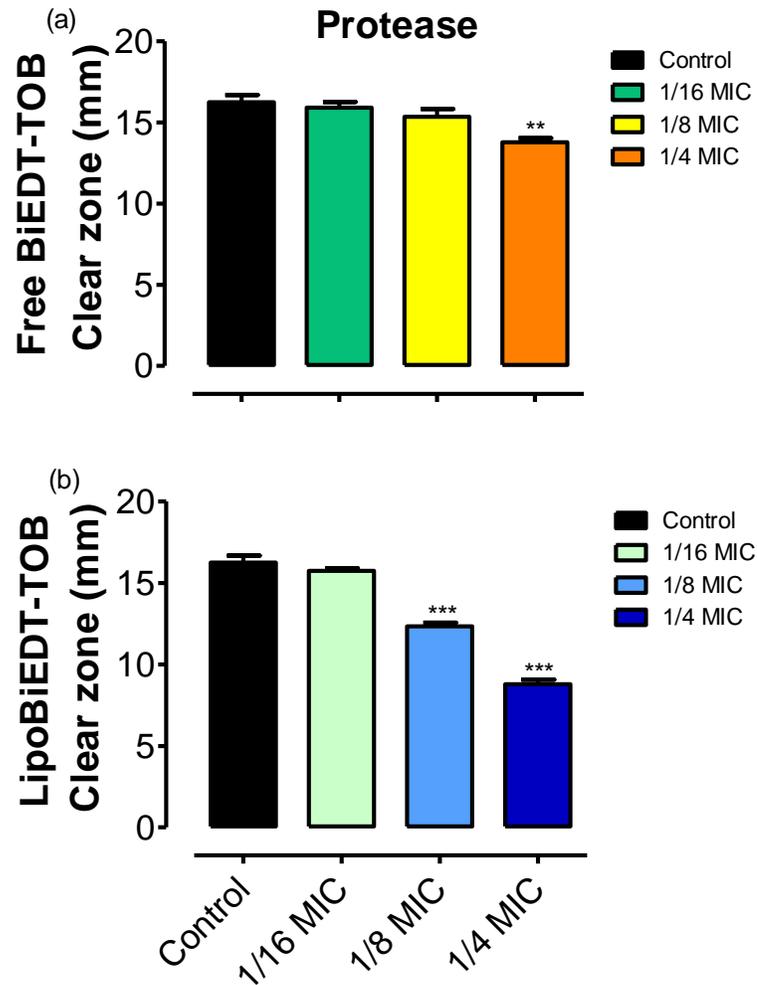
**Figure 3: Production of the QS molecules as measured by  $\beta$ -galactosidase activity: (a) in the presence of free BiEDT-TOB or (b) in the presence of LipoBiEDT-TOB at 1/16 -1/4 MICs. *P. aeruginosa* was exposed to free and LipoBiEDT-TOB then the supernatants were collected and incubated with *A. tumefaciens* (A136).  $\beta$ -Galactosidase activities were measured in miller units. Each bar represents the mean  $\pm$  S.E.M. of three independent experiments. P values were considered significant when compared with the control and between groups: \*\*\*P<0.001, \*\*P<0.01, and \*P<0.05.**



**Figure 4: Lipase activities in supernatant from PA-489122. Cultures grown either without antibiotics as control and (a) in the presence of 1/4 MIC of free BiEDT-TOB[1 mg/L of TOB][1  $\mu$ M of BiEDT], 1/8 MIC of free BiEDT-TOB[0.5 mg/L of TOB][0.5  $\mu$ M of BiEDT] or 1/16 MIC of free BiEDT-TOB[0.25 mg/L of TOB][0.25  $\mu$ M of BiEDT] or (b) in the presence of 1/4 MIC of LipoBiEDT-TOB[0.25 mg/L of TOB][0.25  $\mu$ M of BiEDT], 1/8 MIC of LipoBiEDT-TOB[0.125 mg/L of TOB][0.125  $\mu$ M of BiEDT], 1/16 MIC of LipoBiEDT-TOB[0.062 mg/L of TOB][0.062  $\mu$ M of BiEDT]. Each bar represents the mean  $\pm$  S.E.M. of three independent experiments. P value was considered significant when compared with the control: \*\*\*p<0.001.**



