The effect of particle size of inhaled tobramycin dry powder on the eradication of *Pseudomonas aeruginosa* biofilms

Reham Aljalamdeh, Robert Price, Matthew D. Jones, and Albert Bolhuis*

Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, United Kingdom

*Corresponding author: email: a.bolhuis@bath.ac.uk, phone +44 (0)1225 383813

Abstract

Pseudomonas aeruginosa is the predominant opportunistic bacterium that causes chronic respiratory infections in cystic fibrosis (CF) patients. This bacterium can form biofilms, which are structured communities of cells encased within a self-produced matrix. Such biofilms have a high level of resistance to multiple classes of antibiotics. A widely used treatment of *P. aeruginosa* lung infections in CF patients is tobramycin dry powder inhalation. The behaviour of particles in the lung has been well studied, and dry powder inhalers are optimised for optimal dispersion of the drug into different zones of the lung. However, one question that has not been addressed is whether the size of an antibiotic particle influences the antibiofilm activity against *P. aeruginosa*. We investigated this by fractionating tobramycin particles using a Next Generation Impactor (NGI). The fractions obtained were then tested in an *in vitro* model on *P. aeruginosa* biofilms. The results indicate that the antibiofilm activity of tobramycin dry powder inhaler can indeed be influenced by the particle size. Against *P. aeruginosa* biofilms of two clinical isolates, smaller tobramycin particles (aerodynamic diameter <2.82 μm) showed better efficacy by approximately 20% as compared to larger tobramycin particles (aerodynamic diameter <11.7 μm) However, this effect was only observed when biofilms were treated for 3 hours, whereas there was no difference after treatment for 24 hours. This suggests that in our model the rate of dissolution of larger particles limits the effectiveness of tobramycin over a 3-hour time period, which is relevant as this is equivalent to the time in which most tobramycin is cleared from the lung.

Keywords: *Pseudomonas aeruginosa*, biofilms, tobramycin, dry powder inhaler, Next Generation Impactor (NGI), particle size

1. Introduction

In patients with cystic fibrosis (CF), the opportunistic pathogen *Pseudomonas aeruginosa* is a major cause of lung infections leading to increased morbidity and mortality rates among these patients. This is attributed partially to the ability of this bacterium to form biofilms (Ciofu et al., 2015; Muheim et al., 2017; Nikaido and Pagès, 2012). These biofilms are highly organized communities of cells that are attached to each other and/or to surfaces, and embedded within a self-synthesized extracellular polymeric substance (EPS) matrix, mainly containing extracellular DNA, polysaccharides, and/or proteins (Furiga et al., 2015). Biofilms are characterized by a high tolerance to both immune defensive mechanisms and to most of the available antibiotic therapies. For instance, it is assumed that cells inside biofilms are 10-1000 times less susceptible to anti-microbial therapies as compared to planktonic free-floating cells (Marshall et al., 2016; Patton et al., 2010; She et al., 2018).

P. aeruginosa biofilms in CF lungs settle and localise in a thick mucus layer in the trachea-bronchial region of the respiratory airways (Geller et al., 2011). Such a location makes the treatment of these infections using the systemic delivery of antibiotic agents challenging, as high doses are required to reach the lung tissue which can lead to adverse reactions. Therefore, the pulmonary delivery of antibiotics is an attractive approach for the treatment of lung CF infections (Geller et al., 2011; Worlitzsch et al., 2002). Inhaled antibacterial drugs that are used in the treatment of *P. aeruginosa* CF lung infections are in two forms, being either nebulized solutions or dry powder formulations (Ambrus et al., 2018). The currently approved inhaled dry powder antibiotics are colistin and tobramycin. For instance, TOBI Podhaler® is an approved dry powder inhalation formulation of tobramycin (McKeage, 2013; Akkerman-Nijland et al, 2020), which most CF patients prefer when comparing to nebulised tobramycin, leading to, for instance, better adherence (Harrison et al., 2014). Tobramycin powder from a capsule is aerosolized using the Podhaler device by the energy of the patient's own inspiration

(Konstan et al., 2011), resulting in the particles in the powder being separated from each other and carried in the airstream to the lungs where they deposited.

