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

Running Title: Efficacy of LiposomalBiEDT-Tobramycin.

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We sought to investigate alterations in 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 in a Liposome-Loaded Tobramycin Formulation (LipoBiEDT-TOB) administered to rats chronically infected with *P. aeruginosa*. Quorum-sensing signal molecule N-acyl homoserine lactone was monitored by a biosensor organism. *P. aeruginosa* virulence factors were assessed spectrophotometrically. Agar beads model of chronic *Pseudomonas* lung infection in rats were 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) reduced lipase, chitinase and protease productions. Twenty four hours 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}/lungs respectively in untreated and in lungs treated with free antibiotic. The antibiotic concentration after the last dose of LipoBiEDT-TOB was 25.1 µg/lungs while no tobramycin was detected in the kidneys. As for the free antibiotic, we found 6.5 µg/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.

**Key words:** cystic fibrosis, aminoglycoside, cytokines, virulence factors, quorum sensing.
1. Introduction:

Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutation in a cystic fibrosis transmembrane regulator (CFTR) gene that affects multi-organs and systems including the lungs, the pancreas, the endocrine system and the gastrointestinal tract (24). Pulmonary injury is the most challenging medical problem and is responsible for the majority of morbidity and mortality in the CF population (55). There are more than 1500 mutations in CFTR genes with different degree of disease severity. Mutation in CFTR caused by deletion of phenylalanine at position 508, known as ΔF508, is more common and causes severe disease due to non-functional chloride ion channels (68). 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 (71). 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 (44, 70) provides a suitable growth environment for bacteria such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* (13, 47, 77). Recurrent *P. aeruginosa* induced pulmonary infection and inflammation is more common and is associated with reduced lung function and disease exacerbation (22, 61).

*P. aeruginosa* is a Gram-negative opportunist human pathogen found in surrounding environments such as fresh water, plants, sinks, hand soaps, and hospitals (64, 65). *P. aeruginosa* cells interact with specific host cell receptors through appendices such as pili type IV that recognise the over-expressed asialoganglioside (GM1) in CF epithelial cells; and its monotrichous flagellum binds specifically to secreted respiratory mucins (10, 30, 46). *P.
P. aeruginosa utilizes mucus as a shield against the host immune system and regulates its cell density, virulence factors production, and biofilm formation through quorum sensing (QS) signaling (15, 26, 45, 67). The pathogen carries two homologues that control the QS system: the lasI/lasR and rhlI/rhlR (53). The autoinducer proteins are responsible for synthesizing specific signal molecules. LasI and RhlI synthesize N-3-oxo-dodecanoylhomoserine lactone (3O-C12-HSL) and N-butanoxyhomoserine lactone (C4-HSL) respectively; whereas, LasR and RhlR function as transcriptional activator proteins (9, 37, 66). Bacteria release 3O-C12-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, 38, 42, 54, 74). Activation of airway epithelial cells signalling 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 neutrophils (11). While neutrophils eradicate bacteria, their toxic products such as elastase and reactive oxygen radicals in the airway damages the lungs tissue as well (31).

Aggressive chemotherapy, through various routes, has been utilized to decrease the persistence of P. aeruginosa in lungs (29, 62). Administration of aminoglycosides such as tobramycin, along with β-lactams, is usually prescribed against P. aeruginosa to reduce infection (1, 60). Tobramycin at sub-inhibitory concentration reduces production of P. aeruginosa virulence factors at translation level by inhibiting the release of C4-HSL and 3O-C12-HSL levels (5, 28). However, since a high dosage and prolonged use of tobramycin are required to eradicate bacteria, a high risk of ototoxicity and nephrotoxicity exists (1). Furthermore, the presence of mucous, overexpression of multidrug efflux pumps, and bacterial transition to biofilm form result in a poor prognosis (41, 43, 59, 78).
Bismuth subsalicylate and bismuth subcitrate have been used for years to treat gastrointestinal disorders associated with *Helicobacter pylori* (69). A combination of bismuth and thiol agents increases the bismuth solubility, lipophilicity, and its anti-microbial activity against Gram-positive and Gram-negative bacteria (19). Huang and Stewart have shown that bismuth dimercaprol was able to reduce biofilm formation by *P. aeruginosa* (40). Bismuth ethandithiol (BiEDT) along with tobramycin have a synergistic effect against *P. aeruginosa* and *Burkholderia cepacia in vitro* (18, 72). The cytotoxic effects of bismuth, however, limit its utility. BiEDT at concentrations 10 and 20 µM render human lung cells in culture nonviable (76). 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 (4, 23).

