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Bismuth-ethanedithiol incorporated in a liposome-loaded tobramycin formulation modulates the alginate levels in mucoid *Pseudomonas aeruginosa*

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Abstract

Objectives This study examined the antibacterial activity, alginate modulation, and deposition of a tobramycin bismuth-ethanedithiol (Tob-Bi) conventional (free) or vesicle-entrapped (lipo) formulation against two mucoid *Pseudomonas aeruginosa* clinical isolates.

Methods The inhibitory, bactericidal and biofilm eradication concentrations (in presence or absence of alginate lyase) were determined. The modulation of alginate was assessed by the carbazole assay and fluorescent-labelling of live alginate-producing biofilms by confocal microscopy. The deposition of the formulations was assessed using the immunogold-labelling technique, transmission electron microscopy, and energy dispersive X-ray spectroscopy (EDS).

Key findings The inhibitory and bactericidal concentrations for lipo Tob-Bi compared with free Tob-Bi were reduced in all strains by 2- to 8-fold, and 2- to 32-fold, respectively. The biofilm eradication concentrations for lipo Tob-Bi compared with free Tob-Bi were reduced by 4- to 32-fold in the mucoid strains. The addition of alginate lyase transiently enhanced eradication for one mucoid strain only. The alginate levels were attenuated by more than half, and free Tob-Bi fared better than lipo Tob-Bi determined by the carbazole assay. Under confocal microscopy, alginate lyase reduced alginate levels and detached mucoid biofilms. Free and lipo Tob-Bi did not detach the bacteria from the surface, but attenuated alginate levels. Tobramycin was detected by immunogold-labelling inside the bacterium, but EDS did not detect bismuth deposits.

Conclusions These findings substantiate a role in which tobramycin, bismuth, and alginate lyase play in eradicating mucoid *P. aeruginosa* growth and modulate alginate levels.
Keywords antibiotics; bacteria; biofilms; cystic fibrosis; exopolysaccharides

Introduction

Pseudomonas aeruginosa infections and their persistence to aggressive antibiotic therapy has been the subject of numerous investigations in the past quarter of this century.[1] This Gram-negative bacillus is an opportunistic pathogen in human diseases such as bacteraemia, burn wound infections, keratitis, and is commonly associated with increased morbidity and mortality in cystic fibrosis populations.[2] Bacterial colonization of cystic fibrosis lungs usually commences with non-mucoid *P. aeruginosa* attachment and expansion on an abnormal viscous epithelial surface of the lung caused by mutations in the cystic fibrosis transmembrane conductance regulator gene.[3] The viscous surface is mostly derived from sputum containing secreted mucins, DNA, and actin from goblet cells and necrotic neutrophils.[4,5] Ultimately, this viscous layer creates a harsh anaerobic environment leading to an eventual mutation and inactivation of the MucA protein which is an inhibitor of AlgU. The hyperactivation of AlgU (regulates alginate production) converts non-mucoid *P. aeruginosa* to an exopolysaccharide-producing mucoid phenotype.[6–8] The activation of MucE by AlgW (a serine protease which regulates MucE) leads to the reduction of MucA levels, which can also promote mucoid conversion.[9,10]

The anionic exopolysaccharides in mucoid *P. aeruginosa* responsible for biofilm formation are primarily composed of linear polymers of randomly O-acetylated β-D-mannuronic acid and its C-5 epimer α-L-guluronic acid.[11] Non-mucoid strains (e.g. PAO1 and PA-14)

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without alginate as a major exopolysaccharide component are also capable of producing biofilms rich in galactose and mannose (*psl*), and glucose (*pel*).[12–14] The overexpression of these exopolysaccharides and formation of mushroom-shaped biofilm complexes retards antibiotic penetration and host immune factors.[1,15,16] This state of persistence and dormancy combined with elevated expressions of multidrug efflux pumps, secretion of several virulence factors (e.g. lipases, proteases) and secondary metabolites (e.g. pyocyanin, pyoverdin) cause the degradation and fibrosis of lung tissue and promote inflammation.[2,17,18] Furthermore, the alginate secretion can promote the persistence of other opportunistic pathogens like *Burkholderia cenocepacia*.[19] The multidrug resistant strains leave few treatment options and a poor prognosis for the patients conferring high resistance towards macrolides, fluoroquinolones, β-lactams, cephalosporins and aminoglycosides.[15] Sub-inhibitory concentrations of some antibiotic classes like the β-lactams and aminoglycosides can also promote resistance, alginate production, and bacterial attachment.[20–23] Despite resistance, the aminoglycosides (particularly tobramycin) are intermittently used to treat pseudomonal respiratory infections and are administered in a nebulized form by inhalation for better targeting to the infection site, which minimize systemic circulation.[1]