Following inhalation, these particles distribute and deposit into different compartments of the lung. Briefly, the lung can be divided into two regions, the conducting and the respiratory zones. The conducting zone includes trachea, bronchi, bronchioles, and terminal bronchioles, whereas the respiratory zone comprises of the respiratory bronchioles, alveolar ducts and alveolar sacs (Hoiby, 2011). Deposition of dry powder particles in these zones depends on several variables such as patient-associated factors, the inhaler device and inhaled powder formulation properties (Tiddens et al., 2014) such as particle shape, density and size.

Drug particle size is one of the important properties that can influence both the deposition and fate of particles in the respiratory airways. Normally, inhaled drug particles are polydisperse in nature with a large particle size range (Deng et al., 2018). Particles with an aerodynamic diameter (d_{ae}) larger than 10 μm are mostly deposited in the oropharyngeal region and do not reach the lungs, and those that are between 3-10 μm are mostly deposited in the trachea-bronchial region. Furthermore, particles at a range of 1-3 μm target the alveolar zone of the lungs (Geller et al., 2011; Nafee et al., 2014; Verbanck et al., 2006), but those that even smaller (<1 μm) are exhaled due to low inertial and gravitational forces which are insufficient to deposit them (Nafee et al., 2014). Once deposited, the particles must dissolve and the rate of this depends on the size of these particles, which in turn can influence drug efficacy (Nafee et al., 2014).

Currently, studies that evaluate the *in vitro* activity of tobramycin rely on testing its efficacy against *P. aeruginosa* biofilms in an aqueous solution. However, there are no biofilm models to test dry powder formulations, and it is thus unclear what the effect of, for instance, particle size is on antibiofilm activity, highlighting the need for a model that can address such issues. To achieve this, we used a Next Generation Impactor (NGI), which is an instrument used to

83 measure *in vitro* behaviour of inhalable dry powder products (Rowland et al., 2018). The NGI
84 sequentially separates drug aerosols into various size categories from larger to smaller particles
85 on the basis of the particles' aerodynamic diameter (Guo et al., 2008; Roberts and Mitchell,
86 2013; Wang et al., 2017). The cut-off aerodynamic diameters of these particles were previously
87 determined at flow rates of 30 and 60 L/min (Marple et al., 2003). Using the NGI and a recently
88 developed aerosol dose collection apparatus, we fractionated tobramycin particles into
89 different sizes and tested these on a *P. aeruginosa* biofilm models to (a), test the feasibility of
90 analysing dry powders on biofilms and (b), to further understand the role of particle size on
91 antibiotic efficacy, which could be very valuable for improving the pharmacological activity
92 of inhaled antibiotics.

2. Materials and Methods

2.1 Chemicals

All chemicals and culture media were purchased from Sigma-Aldrich (Gillingham, UK) or Fisher Scientific (Loughborough, UK), unless stated otherwise. The TOBI Podhaler® and 28 mg tobramycin inhalation powder (TIP) capsules were purchased from Novartis Pharmaceuticals (Camberley, UK). Tobramycin that was used to determine the minimal inhibitory concentration (section 2.3) and a calibration curve (section 2.8) was purchased from Fisher Scientific (97% purity).

2.2 Bacterial strains and growth media

The bacterial strains used in this study are laboratory strain *P. aeruginosa* PAO1 (Stover et al., 2000) and three clinical CF isolates LMG 27648, LMG 27643, and LMG 27649. These clinical *P. aeruginosa* isolates were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM, Brussels, Belgium). Strains were routinely grown on Mueller Hinton (MH; Oxoid) broth. Artificial sputum media (ASM) and minimal MOPS medium (MMM) were prepared as described elsewhere (Kirchner et al., 2012; LaBauve and Wargo, 2012).