**Figure 5: Chitinase activities in supernatant from PA-489122. Cultures grown either without antibiotics as control and (a) in the presence of 1/4 MIC of free BiEDT-TOB[1 mg/L of TOB][1  $\mu$ M of BiEDT], 1/8 MIC of free BiEDT-TOB[0.5 mg/L of TOB][0.5  $\mu$ M of BiEDT] or 1/16 MIC of free BiEDT-TOB[0.25 mg/L of TOB][0.25  $\mu$ M of BiEDT] or (b) in the presence of 1/4 MIC of LipoBiEDT-TOB[0.25 mg/L of TOB][0.25  $\mu$ M of BiEDT], 1/8 MIC of LipoBiEDT-TOB[0.125 mg/L of TOB][0.125  $\mu$ M of BiEDT], 1/16 MIC of LipoBiEDT-TOB[0.062 mg/L of TOB][0.062  $\mu$ M of BiEDT]. Each bar represents the mean  $\pm$  S.E.M. of three independent experiments. P values were considered significant when compared with the control and between groups: \*\*\*P<0.001, \*\*P<0.01.**



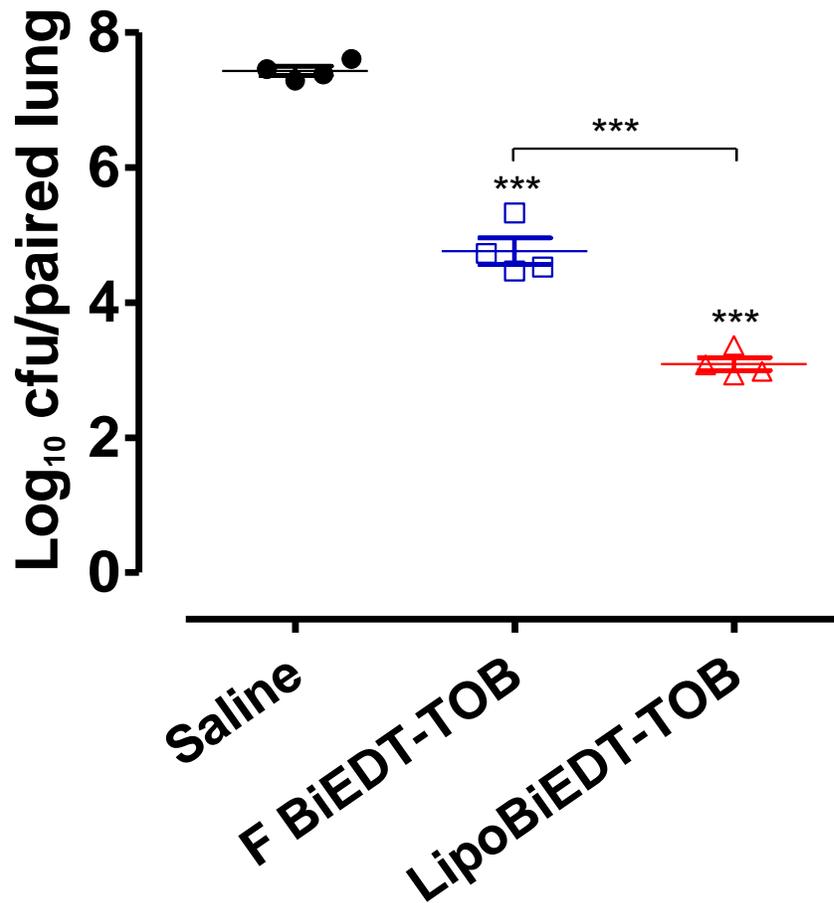
**Figure 6: Protease activities in supernatant from PA-489122. Cultures grown either without antibiotics as control and (a) in the presence of 1/4 MIC of free BiEDT-TOB[1 mg/L of TOB][1  $\mu$ M of BiEDT], 1/8 MIC of free BiEDT-TOB[0.5 mg/L of TOB][0.5  $\mu$ M of BiEDT] or 1/16 MIC of free BiEDT-TOB[0.25 mg/L of TOB][0.25  $\mu$ M of BiEDT] or (b) in the presence of 1/4 MIC of LipoBiEDT-TOB[0.25 mg/L of TOB][0.25  $\mu$ M of BiEDT], 1/8 MIC of LipoBiEDT-TOB[0.125 mg/L of TOB][0.125  $\mu$ M of BiEDT], 1/16 MIC of LipoBiEDT-TOB[0.062 mg/L of TOB][0.062  $\mu$ M of BiEDT]. Each bar represents the mean  $\pm$  S.E.M. of three independent experiments. P values were considered significant when compared with the control and between groups: \*\*\*P<0.001, \*\*P<0.01.**