Other suggestive therapies for *P. aeruginosa* being investigated *in vitro* include bismuth, a trivalent metal which has been in the market for years as bismuth subsalicylate or bismuth subcitrate currently used in the treatment of certain gastrointestinal disorders and *Helicobacter pylori*.[24–27] The ligation of bismuth to lipophilic dithiols (e.g. 1,2-ethanedithiol) have also gained prominence with greater antimicrobial activity.[28,29] At subinhibitory concentrations *in vitro*, bismuth-dithiols have been shown to interfere with *P. aeruginosa* adherence to abiotic and biotic surfaces, iron uptake, virulence factors secretions, and also suppress exopolysaccharide expression and biofilm formation.[28,30,31] At the moment, the killing mechanism of bismuth is believed to be its ability to induce blebbing of the cellular membrane.[32] Evidently, bismuth-dithiols have a disadvantage with narrow therapeutic indices due to their high toxicity, suggesting they may be more valuable at lower concentrations in synergism with other antimicrobials.

Alginate lyase is another promising anti-pseudomonal enzyme.[33] In-vitro experiments have shown that alginate lyase is capable of attenuating mucoid *P. aeruginosa* attachment and reduce biofilm structures.[34,35] The enzyme which is produced by marine algae, and other microorganisms, including *P. aeruginosa* degrades poly-mannuronate substrates via β-elimination.[36] Although *P. aeruginosa* secretes alginate lyase in to the periplasm, the enzyme does not breakdown its own alginate on the bacterial surface as the mannuronic acids are randomly O-acetylated to a less preferred substrate.[37,38] *P. aeruginosa* alginate lyase, however, is in involved in production and elongation of alginate, and providing alginate primers for polymerization.[11,36]

An alternative method of tobramycin and bismuth-dithiol targeting is liposomal drug delivery.[39] Liposomes are vesicles consisting of one or more lipid bilayers incorporated with bismuth-dithiols, encircling an aqueous centre loaded with the hydrophilic tobramycin. Previously, we developed a synergistic approach consisting of vesicle-entrapped tobramycin bismuth-ethanedithiol (Tob-Bi) intended to increase activity against *P. aeruginosa* quorum sensing, virulence factors production, and biofilm formation, while having reduced toxicity against human A549 lung cells *in vitro*.[39,40] Furthermore, alginate lyase in the presence of liposomal tobramycin increased susceptibility of *P. aeruginosa* biofilms, and acted in synergy with tobramycin to eradicate endogenous bacteria in patient-expectorated sputum.[41]

In this study, we have assessed whether a novel formulation of tobramycin and bismuth, either in the conventional form (free) or co-encapsulated in liposomes (lipo), and in synergy with alginate lyase could modulate alginate production by mucoid *P. aeruginosa*. We found that tobramycin and bismuth in a liposomal formulation in synergy with alginate lyase increased the susceptibility of mucoid *P. aeruginosa* biofilms, and attenuated the alginate production.

Materials and Methods

Chemicals

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was obtained from Northern Lipids Inc. (Burnaby, BC, Canada). Tobramycin was obtained from Sandoz Laboratories (Boucherville, QC, Canada). The green fluorescent SYTO 9 nucleic acid stain and the orange-red fluorescent tetramethylrhodamine conjugate of concanavalin A (ConA) were purchased from Invitrogen (Burlington, ON, Canada). Obtained bismuth nitrate ($Bi(NO_3)_3 \cdot 5H_2O$), cholesterol, 1,2-ethanedithiol (EDT), carbazole reagent, alginate lyase from *Flavobacterium* sp. (29 U/mg), and all other products were from Sigma-Aldrich (St Louis, MO, USA).

Liposomal tobramycin-bismuth preparation

Multilamellar liposomes composed of DSPC and cholesterol (2 : 1 molar ratio) with bismuth-ethanedithiol (1 : 1 molar ratio) incorporated in the lipid bilayer and tobramycin loaded in the aqueous layer were prepared by a dehydration-rehydration technique as previously reported.[40] The entrapment efficiency and stability of this formulation have been published previously.[39,40]

Bacterial strains and culture condition

Two clinical *P. aeruginosa* strains (PA 48913 and PA 136412) had been isolated previously from the lungs of cystic fibrosis patients at Sudbury Regional Hospital (Sudbury, ON, Canada) and their ability to produce alginate was confirmed by the carbazole assay described further below. A reference strain of *P. aeruginosa* (ATCC 27853) was purchased from PML Microbiologicals (Mississauga, ON, Canada). Strains were grown in a minimal medium composed of (in mM): 27 $(NH_4)_2SO_4$, 30 $Na_2HPO_4 \cdot 2H_2O$, 20 KH_2PO_4, 47 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 0.01 $FeCl_2$, 0.05% glucose (w/v), and 0.05% casamino acids (w/v). The medium was stored at room temperature away from direct sunlight, and supplemented with antibiotics when it was appropriate. All experiment incubations were conducted at 36°C, with exception of confocal microscopy, which was at 20–22°C.