Liposomes are small lipid vesicles with a size ranging from nanometers to a 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 (73). 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 (6, 73). Previous studies in our laboratory have shown that co-encapsulation of BiEDT with tobramycin in liposomes resulted in eliminating the BiEDT toxic effect on human lung cells while increasing its antibacterial efficacy against *P. aeruginosa* and *B. cepacia* (34, 35).

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. **Materials and methods:**

   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$_3$)$_3$·5H$_2$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$_{24}$H$_{39}$O$_4$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. **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 (NH₄)₂SO₄, 30 mM Na₂HPO₄·2H₂O, 20 mM KH₂PO₄, 47 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.01 mM FeCl₂, 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 (Ti-) (pCF218) (pCF372) was used as the biosensor for the detection of AHL and cultured in Luria-Bertani (LB) broth at 30°C.

### 2.3. LipoBiEDT-TOB preparation

A dehydration-rehydration method was used to prepare liposomal bismuth tobramycin. To prepare BiEDT, Bi(NO₃)₃ 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 forming a thin layer of lipid. The lipid film was rehydrated by 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 incubated for 30 min at 45°C; then PBS was added to form the original volume. The solution was centrifuged (Beckaman 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 (34). The size of liposomes was determined by a Submicron Particle Sizer, Model 270 (Nicomp, Santa Barbara, CA, USA).

2.4. Tobramycin encapsulation efficiency (E.E.) 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 a McFarland 0.5 (1.5×10⁸ bacterial/mL). The bacterial suspension in warm (45°C) Muller–Hinton agar was then poured into a sterile glass plate (460mm×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% Triton X-100 (v/v, 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 12.5µg/ml, the coefficients of variation 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 12.5µg/ml gave a correlation coefficient greater than 0.999. Concentration measurements are the mean of at least three independent experiments each experiment was measured in triplicate.

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 2.4) by the concentration of free tobramycin used in the original preparation of these liposomes.

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 was 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 µM for BiEDT in the LipoBiEDT-TOB
and free BiEDT-TOB as reported previously (34). Drug-free bacterial cultures and an ABt broth medium alone were used as positive and negative controls respectively.

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 (34). To simplify, samples were lyophilized, weighed, and then transferred into Teflon digestion vessels. A total of 1 mL H₂O₂ (30%, w/w) and 4 mL HNO₃ 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 times dilution with 2% HNO₃. Samples were then analyzed by GFAAS (AAnalyst 600, Perkin Elmer Precisely, Woodbridge, ON, Canada).

2.7. Evaluation of QS and virulence factors 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 (OD₆₀₀=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 OD₆₀₀=0.26, they were exposed to an equal volume of free or liposomal BiEDT-TOB (1/16-1/2 the 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
To test that there are no killing effect of the antibiotic on bacteria at concentrations below the MICs, free or liposomal BiEDT-TOB (1/4 and 1/2 the MIC) 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$_{600}$) for 8 h.

### 2.8. Bioassay for AHL production

Supernatant samples were screened for AHL production as described previously with some modification (49). *A. tumefaciens* strain A136 (Ti-) (pCF218) (pCF372) equal to $10^6$ with β-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 µl) from control or treated supernatant samples were transferred to the wells. The Petri dishes were incubated for 48 h at 30°C. AHL productions were confirmed by blue pigmentation around the wells.

### 2.9. β-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 β-galactosidase in the reporter strain *A. tumefaciens* (A136) as described previously (79). 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$_{600}$) before centrifugation. The supernatants were removed, and the pellets were suspended in an equal
volume of Z buffer (0.06 M Na₂HPO₄·7H₂O, 0.04 M NaH₂PO₄·H₂O, 0.01 M KCl, 0.001 M MgSO₄·7H₂O, 0.05 M β-mercaptoethanol, PH 7.0). The cells were then permeabilized by a solution of 200 µL of chloroform and 100 µL of 0.1 % sodium dodecyl sulphate prior to the addition of 0.4 mL of O-nitrophenol-β-D-galactopyranoside (4 mg/mL in PBS). After the development of yellow color, 1 mL of 1M Na₂CO₃ was added to stop the reaction. Optical density of the reaction samples was measured at 420 and 550 nm. Miller units of β-Galactosidase were calculated as \( (1000 \times A_{420nm}) \times (1.75 \times A_{550nm}) \div (\text{time} \times \text{volume} \times A_{600nm}) \) as described previously (51).

2.10. Virulence factors 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 CaCl₂, 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 (OD₄₀₀). 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 (OD₂₉₀). 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 (OD₆₀₀). For the protease assay, 100 µL 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.