### 2.3.7. LipoBiEDT-TOB or BiEDT-TOB activity against infected rats lungs

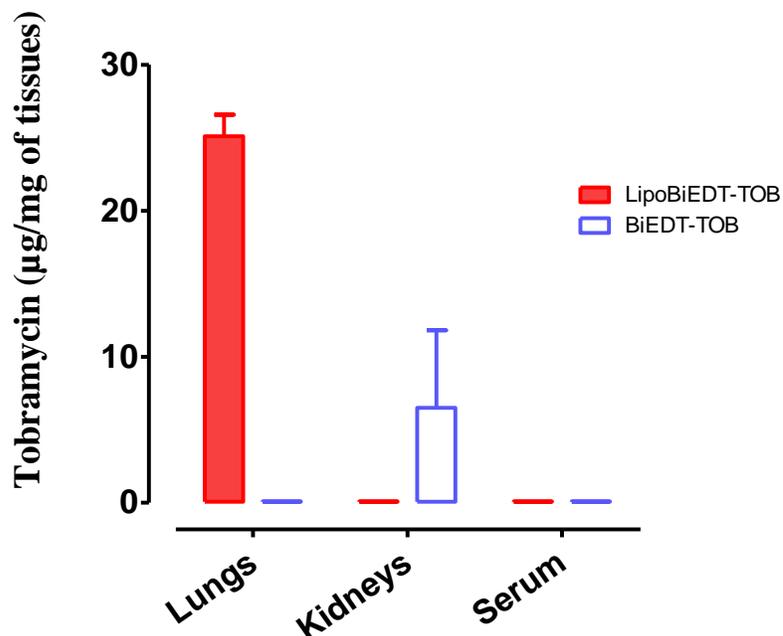
The number of bacteria loaded in agar beads was  $8.4 \pm 0.1 \log_{10}$  CFU/mL. We instilled a total of  $10^6$  CFU in 50- $\mu$ L volume in the lungs of each rat. The number of CFU enumerated following 24 h of the last treatment with saline was  $7.4 \pm 0.2 \log_{10}$  CFU/lungs. The bacterial load in the lungs of the rats after three doses of 300  $\mu$ g for tobramycin and 300  $\mu$ M for BiEDT in free or liposomal formula was significantly lower ( $P < 0.001$ ) than the control (Fig. 7). The effect of liposomal formulation in lowering bacterial load was significantly higher than that of the free formulation ( $3.1 \pm 0.1 \log_{10}$  CFU/lungs vs.  $4.7 \pm 0.3 \log_{10}$  CFU /lungs;  $P < 0.001$ ) as shown in Fig. 7.

### 2.3.8. Levels of active antibiotic in the lungs, the kidneys and the sera of treated rats

The tobramycin concentration was  $25.1 \pm 1.5 \mu\text{g}/\text{mg}$  of lungs after 24 h of administering the last doses of LipoBiEDT-TOB. We did not detect tobramycin in the kidneys or sera of the rats treated with the liposomal formulation. In addition, we did not detect any active tobramycin in the lungs or sera, but we found  $6.5 \pm 5.3 \mu\text{g}/\text{mg}$  tobramycin in the kidneys of the rats treated with free BiEDT-TOB (Fig. 8).