Inhibitory, bactericidal, and biofilm eradication concentrations

The minimum inhibitory concentrations (MICs) were measured by the microbroth dilution method according to CLSI guidelines in triplicates. Serial 2-fold dilutions in minimal medium of either free or lipo Tob-Bi at final concentrations of 256 μg/ml tobramycin and 410 μM bismuth to 0.031 μg/ml tobramycin and 0.05 μM bismuth were prepared. The ratio of tobramycin to bismuth used in the free formulation was based on the concentration of tobramycin and bismuth which were co-encapsulated in liposomes. *P. aeruginosa* at 1×10^6 colony forming units (CFU)/ml (final concentration) was added to each well along with positive and negative controls and incubated at 36°C for 18 h. The minimum bactericidal concentrations (MBCs) were determined by sub-culturing from the MIC experiments on Mueller-Hinton agars and incubating at 36°C for 48 h. Biofilm growth in the Calgary Biofilm Device plates and minimum biofilm eradication concentrations (MBECs) were obtained as described previously.[41]

Carbazole assay: modulation of alginate

The mucoid *P. aeruginosa* strains were exposed to sub-MIC levels of free or lipo Tob-Bi. Nontreated cultures were used as controls. The non-mucoid strain did not produce substantial levels of uronic acids and further experiments were not completed on this strain (data not shown). The mucoid PA 136412 was grown in medium for 48 h in an incubator at 36°C with mild shaking, while the mucoid PA 48913 was grown on 2% agarose-supplemented medium for 96 h ensuring higher production of alginate for this specific strain (data not shown). At 24-h intervals, 5 ml samples of bacteria were either aspirated (for PA 136412) or scraped from the agar (for PA 48913), vigorously vortexed and centrifuged. The concentration of alginate in the supernatant was determined immediately by the carbazole assay. Briefly, 30 μl of the supernatant was mixed with 1 ml borate-sulphuric acid reagent (10 mM H_3BO_3 in concentrated H_2SO_4) and 30 μl carbazole reagent (0.1% in ethanol). The solution was heated in a water bath at 55°C for 30 min producing a pink colour and then read by a spectrophotometer (OD_{530}). Three independent experiments were completed in duplicates. All data were normalized by dividing the carbazole absorbance by bacterial turbidity (OD_{600}). The alginate percentage was determined in relation to control samples.

Confocal microscopy: attenuation and staining of *P. aeruginosa* alginate

To visualize the formation of alginate on bacterial surface and its modulation in presence of antibiotics, PA 48913 strain (able to produce substantial quantities of alginate upon adherence to a surface) was grown in a Stovall flow cell apparatus (channel dimensions 1 mm (diam.) × 4 mm (width) × 40 mm (length)). The #1 glass cover slip was inoculated with 0.2 ml bacteria (1×10^6 CFU/ml), and left for 4 h to attach without flow. The unattached bacteria were removed by a continuous flow of medium (with or without antibiotics) with mild stirring of medium (for consistent flow of antibiotics) into the cell apparatus for up to 96 h at 20–22°C with a rate of 5 ml/h. Attached bacteria were stained with SYTO 9 (10 μM) and ConA (100 μg/ml) for 30 min and excess dye was washed with medium alone for 15 min. Immediately, random spots where bacterial growth was noticed were chosen and images were taken with the scanning confocal laser microscope (Nikon Eclipse C1 Plus, Mississauga, ON, Canada) using the appropriate filters.

Deposition of Tob-Bi in a mucoid strain

To determine the ability of free or lipo Tob-Bi to interact, fuse and accumulate in bacteria, the immunogold-labelling technique was applied as described previously.[42] The images were taken from the PA 48913 strain incubated in medium alone or in the presence of free and lipo Tob-Bi at 1 MIC for 6 h. Deposition of bismuth was determined by visual inspection of dark spots within the bacterium and then verified with energy dispersive X-ray spectroscopy (EDS).

Data analysis

Comparisons of groups were made by one-way analysis of variance using InStat 3 from GraphPad (GraphPad Software Inc., Version 5.0) followed with Dunnet post *t*-test. $P < 0.05$ was considered significant.