2.11. Preparation of agar beads

Agar beads were prepared as described previously with some modifications (14). 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 10-fold serial dilutions in phosphate-buffered saline (PBS) on Mueller-Hinton agar plates as described previously (56).

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 anesthetised 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 µL 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 of 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 µM. Saline (90 µL) was administered to the infected control animals.
Twenty four hours after the last treatment, the animals were euthanized by CO2. 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 homogenising 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. 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 log10 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 µL of sterile PBS. The presence of active tobramycin was detected by an agar diffusion assay as described above in 2.4. section.

2.13. **IL-8 assay**

Supernatants from sera and the lungs 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 ×g and 22°C. The supernatants were added to the well for two hours while shaking. The plate was 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 TMB Substrate Solution C was added for 15 min in the dark without shaking. A 2N H₂SO₄ 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.

2.14. Data analysis

The data presented as mean ± 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 were considered as statistically significant.

3. Results:

3.1. LipoBiEDT-TOB characterization

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

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 lowers 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 µM for BiEDT in free BiEDT-TOB compared to 1 mg/L for tobramycin and 1 µM for BiEDT in LipoBiEDT-TOB.

3.3. Effect of sub-inhibitory concentrations of free or LipoBiEDT-TOB

The effect of concentrations (1/4-1/2 the MICs) of free BiEDT or LipoBiEDT-TOB on bacterial growth are shown in (Fig. 1). The rate of growth of cells treated with 1/2 The MIC of liposomal formulation is inhibited, 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 the MICs of free or LipoBiEDT-TOB.
3.4. **QS molecules 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 the MICs. Both formulas have reduced AHL production up to 1/16 the MICs tested compared to control, but did not prevent the production completely (Fig. 2). However, LipoBiEDT-TOB reduced AHL production at sub-inhibitory concentrations four times lower than 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.

3.5. **AHL quantification**

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

3.6. **Reduction of virulence factors by BiEDT-TOB**

We compared the effects of free and liposomal BiEDT-TOB at 1/16-1/4 the MICs on production of 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 the 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), liposomal formulation was able to reduce chitinase at eight times lower than the concentration of 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 the MICs ($P<0.01$) compared to the control whereas LipoBiEDT-TOB attenuated significantly at 1/4 the 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).

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

The number of bacteria loaded in agar beads was $8.38 \pm 0.09$ log$_{10}$ CFU/mL. We instilled a total of $10^6$ CFU in 50 µL in the lungs of each rat. The number of CFU enumerated following 24 h of the last treatment with saline was $7.36 \pm 0.17$ log$_{10}$ CFU/lungs. The bacterial load in the lungs of the rats after three doses of 300 µg for tobramycin and 300 µM 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 ($3.06 \pm 0.13$ log$_{10}$ CFU/lungs vs. $4.67 \pm 0.33$ log$_{10}$ CFU/lungs, $P<0.001$) as shown in (Fig. 7).
3.8. Levels of active antibiotic in the lungs, the kidneys and the sera of treated rats

The tobramycin concentration was $25.1 \pm 1.48 \, \mu g/mg$ 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 $6.5 \pm 5.3 \, \mu g/mg$ tobramycin in the kidneys of the rats treated with free BiEDT-TOB (Fig. 8).

3.9. Effect of LipoBiEDT-TOB on IL-8 production

We investigated whether LipoBiEDT-TOB would reduce the level of IL-8. The level of IL-8 reduced from $72.93 \pm 28.81 \, pg/mL$ in lungs’ treated group with saline to $9.50 \pm 1.31$ and $6.92 \pm 2.13 \, pg/mL$ in treated group with LipoBiEDT-TOB and free BiEDT-TOB respectively (Fig. 9a). Free BiEDT-TOB slightly reduced IL-8 that released in sera to $34.29 \pm 14.80 \, pg/mL$ compared to $58.75 \pm 9.86 \, pg/mL$ measured in sera saline-treated group whereas only $0.44 \pm 0.29 \, pg/mL$ of IL-8 was detected in sera of LipoBiEDT-TOB (Fig. 9b).