2.3 Minimum inhibitory concentration (MIC)

MIC tests were performed in MH, MMM and ASM using the macro-dilution protocol as described elsewhere (Andrews, 2001). The MIC is defined as the lowest concentration of an antibiotic agent that shows no visible growth of a microorganism after overnight incubation.

2.4 Colony biofilm assay

Colony biofilms of *P. aeruginosa* were grown as described (Merritt et al., 2005). Briefly, sterile semipermeable polycarbonate membranes (Whatman, Little Chalfont, UK; 0.2 µm pore size, 25 mm) were placed on the surface of MH agar plates. Then an aliquot of 50 µl of overnight culture, adjusted to an optical density (OD) at 600 nm of 0.05, was spotted on each membrane. After that, the inoculated membranes were incubated for 48 h at 37°C to permit biofilm formation. The polycarbonate membranes were moved to fresh agar plates with sterile forceps on a daily basis. On the third day, a 30 µg tobramycin disc (Oxoid), or a glass fibre filter with TIP of various sizes (see section 2.6) were placed on the biofilms using sterile forceps. In case of the glass fibre filter, TIP particles were in direct contact with the top of the biofilms, and controls were covered with a filter without tobramycin. A schematic of the colony biofilm with tobramycin filter is shown in Figure 1. The biofilms were then incubated for a further 3 h or 24 h, after which cells were harvested by resuspension in 5 mL of sterile phosphate buffered saline (PBS). Cells were dispersed by vigorously vortexing, and the colony forming units were determined by serial dilution and plating.

2.5 Tobramycin capsule filling and humidity control

To collect similar amounts of TIP with different particle sizes, we adjusted the mass of TIP aerosolised into the NGI. Before each experiment, hydroxypropyl methylcellulose capsules (HPMC; transparent; size #3; Capsugel, Colmar, France) were filled manually with TIP extracted from TOBI Podhaler® capsules and weighed using a four-place analytical balance (Sartorius, Epsom, UK). Initial experiments found unacceptable variation in NGI deposition when capsules were filled immediately before use. Therefore, capsules were stored for 24 h in a sealed desiccator under a controlled temperature of 25°C and relative humidity (RH) of 43% before testing, resulting in acceptable reproducibility. This RH was produced using a saturated

140 salt solution of potassium carbonate (K_2CO_3) (Miller et al., 2017). Temperature and relative
141 humidity were monitored using a thermohygrometer placed inside the desiccator, and the
142 following day these capsules were aerosolised through the NGI as described below (section
143 2.6).

144

145 **2.6 Operation of the NGI**

146 The NGI was used by applying conditions described elsewhere (Meenach et al., 2013). Briefly,
147 before testing, the pre-separator was filled with 15mL Milli-Q water. The NGI stages were
148 coated with a solution of 1% (v/v) glycerol in methanol (VWR Chemicals) to minimize particle
149 bounce. The NGI was connected to twin vacuum pumps (GAST 1023 series, connected in
150 parallel) via a critical flow controller (TPK, Copley, Nottingham, UK), which was fixed before
151 each experiment at 30 L/min or 60 L/min flow rates using a digital flow meter (DFM2000,
152 Copley Scientific, Nottingham, UK). A TIP capsule for each experiment was aerosolised from
153 a Podhaler through the NGI for 10 seconds, which was chosen as it is sufficient for complete
154 dispersion of the powder from the capsule.

155 Initial experiments were carried out to determine the aerodynamic particle size distribution of
156 TIP when aerosolised from the Podhaler at 30 L/min and 60 L/min. These experiments utilised
157 all eight stages of the NGI and the aerosolization of 28 mg TIP from a single as supplied TOBI
158 capsule (n=5). Following each aerosolization, the mass of tobramycin collected on the
159 induction port, pre-separator, stages 1 to 7, and MOC was determined (section 2.8).