Results

MICs, MBCs and MBECs of Tob-Bi

The values for the MICs (2- to 8-fold) and MBCs (2- to 32-fold) of lipo Tob-Bi were all significantly lower than that of free Tob-Bi in all strains (Table 1). The formation of biofilms greatly increased the MBECs when compared with the MICs (4- to 512-fold). The MBECs were reduced 4- to 32-fold for lipo Tob-Bi compared with free Tob-Bi in the mucoid strains, but no differences were seen for the non-mucoid strain. The presence of alginate lyase enhanced the eradication of PA 48913 biofilms for free Tob-Bi (32-fold) and lipo Tob-Bi (16-fold), but did not enhance the MBECs for the other two strains. Alginate lyase did not detach the non-mucoid ATCC 27853 biofilms (9.7×10^6 CFU/ml for control and 1.5×10^7 CFU/ml for alginate lyase-treated), but detached bacteria by 2- and 4-fold for PA 136412 (1.6×10^6 CFU/ml for control and 8.2×10^5 CFU/ml for alginate lyase-treated) and PA 48913 (2.9×10^7 CFU/ml for control and 6.8×10^6 CFU/ml for alginate lyase-treated).

Carbazole assay: Tob-Bi modulates alginate production

The modulation of alginate levels in PA 136412 at 24 and 48 h (Figure 1a and 1b), and for PA 48913 at 48 and 96 h (Figure 1c and 1d) were determined. Both free and lipo Tob-Bi were effective in attenuating alginate levels in both mucoid strains. The alginate levels in cultures exposed to free and lipo Tob-Bi were significantly attenuated by more than 50% ($P \leq 0.05$). However, the alginate attenuation was not maintained and at 48 or 96 h, lipo Tob-Bi had no significant effect on alginate levels ($P \geq 0.05$).

Confocal microscopy: alginate lyase and Tob-Bi attenuate *P. aeruginosa* alginate

The mucoid PA 48913 strain was chosen for confocal microscopy because of its high MBEC values, its response to

Table 1 Activity of free or liposomal tobramycin bismuth-ethanedithiol against three *Pseudomonas aeruginosa* strains

Strain	Formulations		Fold change (significance)
	Free Tob-Bi	Lipo Tob-Bi	
ATCC 27853			
MIC	(0.25) [0.4]	(0.125) [0.2]	2 ($P > 0.05$)
MBC	(0.5) [0.8]	(0.25) [0.4]	2 ($P > 0.05$)
MBEC	(2) [3.2]	(2) [3.2]	0
+Alginate lyase	(2) [3.2]	(2) [3.2]	0
PA 136412			
MIC	(0.5) [0.8]	(0.063) [0.1]	8 ($P < 0.05$)
MBC	(8) [12.8]	(1) [1.6]	8 ($P < 0.05$)
MBEC	(2) [3.2]	(0.5) [0.8]	4 ($P < 0.05$)
+Alginate lyase	(2) [3.2]	(0.5) [0.8]	4 ($P < 0.05$)
PA 48913			
MIC	(1) [1.6]	(0.5) [0.8]	2 ($P > 0.05$)
MBC	(128) [205]	(4) [6.4]	32 ($P < 0.05$)
MBEC	(512) [820]	(16) [25.6]	32 ($P < 0.05$)
+Alginate lyase	(16) [25.6]	(1) [1.6]	16 ($P < 0.05$)

Serial 2-fold dilutions of free or liposomal (lipo) tobramycin bismuth-ethanedithiol (Tob-Bi) in medium were added to planktonic, or biofilm populations (with or without alginate lyase at 20 U/ml) and inhibitory concentrations were measured according to CLSI guidelines. Tobramycin (Tob) concentrations are in (μg/ml) and bismuth (Bi) concentrations are in (μM). An increase in susceptibility more than 2-fold was considered significant ($P < 0.05$) when comparing lipo Tob-Bi to free Tob-Bi. MBC, minimum bactericidal concentrations; MBEC, minimum biofilm eradication concentration; MIC, minimum inhibitory concentration.

alginate lyase treatment and its ability to produce substantial quantities of alginate upon adherence to a surface (Figure 2). The bacteria in each condition were grown for a total of 96 h. Untreated controls produced live bacteria (green fluorescence) with a mucoidal surface (red fluorescence). Alginate lyase treatment at 10 U/ml for 1 h after the biofilms were formed slightly detached bacteria and reduced alginate levels indicated in the merged image. Treatment with 20 U/ml for 2 h drastically reduced bacterial density and alginate levels. The mucoid strain was also attached to the surface for 4 h, and allowed to grow for a total of 96 h in the presence of free or lipo Tob-Bi at half MIC. Although the formulations did not detach the bacteria from the surface, the alginate levels were reduced compared with controls.