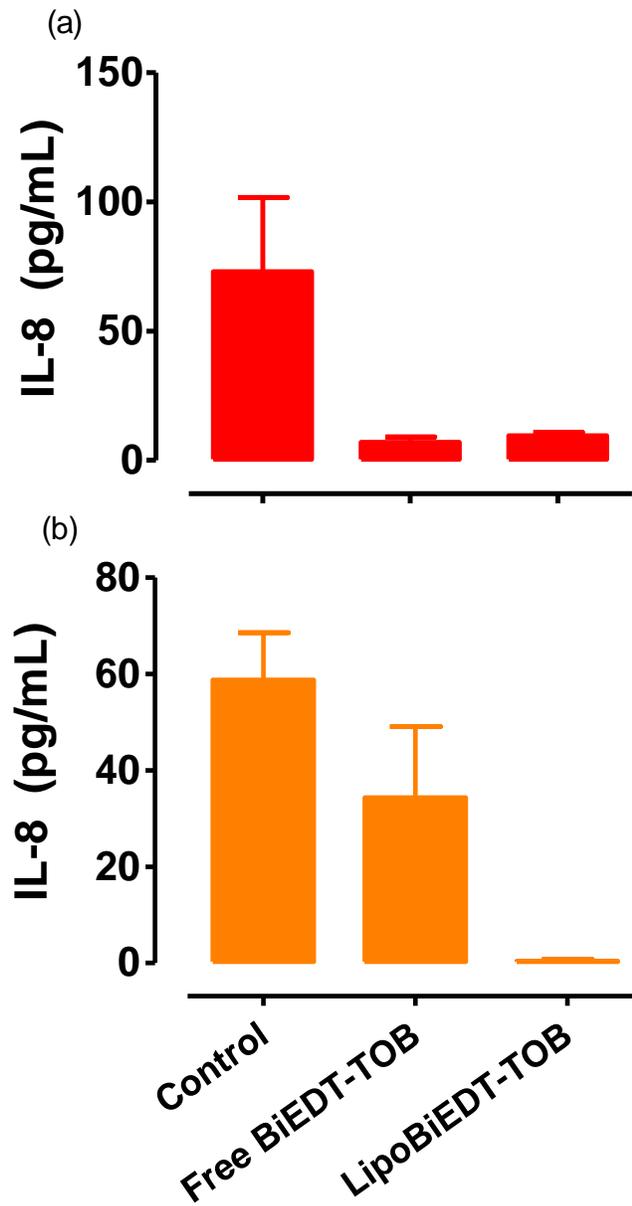
**Figure 7: Effect of free BiEDT-TOB or LipoBiEDT in chronic lung infection model. Rats were inoculated with agar beads containing  $10^6$  CFU of *Pseudomonas aeruginosa*. After the bacteria were grown for four days, saline (filled circles) as well as free BiEDT-TOB (open squares) or LipoBiEDT-TOB (filled triangle) was intratracheally-administered at 300  $\mu\text{g}/\text{mL}/\text{kg}$  for three days. Lungs were then harvested and homogenized for analysis. Each column represents mean  $\pm$  S.E.M. of four animals. P values were considered significant when compared with the control as well as between groups \*\*\* $P < 0.001$ .**



**Figure 8: Measurement of active tobramycin. Tobramycin concentrations in the lungs and the kidneys homogenates of rats chronically infected with *P. aeruginosa* were evaluated by microbiological assay. Tissues were removed at 24 h after that last treatment of LipoBiEDT-TOB (filled bar) or free BiEDT-TOB (clear bar). Bars represent the mean  $\pm$  S.E.M. of four animals.**

### 2.3.9. Effect of LipoBiEDT-TOB on IL-8 production

We investigated whether LipoBiEDT-TOB would reduce the level of IL-8. In the lungs, the level of IL-8 decreased from  $72.9 \pm 28.8$  pg/mL for the group treated with saline to  $9.5 \pm 1.3$  and to  $6.9 \pm 2.1$  pg/mL in groups treated with LipoBiEDT-TOB and free BiEDT-TOB, respectively (Fig. 9a). Free BiEDT-TOB slightly reduced IL-8 released in sera to  $34.3 \pm 14.8$  pg/mL compared to  $58.8 \pm 9.9$  pg/mL measured in sera for the saline-treated group whereas only  $0.4 \pm 0.3$  pg/mL of IL-8 was detected in sera of LipoBiEDT-TOB treated animals (Fig. 9b).