160 Subsequently, TIP particles of different sizes were collected from either stage 2 or 4 using an
161 Aerosol Dose Collection (ADC) device (Price et al., 2020). The ADC allows particles to be
162 collected on a glass fibre filter without the formation of *in situ* agglomerates, which can affect
163 their subsequent dissolution behaviour. In the experimental set up with the ADC, a rubber

stopper was placed in the NGI air outlet from stage 2 or stage 4 to disrupt airflow and ensure the collection of all TIP particles on the filter. Second, a glass fibre filter (Copley®, 25 mm diameter, 1 μm pore size) was mounted in the ADC to collect TIP particles. The glass fibre filter was replaced for each repeat of the experiment and the flow rate was adjusted for each experiment after placing the filter in the ADC. These filters were either applied directly to biofilms (section 2.4), particles were visualised by electron microscopy (section 2.7), or the mass of TIP collected was determined (section 2.8).

2.7 Scanning Electron Microscopy (SEM)

To demonstrate that the ADC apparatus had successfully captured TIP particles of different sizes, their geometric particle size distribution was determined using SEM. TIP particles on glass fibre filters were analysed by applying conditions stated elsewhere (Li et al., 2014). TIP samples were fixed into aluminium stubs (Agar Scientific, Stansted, UK) using double-sided adhesive carbon tabs (Agar Scientific). Then, the samples were coated with a thin film of gold using a sputter coater (Sputter Coater S 150B, Edwards, Burgess Hill, UK). The coating process was operated at 1 kV of voltage for 3 min. The images were captured using Jeol SEM (Jeol Jsm-6480LV Scanning Electron Microscope; Jeol Ltd, Welwyn Garden City, UK), and several magnifications levels were used. The captured images were further analysed for geometric particle size determination using the software package ImageJ (Schneider et al., 2012). For every ImageJ analysis, manual particle size measurements were performed, and for every measurement fixed criteria were used: all particles in the given image might be measured, even the small particles in front of large particles; a specific number (100) of particles were selected randomly and the same magnification (x5000) was used for all images. As TOBI Podhaler® particles are spherical (McKeage, 2013), particle size was not sensitive to the direction of

measurement, so all diameters were measured in the vertical direction. Particle size distributions were summarised by the median diameter and span, which was defined the difference between the ninetieth and tenth centile diameters, divided by the median diameter.

2.8 High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) quantification

To determine the mass of tobramycin collected on parts of the NGI or glass fibre filters, they were rinsed with known volumes of Milli-Q water and sonicated for 10 min in an ultrasonic water bath to ensure complete dissolution of TIP. To quantify the amount of tobramycin, an HPLC-MS method was developed and validated. The chromatographic system consisted of a pentafluoro phenyl F5 column (2.6 μM, 2.1 x 100 mm; Phenomenex, Macclesfield, UK) as the stationary phase, which was used with a flow rate of 0.3 mL/min at 25°C and an injection volume of 10 μL of each sample was injected in triplicate. The mobile phase involved utilizing two solvents, which were 100% water with 0.1% (v/v) formic acid as solvent A and 100% methanol with 0.1% (v/v) formic acid as solvent B. The proportion of these solvents in the mobile phase was controlled during the analysis by the ultra HPLC instrument. Elution was carried out with 0% mobile phase B for 3 min followed by a linear gradient to 100% B for 7 min. The mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) was operated in electrospray time of flight (ESI) positive-ion MS mode, and the following conditions were used during the MS analysis. The capillary voltage was set to 4500 V, nebulizing gas at 4 bar, and drying gas at 12 L/min at 220°C. The concentration of tobramycin in injected samples (and thus the mass of deposited tobramycin) was determined by constructing a calibration curve using kanamycin as an internal standard. Following the addition of kanamycin (final concentration 4 μg/mL), samples were vigorously vortexed prior to HPLC-MS analysis. All

212 stock solutions were prepared on the same day of the experiment, and HPLC-MS analysis was
213 performed with 24 h. The calibration curve was linear (r^2= 0.998) over the range of 5 to 20
214 µg/ml. Based on the standard deviation of y-intercepts of the regression line (International
215 Conference on Harmonisation, 1996), the estimates for the limit of detection was 1.4 µg/ml
216 and the limit of quantification was 4.4 µg/ml.