Transmission electron microscopy: deposition of tobramycin but not bismuth in mucoid *P. aeruginosa*

To determine the interaction and fusion of Tob-Bi with mucoid PA 48913, the immunogold-labelling technique was applied (Figure 3). The images were taken from the strain incubated with no antibiotic (Figure 3a), free Tob-Bi (Figure 3b) and lipo Tob-Bi (Figure 3c). The tobramycin and bismuth within the bacterium were indicated by colloidal gold particles (10 nm size) and dark spots in the bacterium, respectively. The uptake of tobramycin seemed to be higher in lipo Tob-Bi than free Tob-Bi indicating the superiority of liposomes to fuse and deliver a higher dose of the antibiotic. Although dark spots were observed within and around the surface membrane of the bacterium, EDS was performed to validate if these dark spots were in fact bismuth penetration of cell membrane or interaction with the alginate. The EDS results indicated that these dark regions were not due to bismuth localization and bismuth levels (within the detection limits of EDS) in the bacterium were absent in both treatments.

Discussion

The eradication of *P. aeruginosa* is more successful in the early stages of colonization, as the pathogen becomes increasingly resistant with recurring infections.[43,44] With better understanding of the *P. aeruginosa* resistance mechanisms, novel modes of therapy are under development to attenuate alginate production and eradicate *P. aeruginosa* biofilms.[45] Following the work by Domenico *et al*.[28] on the activity of bismuth-thiols against *P. aeruginosa*, our laboratory produced a tobramycin and bismuth-ethanedithiol formulation which attenuated several virulence factors, quorum sensing, bacterial attachment and biofilm formation.[40] However, the bismuth-ethanedithiol alone was toxic to normal human bronchial epithelial cells and higher concentrations required for mature biofilm eradication were subsequently toxic to adenocarcinomic human alveolar epithelial cells.[32,39,40] When the two drugs were entrapped in liposomes, the concentration required to attenuate *P. aeruginosa* pathogenicity was lowered, along with reduced cytotoxicity.[39,40] Huang and Stewart[31] and Wu *et al*.[32] demonstrated that bismuth-dithiol significantly attenuated alginate levels in *P. aeruginosa* and *P. syringae*. We expanded on those findings to determine if our liposomal formulation could also attenuate the alginate in mucoid *P. aeruginosa* isolates from cystic fibrosis patients. Furthermore, the addition of alginate lyase in synergy with the formulation was examined.

We chose the glucose and casamino acid enriched medium with a concentration ten times lower than our previous work.[40] This reduction was necessary to improve the staining