4. Discussion:

Many studies described the efficacy of inhaled tobramycin on lowering $P. \, aeruginosa$ pulmonary infection in CF patients (63). 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 (3, 36). Bismuth has emerged as a
therapeutic agent against gastrointestinal infection caused by *H. pylori* (20). Introducing BiEDT at a sub-inhibitory concentration resulted in reducing alginate and lipopolysaccharides production as well as inhibiting adherence of *P. aeruginosa* to epithelial cells and secretion of virulence factors (76). 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 diminishes AHL production and bacterial adherence to human lung epithelial cells (34, 35). Herein, we demonstrate that LipoBiEDT-TOB at sub-inhibitory concentration is able to debilitate QS signaling molecules 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 MICs 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 resistance Gram-negative strains to liposomal polymyxin B (3). 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 inhibiting clinical isolate *P. aeruginosa* communication and virulence factors 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 the MICs (Fig. 3). LipoBiEDT-TOB was able to reduce AHL production 29% at 1/8 the MIC, whereas 19% was reduced by free BiEDT-TOB at 1/8 the MIC compared to control. Exposing *P. aeruginosa* to free BiEDT-TOB at 1/4 the MIC showed 50%
reduction in AHL, whereas LipoBiEDT-TOB at 1/4 the MIC exhibited approximately 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 (5, 28). Another study reported the improved efficacy of tobramycin with BiEDT in liposomes (35), thereby LipoBiEDT-TOB is more advantageous in reducing 3-oxo-dodecanoylhomoserine lactone and N-butanoylhomoserine lactone productions by enhancing tobramycin penetration inside the cell to interact with ribosome. This interaction might result in down-regulation of QS gene (5).

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 their 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* (5). BiEDT is known to inhibit alginate and lipopolysaccharides as well as causing blebbing of the *P. aeruginosa* cell wall (76). 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* (2). Collectively, BiEDT in liposome form facilitates the uptake of loaded antibiotic, thereby might promote down-regulation of QS and virulence factors gene expression or reduce their post-transcription synthesis (2).

Many investigators have reported intratracheal administration of liposome-loaded drugs such as deguelin (75), insulin (8), tobramycin (48), siRNA, antisense oligonucleotides, and anticancer
drugs (27) into the lungs of rodents. The liposomal delivery system and intratracheal route satisfy three therapeutically preferred goals in pulmonary infection: (i) sustained release of antibiotic from liposomes, increases the residence time of the drug; (ii) reduce antibiotic toxicity; and (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 LipoBiEDT-TOB (300 mg/L/kg for tobramycin and 300 µM/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 defences typical of CF. Bacterial counts in the lungs showed 2.7 log reduction in CFU of free BiEDT-TOB treated group compared to control, whereas LipoBiEDT-TOB reduced the bacterial counts approximately by 4.3 log compared to control. The increased efficacy of LipoBiEDT-TOB can be explained by enhanced penetration of encapsulated formula through the bacterial outer membrane, likely through the mechanism of fusion (3). Previous works by others have shown improved the bactericidal activity of liposome-encapsulated antibiotics specific to *P. aeruginosa* (50, 52).

The microbiological analysis of the liposomal antibiotic in the lungs indicated the presence of 25 x MIC 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 of the MIC was detected in the lungs, the animals’ lungs treated with LipoBiEDT-TOB remained infected. Previous study speculated that the persistent infection with liposomal antibiotic treatment might be due to the high stability of liposomes lipid composition, the protection of bacteria by agar beads, or part of the agar beads injection being preserved in the bronchial tree.
Since our formula consists of DSPC and cholesterol with a phase transition temperature of 55°C (21), 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 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 (17, 56) 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 (39, 56). 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 (7, 57) and could suggest an advantage in reducing the nephrotoxicity associated with tobramycin treatment (7).

Tobramycin is known to have both antibacterial and anti-inflammatory activity (16, 32). Our results indicate the beneficial of administration LipoBiEDT-TOB intratracheally on P. aeruginosa and show a reduction in inflammation by reducing IL-8 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 (12, 33). However, since the local inflammatory response is in agreement with pulmonary infection (58), the significant decrease in the P. aeruginosa counts in lungs may explain the beneficial aspects of LipoBiEDT-TOB.
In conclusion, LipoBiEDT-TOB modulated the production of QS, virulence factors and IL-8 and could highly enhance the treatment of chronic pulmonary infection in CF patients.

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Transparency declarations

None to declare.
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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 µM of BiEDT] (open circle), 1/4 MIC of LipoBiEDT-TOB [0.25 mg/L of TOB][0.25 µM of BiEDT] (filled triangle), 1/2 MIC of free BiEDT-TOB [2 mg/L of TOB][2 µM of BiEDT] (filled squares), or 1/4 MIC of free BiEDT-TOB [1 mg/L of TOB][1 µM of BiEDT] (open triangle).
Figure 2. Effect of sub-inhibitory concentrations of free or LipoBiEDT-TOB (1/16-1/4 the MICs) on QS. LB agar containing *Agrobacterium tumefaciens* and β-D-galactopyranoside was poured in Petri dishes. Holes were made in the agar by a vacuum device and 80 µl from control or treated supernatant samples were transferred to the wells. The plates were incubated for 48 h at 30°C.
Figure 3. Production of the QS molecules as measured by β-galactosidase activity: (a) in the presence of free BiEDT-TOB or (b) in the presence of LipoBiEDT-TOB at 1/16 - 1/4 the MICs. *P<0.05, **P<0.01, and ***P<0.001.