**Figure 9:** The concentration of IL-8: (a) in lungs and (b) in sera of rats infected with *P. aeruginosa* by enzyme-linked immunosorbent assay (ELISA). Each column represents mean  $\pm$  S.E.M. of four animals.

## 2.4. Discussion

Many studies described the efficacy of inhaled tobramycin on lowering *P. aeruginosa* pulmonary infection in CF patients (59). The high dose required and the prolonged use of tobramycin raise investigators' concerns about its toxicity. Encapsulation of antimicrobial agents in liposomes has been proven to increase their efficacy (60, 61). Bismuth has emerged as a therapeutic agent against gastrointestinal infection caused by *H. pylori* (62). Introducing BiEDT at a sub-inhibitory concentration resulted in reducing alginate and lipopolysaccharide production, as well as inhibition of adherence of *P. aeruginosa* to epithelial cells and secretion of virulence factors (47). Furthermore, previous results from our laboratory indicated that co-encapsulation of BiEDT into liposomal-loaded tobramycin increases the killing effect on *P. aeruginosa*, as well as diminishing AHL production and bacterial adherence to human lung epithelial cells (52, 53). Herein, we have demonstrated that LipoBiEDT-TOB at sub-inhibitory concentration is able to debilitate QS signaling molecule production and secretion of virulence factors, including protease, chitinase and lipase *in vitro*. In addition, we examine *in vivo* bactericidal efficacy and the anti-inflammatory property of LipoBiEDT-TOB in a rat model of pulmonary infection.

The MIC results reported here indicate significant differences between free and liposomal BiEDT-TOB. The MIC of LipoBiEDT-TOB was 16-fold lower than the MIC of tobramycin alone and fourfold lower than the MIC of free BiEDT-TOB. These values are in agreement with previous observation on improved susceptibility of resistant Gram-negative strains to liposomal polymyxin B (60). Since exposing bacteria to the sub-inhibitory concentration of free or LipoBiEDT-TOB did not prevent *P. aeruginosa* to grow (Fig. 1), we investigated their potential effect on inhibition of clinical isolate *P. aeruginosa* communication and virulence factor production. The secretion of AHL molecules that play an important role in regulating the

production of several virulence factors was reduced compared to the control at both free and LipoBiEDT-TOB up to 1/16 MICs (Fig. 3). LipoBiEDT-TOB was able to reduce AHL production 29% at 1/8 MIC, whereas production was reduced 19% by free BiEDT-TOB at 1/8 MIC compared to the control. Exposing *P. aeruginosa* to free BiEDT-TOB at 1/4 MIC showed 50% reduction in AHL, whereas LipoBiEDT-TOB at 1/4 MIC led to an approximate 71% reduction compared to the control. However, comparing free and liposomal formulations, LipoBiEDT-TOB was found to be more effective at concentrations four times lower than free BiEDT-TOB based on qualitative (Fig. 2) and quantitative measurements (Fig. 3). Studies have reported that tobramycin at sub-inhibitory concentrations was able to decrease *N*-3-oxo-dodecanoylhomoserine lactone and *N*-butanoylhomoserine lactone once tobramycin gains access to interact with bacterial ribosome (36, 37). Another study reported the improved efficacy of tobramycin with BiEDT in liposomes (53); thus, LipoBiEDT-TOB provides greater advantage in reducing 3-oxo-dodecanoylhomoserine lactone and *N*-butanoylhomoserine lactone production levels by enhancing tobramycin penetration into the cell to interact with ribosomes. This interaction might result in down-regulation of the QS gene (36).

LipoBiEDT-TOB also reduced the level of virulence factors including lipase (Fig. 4), chitinase (Fig. 5), and protease (Fig. 6) at a concentration four to eight times lower than free BiEDT-TOB with respect to the corresponding untreated control levels. It is not yet clear, however, how LipoBiEDT-TOB exerts its effect to reduce virulence factors. Tobramycin inhibits protein synthesis in *P. aeruginosa* (36). BiEDT is known to inhibit alginate and lipopolysaccharides, as well as causing blebbing of the *P. aeruginosa* cell wall (47). Furthermore, transmission electron microscopy has provided evidence of the fusion of LipoBiEDT-TOB and the penetration of tobramycin into the cell wall of *P. aeruginosa* (63).