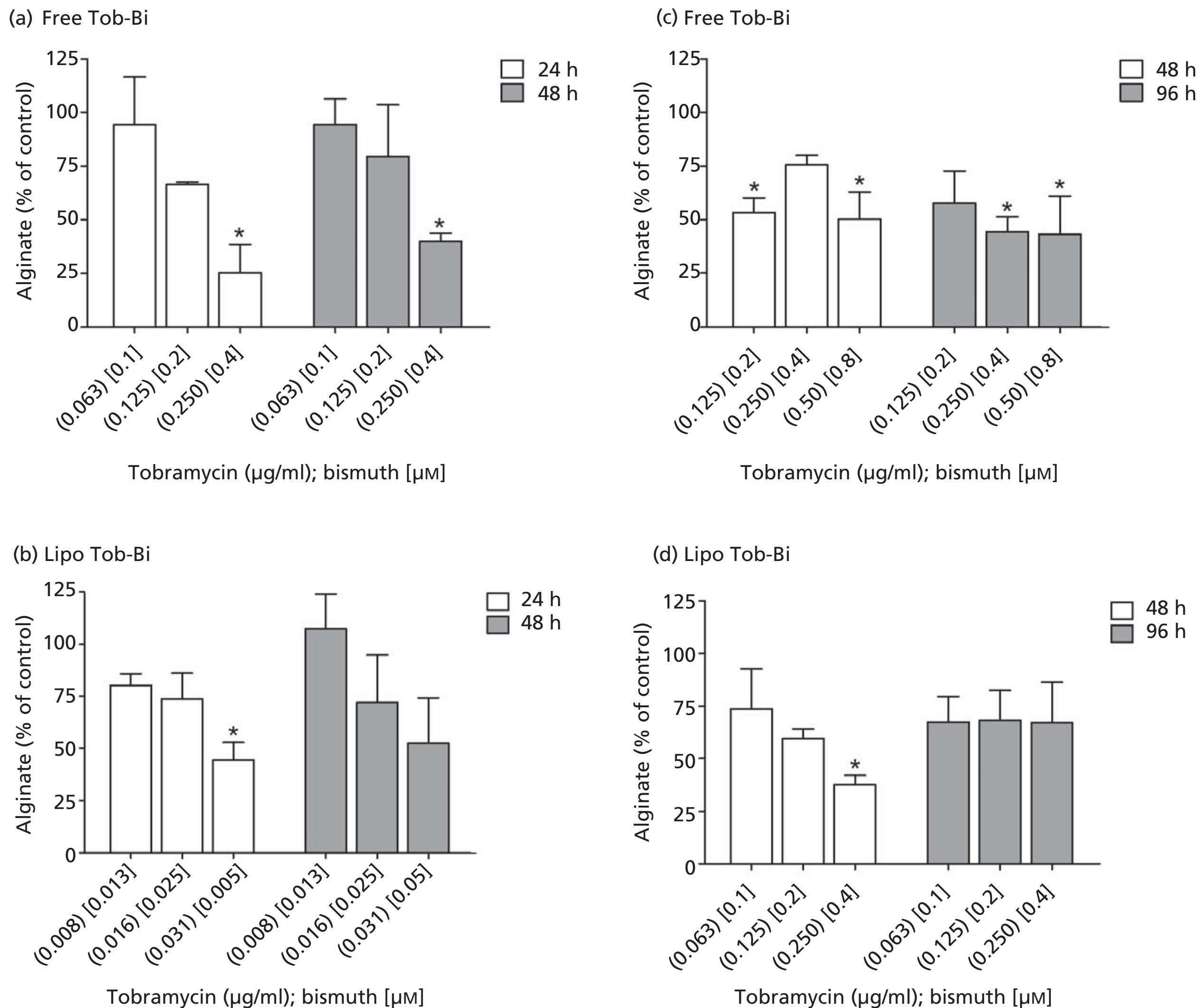

Figure 1 Subinhibitory concentrations of tobramycin bismuth-ethanedithiol modulate alginate production. The production of alginate in (a, b) the *Pseudomonas aeruginosa* strain PA 136412 for 48 h (in medium) and (c, d) the PA 48913 strain for 96 h (on medium with 2% agarose) was examined in presence of 1/8, 1/4, and 1/2 the minimum inhibitory concentration of free or liposomal (lipo) tobramycin bismuth-ethanedithiol (Tob-Bi). Alginate production increased with time in nontreated samples. Alginate concentrations were measured by carbazole assay (OD_{530}) and based on percentage of control for each time point. All experiments were conducted with three repeats and two replicates, $^*P < 0.05$.

of the alginate with lectins for confocal microscopy.[46] The growth conditions greatly enhanced alginate production as well, and for the carbazole assay the PA 136412 strain was grown in the medium, while PA 48913 was grown on the surface of agarose-supplemented medium. The values for MICs and MBCs against a susceptible non-mucoid ATCC strain and two mucoid clinical isolates of *P. aeruginosa* showed that lipo Tob-Bi fared better than free Tob-Bi in inhibiting and eradicating all three strains (Table 1). This was in agreement with our previous work that *P. aeruginosa* was significantly more susceptible to Tob-Bi in a liposomal formulation. To establish the importance of alginate on biofilm resistance and the possible synergy between alginate lyase and Tob-Bi, the MBECs were also determined. The formation of biofilms on the pegs significantly increased the MBEC of PA 48913 compared with the other strains, and the addition of alginate lyase significantly reduced the MBEC. Since PA 48913 strain produced more alginate on agar than PA 136412, we correlated the resistance of this strain to the production of alginate. Alginate lyase also detached the bacteria from the biofilm pegs by 4-fold compared with nontreated controls. The increased susceptibility of the bacteria to treatment was attributed to the alginate lyase cleaving by polymannuronate and bacterial detachment on one hand and by biofilm penetration by Tob-Bi on the other hand.

Alginate is a major contributor to *P. aeruginosa* resistance. The modulation of alginate in the presence of free and lipo Tob-Bi demonstrated that sub-MIC levels of both formulations significantly ($P < 0.05$) reduced alginate production measured by the carbazole assay (Figure 1). Considering that