P. aeruginosa was exposed to free and LipoBiEDT-TOB then the supernatants were collected and incubated with A. tumefaciens (A136). β-Galactosidase activities were measured in miller unit. Each bar represents the mean ± 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 µM of BiEDT], 1/8 MIC of free BiEDT-TOB[0.5 mg/L of TOB][0.5 µM of BiEDT] or 1/16 MIC of free BiEDT-TOB[0.25 mg/L of TOB][0.25 µM of BiEDT] or (b) in the presence of 1/4 MIC of LipoBiEDT-TOB[0.25 mg/L of TOB][0.25 µM of BiEDT], 1/8 MIC of LipoBiEDT-TOB[0.125 mg/L of TOB][0.125 µM of BiEDT], 1/16 MIC of LipoBiEDT-TOB[0.062 mg/L of TOB][0.062 µM of BiEDT]. Each bar represents the mean ± 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 µM of BiEDT], 1/8 MIC of free BiEDT-TOB[0.5 mg/L of TOB][0.5 µM of BiEDT] or 1/16 MIC of free BiEDT-TOB[0.25 mg/L of TOB][0.25 µM of BiEDT] or (b) in the presence of 1/4 MIC of LipoBiEDT-TOB[0.25 mg/L of TOB][0.25 µM of BiEDT], 1/8 MIC of LipoBiEDT-TOB[0.125 mg/L of TOB][0.125 µM of BiEDT], 1/16 MIC of LipoBiEDT-TOB[0.062 mg/L of TOB][0.062 µM of BiEDT]. Each bar represents the mean ± 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 µM of BiEDT], 1/8 MIC of free BiEDT-TOB [0.5 mg/L of TOB] [0.5 µM of BiEDT] or 1/16 MIC of free BiEDT-TOB [0.25 mg/L of TOB] [0.25 µM of BiEDT] or (b) in the presence of 1/4 MIC of LipoBiEDT-TOB [0.25 mg/L of TOB] [0.25 µM of BiEDT], 1/8 MIC of LipoBiEDT-TOB [0.125 mg/L of TOB] [0.125 µM of BiEDT], 1/16 MIC of LipoBiEDT-TOB [0.062 mg/L of TOB] [0.062 µM of BiEDT]. Each bar represents the mean ± 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 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 µg/mL/kg for three days. Lungs were then harvested and homogenized for analysis. Each column represents mean ± 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 the last treatment of LipoBiEDT-TOB (filled bar) or free BiEDT-TOB (clear bar). Bars represent the mean ± S.E.M. of four animals.
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 ± S.E.M. of four animals.
Figure 1. Sub-inhibitory concentrations of BiEDT-TOB do not affect growth of PA-489122.
**Figure 2.** Effect of sub-inhibitory concentrations of free or LipoBiEDT-TOB (1/16-1/2 the MICs) on QS

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Free BiEDT-TOB</th>
<th>LipoBiEDT-TOB</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/4 MIC</td>
<td>Tob 1 mg/L, Bi 1 µM</td>
<td>Tob 0.25 mg/L, Bi 0.25 µM</td>
</tr>
<tr>
<td>1/8 MIC</td>
<td>Tob 0.5 mg/L, Bi 0.5 µM</td>
<td>Tob 0.125 mg/L, Bi 0.125 µM</td>
</tr>
<tr>
<td>1/16 MIC</td>
<td>Tob 0.25 mg/L, Bi 0.25 µM</td>
<td>Tob 0.062 mg/L, Bi 0.062 µM</td>
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Control
Figure 3. Production of the QS molecules as measured by β-galactosidase activity
Figure 4. Lipase activities in supernatant from PA-489122.
Figure 5. Chitinase activities in supernatant from PA-489122.
Figure 6. Protease activities in supernatant from PA-489122.
Figure 7. Effect of free BiEDT-TOB or LipoBiEDT in chronic lung infection model.
Figure 8. Measurement of active tobramycin.

Tobramycin (µg/mg of tissues)

- Lungs
- Kidneys
- Serum

LipoBiEDT-TOB

BEdT-TOB

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Figure 9. The concentration of IL-8.