Collectively, BiEDT in liposome form facilitates the uptake of loaded antibiotic, and it might thereby promote down-regulation of QS and virulence factors gene expression or reduce their post-transcription synthesis (63).

Many investigators have reported intratracheal administration of liposome-loaded drugs such as deguelin (64), insulin (65), tobramycin (66), siRNA, antisense oligonucleotides, and anticancer drugs (67) into the lungs of rodents. The liposomal delivery system and intratracheal route satisfy three therapeutically preferred goals in pulmonary infection: (i) sustained release of an antibiotic from liposomes, which increases the residence time of the drug; (ii) reduction of antibiotic toxicity; (iii) direct aiming of the drug at the site of infection. The data reported here manifest that chronic respiratory infection caused by *P. aeruginosa* can be decreased by *in situ* administration of liposome co-encapsulated BiEDT and tobramycin. Three treatments of LipoBiEDT-TOB (300 mg/L/kg for tobramycin and 300  $\mu$ M/kg for BiEDT) reduced *P. aeruginosa* in the lungs. We used a clinical isolate strain embedded in agar beads to initiate a chronic lung infection. Such retention apparently prevents physical elimination of bacteria and ensures stimulation of host defense typical of CF. Bacterial counts in the lungs showed 2.7  $\log_{10}$  units reduction in CFU of the free BiEDT-TOB treated group compared to the control, whereas LipoBiEDT-TOB reduced the bacterial counts approximately by 4.3  $\log_{10}$  units compared to the control. The increased efficacy of LipoBiEDT-TOB can be explained by the enhanced penetration of encapsulated formula through the bacterial outer membrane, likely through the mechanism of fusion (60). Previous work by others has shown improved bactericidal activities of liposome-encapsulated antibiotics specific to *P. aeruginosa* (68, 69).

The microbiological analysis of the liposomal antibiotic in the lungs indicated the presence of 25 times the MIC of active tobramycin after 24 h of antibiotic therapy. However, no active

tobramycin was detected at 24 h when the animals were treated with the free drug. Despite the fact that 25 times the MIC was detected in the lungs, the animals' lungs treated with LipoBiEDT-TOB remained infected. A previous study speculated that the persistent infection with liposomal antibiotic treatment might be due to the high stability of the liposomes' lipid composition, the protection of bacteria by agar beads, or a portion of the agar beads injected being preserved in the bronchial tree (58). Since our formula consists of DSPC and cholesterol with a phase transition temperature of 55°C (70), the high stability of the vesicle might not allow the release of tobramycin at sufficient concentration to ensure a complete eradication. Also, using agar beads to induce chronic infection might contribute to the presence of infection. The microbiological assay also showed no active tobramycin in the kidneys of the LipoBiEDT-TOB treated group, but we found tobramycin accumulation in the kidneys of the free BiEDT-TOB treated group. There was no active tobramycin detected in plasma when the antibiotic was administered in liposomes. Our findings agree with previously published reports (58, 71) and with the notion that the half-life of tobramycin in sera of human and rodents is around 2 h after intravenous and intratracheal administration (58, 72). Likewise, our liposomal formulation results are in agreement with data reported by other researchers who investigated the efficacy of liposomal antibiotics against *P. aeruginosa* respiratory infection (73, 74) and this could suggest an advantage in reducing the nephrotoxicity associated with tobramycin treatment (73).

Tobramycin is known to have both antibacterial and anti-inflammatory activity (75, 76). Our results indicate the benefit of administering LipoBiEDT-TOB intratracheally on *P. aeruginosa* infection and showed lowered inflammation by reduced IL-8 levels in lungs and sera. Although the exact mechanism of tobramycin as an anti-inflammatory drug is not well known, tobramycin have been shown to protect epithelial lung cells against myeloperoxidase by binding to anionic

cell surfaces and neutralizing hypochlorous acid that participate in tissue damage (77, 78). However, since the local inflammatory response is in agreement with pulmonary infection (79), the significant decrease in the *P. aeruginosa* counts in lungs may be explained by the beneficial aspects of LipoBiEDT-TOB.