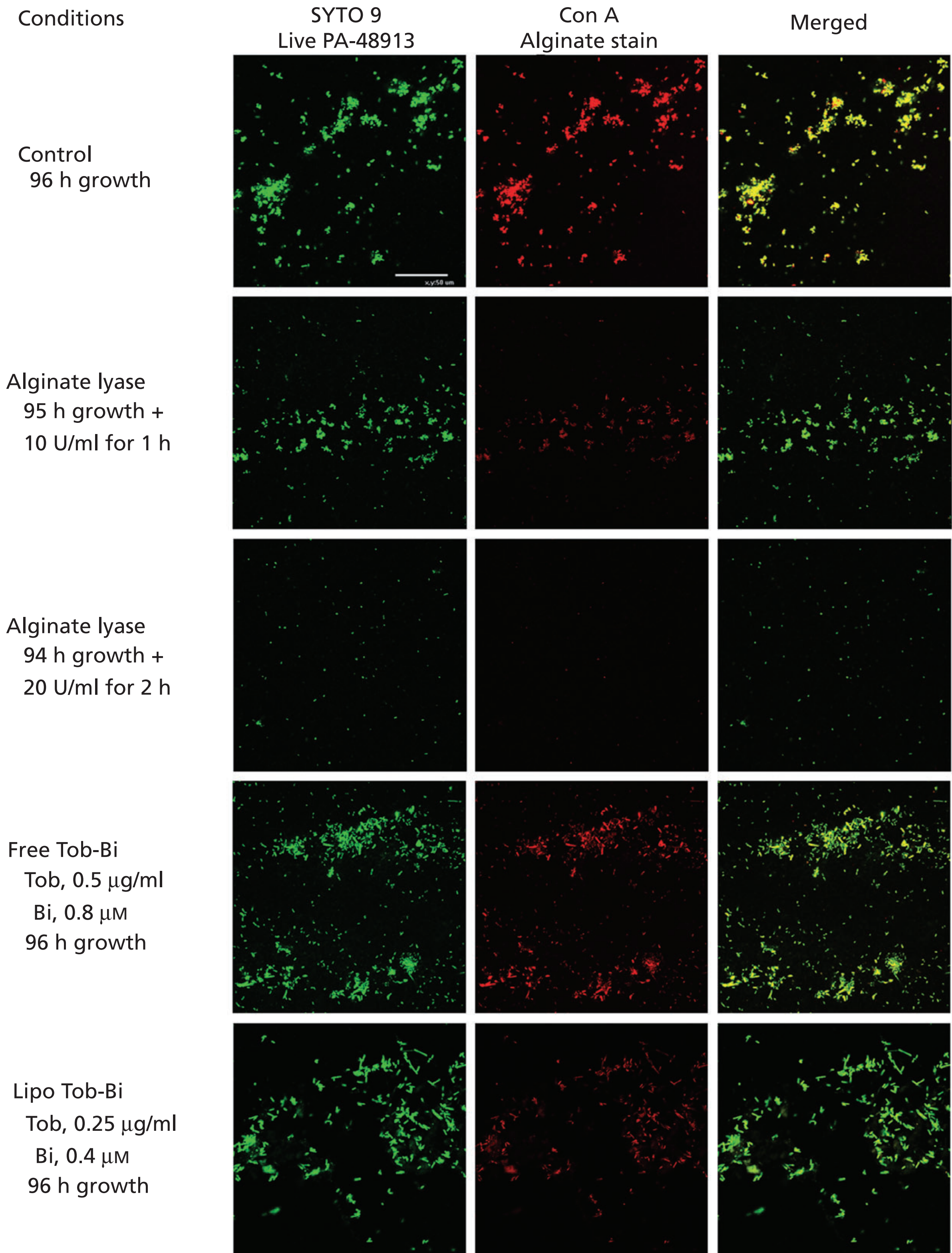

Figure 2 Tobramycin and bismuth modulate alginate production of the *Pseudomonas aeruginosa* strain PA 48913 viewed under confocal microscopy. PA 48913 strain was grown for 96 h in a flow cell with a rate of 4 ml/h at 20–22°C in medium; alginate lyase at 10 U/ml for 1 h; alginate lyase at 20 U/ml for 2 h; free tobramycin bismuth-ethanedithiol modulate (Tob-Bi) at 1/2 the minimum inhibitory concentration (MIC); or liposomal (lipo) Tob-Bi at 1/2 the MIC. Biofilms were stained with SYTO 9 for live cells and concanavalin A (ConA) for alginate. Images were taken with a scanning confocal laser microscope using the appropriate filters. Bar indicates 50 μm.