217

218 **2.10 Statistical analysis**

219 Data were presented as the mean ± standard error of the mean (SEM) of n ≥ 3 independent
220 biological repeats. Results were analysed using GraphPad Prism 7 by applying the Student *t*-
221 test. Values of $p< 0.05$ were considered statistically significant.

222

3. Results

3.1 The *in vitro* activity of tobramycin against *P. aeruginosa*

The MICs (Table 1) of tobramycin against one laboratory strain (*P. aeruginosa* PAO1) and three clinical CF isolates (*P. aeruginosa* LMG 27648, LMG 27643, and LMG 27649) were determined in MH broth, MMM and ASM by the macro-dilution method. The results showed that MIC values in in MH broth and MMM were fairly similar and differed by at most one doubling dilution, in the range of 0.25-1 µg/mL The MIC was, for all four strains, 4 µg/mL when ASM was used. All strains were susceptible to tobramycin according to BSAC breakpoints.

To determine the activity of tobramycin against *P. aeruginosa* biofilms, which is the state that the cells are in during a lung infection, a colony biofilm assay was used. This model was chosen as the biofilm grow on a semi-solid surface with an air interface, which is probably more representative of biofilms in the lung as compared to the more standard 96-well plate assay in which biofilms are completely immersed in liquid. The results showed that in all tested strains, tobramycin reduced the viable count in the biofilms moderately (Fig 2). However, complete eradication was not achieved and the reduction in viable count was, on average, approximately 60%.

3.2 Aerodynamic particle size distribution of tobramycin inhalation powder

The aerodynamic particle size distributions of TIP aerosolised from the Podhaler at both 30 L/min and 60 L/min are shown in Fig 3.

3.3 Tobramycin masses collected with the ADC mounted on stages 2 or 4 of the NGI

To determine the effect of particle size on eradication of *P. aeruginosa* biofilms, it was necessary to collect the same amount of TIP but with different particle sizes. The parameters to obtain approximately 0.5 mg TIP per filter were determined in an empirical manner. To this purpose we used the ADC mounted onto the NGI and determined that, at stage 2 at 30 L/min and TIP capsule mass of 4.4 mg, we collected a very similar mass as when using stage 4 at 60 L/min and TIP capsule mass of 4.3 mg. This resulted in a mean mass of 0.51 mg with SD value of 0.05 for larger particles (stage 2 at 30 L/min, d_{ae}<11.7 μm) and 0.48 mg with SD value of 0.12 for smaller particles (stage 4 at 60 L/min, d_{ae}<2.82 μm), with a difference between those masses of 7.8%. Statistically, the difference between the masses was not significant (p=0.15).

3.4 SEM analysis

Before testing fractionated tobramycin particles on biofilms, a number of tests were performed. Firstly, SEM analysis was used to image TIP particles that had been extracted from the NGI stages using the method outlined in section 2.6. These SEM images were further analysed to obtain geometric particle size measurements using ImageJ software. Representative SEM micrographs for TIP particles (Fig 4) show polydisperse, approximately spherical, and porous microparticles. The geometric particle size distributions determined from these images, using ImageJ, were approximately log-normal (Fig 5) and showed that on stage 2 at 30 L/min, the particle size distribution included some coarse particles, with a median geometric diameter of 5.6 μm and span of 1.5. The particles collected at stage 4 at 60 L/min had a smaller median geometric diameter of 1.4 μm and span of 1.2.