In conclusion, LipoBiEDT-TOB modulated the production of QS molecules, virulence factors and IL-8 and could highly enhance the treatment of chronic pulmonary infection in CF patients.

### **Acknowledgements**

This work was supported by a research grant from the Ministry of Higher Education of the Kingdom of Saudi Arabia represented by the Saudi Cultural Bureau in Ottawa for funding (M.A.).

### **Transparency declarations**

None to declare.

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## Chapter 3

### 3.1. Conclusion

Chronic pulmonary infection caused by Gram-negative *Pseudomonas aeruginosa* is the main cause of morbidity and mortality among Caucasians with CF. *Pseudomonas aeruginosa* regulates its gene expression and virulence factors through cell-to-cell communication molecules to colonize the lung successfully. Tobramycin, an aminoglycoside antibiotic, is the most effective regimen of treatment; however, due to its elevated toxicity profile and the increased resistance of *Pseudomonas aeruginosa* to currently available antibiotics, a carrier system such as liposomes is required to improve bactericidal activity and reduce toxicity.

Liposomes have also drawn great interest from research scientists as a suitable model for the investigation of the structure and function of biological membranes. Several of the liposomes' biological and physicochemical properties including biocompatibility and biodegradability contribute to their success in the pharmaceutical industry. Furthermore, encapsulated agents in liposomes can be protected from the host's metabolic enzymes and other internal environmental elements. Liposomes can also prolong the drug's effect by providing a sustained release of the bioactive compound in the body. In addition, they can be administered through different routes depending on the affected body system. Most importantly, they can carry any compound regardless of their chemical affinity as hydrophilic drugs can be encapsulated in the aqueous core, whereas lipophilic agents are carried within the lipid bilayers. The ability of liposomes to co-encapsulate hydrophobic as well as hydrophilic chemicals facilitates development of synergistic therapeutics. Recent studies showed the superiority of liposomal encapsulated bismuth-ethanedithiol and tobramycin formulation against *Pseudomonas*

*aeruginosa*. The main objective of this study was to evaluate the efficacy of LipoBiEDT-TOB *in vitro* and *in vivo*.

We demonstrated that LipoBiEDT-TOB, below the bactericidal concentration, reduced QS signaling molecules produced by *P. aeruginosa* compared to the untreated samples and exhibited four times higher activity than the free formulation. LipoBiEDT-TOB also significantly decreased the level of virulence factors-secreted enzymes including lipase, chitinase and protease at an encapsulated tobramycin concentration eight times lower than that of free tobramycin *in vitro*.

Intratracheal administration of LipoBiEDT-TOB into the infected lungs of rodents resulted in the presence of the active agents at 25 times higher the concentration required to inhibit the bacteria. In other words, the liposomal BiEDT formulation improved the antimicrobial activity of tobramycin and provided a sustained, as well as direct, release of antibiotic at the site of infection. Furthermore, the liposomal formulation provided an advantage in reducing the nephrotoxicity associated with aminoglycoside treatments. Administration of LipoBiEDT-TOB also showed a beneficial reduction of inflammatory response by reducing IL-8.

This research demonstrates the increase efficacy of bactericidal agents such as bismuth-ethanedithiol when they are encapsulated in a liposomal formulation while reducing its adverse side effects.

### 3.2. Future directions

We have examined the efficacy of liposomal formulations on cell-to-cell communication and secreted virulence factors *in vitro*; however, their exact mechanism of action on reducing QS and virulence factors has not been fully explored yet. Thereby, investigation of the antimicrobial

effectiveness on *P. aeruginosa* gene expressions, which are involved in production signaling molecules and virulence factors *in vivo*, could explain this mechanism.

A single dose of LipoBiEDT-TOB against chronic pulmonary infection caused by *P. aeruginosa* was investigated and showed an improvement in the pharmacokinetic and efficacy against infection. However, it would be important to test in future work the efficacy of a multiple dose regimen to determine whether or not this would eliminate bacterial infection completely.

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