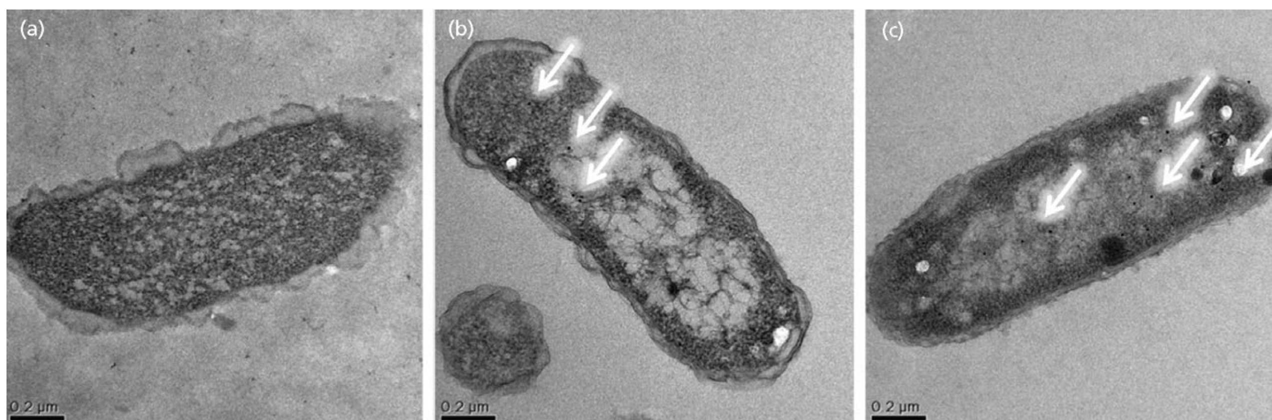

Figure 3 Deposition of tobramycin in the *Pseudomonas aeruginosa* strain PA 48913 after 6 h. In comparison with (a) medium alone, the interaction between (b) free or (c) liposomal tobramycin bismuth-ethanedithiol modulate (Tob-Bi) with bacterial membrane using immunocytochemistry was examined. Exopolysaccharide matrix is visible as a hazy cloud surrounding the bacterium. Samples were analysed using a JEOL STEM (2011), and images were captured with a Gatan Ultrascan digital camera.

the starting concentrations of the lipo Tob-Bi formulation were 2- to 8-fold lower than the MIC of the free Tob-Bi, alginate production was significantly attenuated at half the MIC but did not fare better overall than the free form in either strain. The alginate levels in the lipo Tob-Bi-treated samples were also increased over time. This increase could have been due to the inability of bismuth entrapped in liposomes at low concentrations to reduce the alginate levels over longer periods. Huang and Stewart[31] examined the activity of bismuth dimercaprol against exopolysaccharide-producing *P. aeruginosa*. Those investigators noted that an inhibitory concentration of 12.5 μM was required to significantly reduce exopolysaccharide levels. Wu *et al*.[32] also examined bismuth-dimercaprol, but found bismuth-ethanedithiol with lower concentrations and toxicity were required to inhibit alginate production. We found that entrapment of tobramycin and bismuth-ethanedithiol reduced the necessary concentrations to inhibit alginate, however the free formulation was more effective.

We sought to determine the response of the mucoid PA 48913 strain in flow cells over 96 h to alginate lyase or Tob-Bi (half the MIC). The untreated control grown for 96 h, displayed live green colonies with red alginate producing a yellow colour when merged (Figure 2). The absence of dispersed alginate could have been due to the removal of the alginate matrix by the continuous flow of medium or that this particular strain of *P. aeruginosa* secreted only cell-associated alginate. When alginate lyase (10 U/ml) was introduced for 1 h after a 95 h growth period, *P. aeruginosa* colonies were detached and alginate was reduced. An increase of alginate lyase concentration (20 U/ml) for 2 h detached the colonies significantly and alginate was virtually eliminated. Since alginate lyase detached the bacteria, cells without alginate were not present under the microscope due to the continuous flow of the medium, which washed away the unattached cells. The removal of colonies under a continuous flow has also been observed by other groups.[34,37] The growth of *P. aeruginosa* in the presence of free or lipo Tob-Bi was not affected and colonies were not detached. However, the formulations attenuated the alginate levels, confirming the carbazole assay results.

Since it was not clear if Tob-Bi (in a free or liposomal form) was accumulated in the mucoid PA 48913 strain to modulate alginate production or bound to alginate on the surface, transmission electron microscopy (TEM) studies were performed (Figure 3). With TEM, bismuth could be observed as dark dense spots, and tobramycin could be labelled with gold-conjugated antibodies. As shown by TEM, the presence of the alginate in the controls and its reduction in presence of the formulations could be viewed as hazy clouds around the bacterium, as observed by others.[47,48] More tobramycin in a liposomal than free form penetrated the bacterium. Although dark spots might be seen, the EDS analyses proved that bismuth localization within the bacterium was absent. It was possible that the bismuth could bind to the alginate in the exterior of the cell and disrupt the membrane, increasing tobramycin penetration. The evidence of bismuth action and localization has been previously only demonstrated in non-mucoid *P. aeruginosa* with the observation of dense spots on the outer membrane with blebbing and aggregation of cytoplasmic material, although no EDS analyses were completed to verify these spots.[32] Other studies involving bismuth adherence to cellular surfaces and penetration into cytoplasmic membrane of *Yersinia enterocolitica*, *Clostridium difficile*, and *H. pylori* have been confirmed by TEM and EDS.[24,27,49]

Conclusions

The production of exopolysaccharides by mucoid *P. aeruginosa* could be modulated by Tob-Bi in a liposome form, but the free Tob-Bi attenuated alginate at higher concentrations. Alginate lyase was important in detaching and reducing alginate levels but could not eradicate bacterial growth. However, a synergy between alginate lyase and Tob-Bi existed, which not only increased bacterial susceptibility but detached bacteria from the surface. While we did not observe any evidence of bismuth action on *P. aeruginosa* during TEM analysis, we

do not doubt its contribution to the formulation's antimicrobial activity. Evaluation of the pharmacokinetics and efficacy of this liposomal formulation against mucoid resistant strains in an animal model of chronic pulmonary infection is necessary.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflict of interest to disclose.

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