3.5 The influence of differently sized tobramycin inhalation powder particles against *P. aeruginosa* biofilms

The fractions of small and large TIP particles were used to challenge *P. aeruginosa* biofilms. These were treated with a dose of 0.5 mg/filter TIP, and filters without tobramycin were used as control. The biofilms were incubated for 3 h, as this period is comparable to the time it takes for tobramycin sputum concentrations to be significantly reduced in people with CF (Hubert et al., 2009; Poli et al., 2007). The 3 h treatment time was not particularly effective in killing cells in our biofilm model but, crucially, there was an approximate 20% reduction of the viable count when applying particles with d_{ae}<2.82 µm as compared with particles with d_{ae}<11.7 µm (Fig 6). For *P. aeruginosa* LMG27649 and LMG27643, particles with d_{ae}<11.7 µm did not have any effect on the biofilms, but there was a statistically significant (LMG27649: p=0.04; LMG27643: p=0.02) reduction with particles with d_{ae}<2.82 µm. For the other two strains, larger particles had a moderate effect on the viable count of cells in biofilms and there was a further reduction in viable count when the biofilms were treated with smaller particles. However, in the latter cases this reduction was statistically not significant (LMG27648: p=0.26; PAO1: p=0.63).

The reduction in viable count after a 3 h treatment of the biofilms was rather poor, so we also tested the effect of a 24 h incubation with TIP. In this case, the reduction in viable count was between 80-90% when comparing samples with the untreated control (Fig 7). However, there was no significant difference in viable count reduction when comparing small and large TIP particles.

4. Discussion

We investigated the influence of differently sized TIP particles against *P. aeruginosa* biofilms by making use of the NGI to separate particles into different fractions. These particles were collected from stage 2 at 30 L/min and stage 4 at 60 L/min, meaning that the collected fractions had d_{ae}<11.7 μm and d_{ae}<2.82 μm, respectively (Marple et al., 2003). The efficiency of the NGI and ADC device at capturing particles of different size ranges was confirmed by the SEM analysis, which found particles captured at stage 2 at 30 L/min had a larger median geometric diameter (5.6 μm) than those captured at stage 4 at 60 L/min (1.4 μm).

An aerosol collection apparatus was used to collect TIP particles from the above-mentioned stages. Without this, deposition of particles from the NGI occurs directly on a solid impactor stage with high-speed deposition of particles in a small area, which results in the formation of strong agglomerates which then behave as larger particles (Price et al., 2020). However, using the ADC apparatus enables a slow and uniform deposition of aerosol particles over a single, large surface area glass fibre filter, so the collected powder subsequently behaves as single particles.

A difficult issue to resolve was that the NGI separates powder into size fractions with different masses for each fraction, while equal masses were required to analyse the effect of particle size only. Moreover, the amount of tobramycin collected did not vary in a linear fashion with the aerosolised dose, so was difficult to predict. We essentially had to use a trial and error process to determine the parameters to collect equal masses of differently sized particles. Another issue was that initially the mass of TIP collected from the ADC device was variable, but results became more consistent when capsules were equilibrated at a constant humidity and temperature, before use.

When antibiotic particles are deposited on a biofilm, they must first dissolve in order to exert their pharmacological activity. This depends on particle size, which is one of the parameters that determines physical properties of a drug (Shekunov et al., 2007; Wang et al., 2017). Accordingly, the influence of TIP particles was investigated by testing differently sized particles for 3 and 24 h. At 24 h, there was a significant reduction in the viable count when comparing treated with untreated samples, but there was no difference between smaller and larger particles. Thus, over 24 h the difference in the rate of dissolution of small and large TIP particles is not a rate limiting step. However, this time is not physiologically relevant, as tobramycin sputum concentrations are significantly reduced after just 3 h in people with CF (Hubert et al., 2009; Poli et al., 2007), with the TOBI Podhaler having a sputum half-life of only 1-2 hours (Geller et al., 2007). An incubation time of 3 h was thus more appropriate. This time period was far less effective in reducing the viable count but, importantly, smaller particles were more effective by approximately 20% when compared to larger particles. Indeed, it is generally recognized that the dissolution rate of small-sized particles can be significantly better than the larger-sized particles, which is attributed to the larger specific surface area of the small particles (Riley et al., 2012; Tay et al., 2018; van der Wiel et al., 2017; Watts and Williams, 2011). We should note, however, that while we observed a difference in the effectivity between small and large particles for all *P. aeruginosa* strains, it was statistically significant only for the two clinical isolates that were the most recalcitrant to a 3 h treatment, (LMG27649 and LMG27643). In these strains, larger particles did not cause any reduction in the viable count, while small particles resulted in a 20% reduction. In case of the other strains (LMG27648 and PAO1), larger particles resulted in approximately 15-20% reduction in viable count, with a further non-significant reduction with smaller particles. Planktonic cells of those four strains all displayed the same sensitivity to tobramycin, but phenotypic and genetic differences between the strains could result in the differences in biofilm formation, such as in composition

of the extracellular matrix or thickness of the biofilms (Wimpenny et al., 2000). For example, the three clinical isolates are reported to be alginate producers (Hoffmann et al., 2005; Leitão et al., 1996; Mathee et al., 2008), whereas the laboratory strain PAO1 does not produce this polysaccharide. We also observed that only LMG 27649 was unable to grow in a minimal growth medium without the addition of casamino acids (data not shown), indicating that this strain is auxotrophic, whereas the other strains are not. Whether this would influence the effects of tobramycin particles is not known, but it does clearly show that the strains differ from each other.

The influence of differently sized particles of other drugs has been evaluated previously, showing better efficacy for smaller sized particles as compared to larger (Jinno et al., 2006; Leach et al., 2009; Liu et al., 2015). For example, for the oral vasodilator cilostazol, smaller cilostazol particles of 2.4 μm had a better rate of dissolution and efficacy than particles of 13 μm (Jinno et al., 2006). This was also observed for inhaled beclomethasone (a corticosteroid), which is more effective in a particle size of 1.1 μm as compared to 4 μm (Leach et al., 2009; Van Schayck and Donnell, 2004; Vanden Burgt et al., 2000). Although these differences have been attributed to varying lung deposition patterns with changing particle size, they may also have been influenced the faster dissolution rate of smaller particles.

We should acknowledge that our study has limitations. Firstly, there was a slight difference in the TIP mass that were collected for small and large sized particles from different stages/flow rates. It was technically difficult to obtain equal masses of differently sized particles, in particular at the amounts required for biofilm assays (0.5 mg/filter). However, the difference in the amounts obtained was statistically not significant ($p > 0.05$). It should be noted that on average we collected slightly less (<8%) of the smaller particles, but these were nevertheless more effective, which only strengthens our conclusion that smaller particle sizes result in more efficient killing of cells in *P. aeruginosa* biofilms.

Another limitation is that with the NGI the maximum particle size that is collected on the filters can be controlled, but not the minimum particle size. Thus, while the average particle size differs between the collected fractions, there is some overlap in particle sizes and effects on antibiofilm activity could have been greater if it was technically possible to control both minimum and maximum sizes. Also, our SEM analysis only measured the diameters of a small number of particles (100). Despite this, we obtained log-normal particle size distributions (Fig 5), so these data provide additional reassurance that the NGI and ADC device collected particles of different sizes.

Our system used the *in vitro* colony biofilm model (Merritt et al., 2005). It is a simple model system but nevertheless it is useful as biofilm grows on a semi-solid surface with an air-interface. It is of course not the same as the conditions found in a lung, but in this study, it was a more useful model than for instance the standard 96-well plate biofilm assay. It should also be noted that 0.5 mg/filter TIP as used here is actually a very large dose when compared to a therapeutic dose of 112 mg spread through the whole surface area of the lungs (Geller et al., 2011). Future studies therefore need to focus on the use of models that, firstly, use an amount of antibiotic that better reflects clinical doses and, secondly, better mimic the *in vivo* lung pathological conditions. The latter could be achieved using, for instance, *ex vivo* models that use porcine lung samples (Harrison and Diggle, 2016), or *in vivo* models (Kukavica-Ibrulj and Levesque, 2008).

Our hypothesis on the effect of particle size was only tested for one dry powder inhaled antibiotic, tobramycin. An important aim of our study was to establish a system to test dry powder inhalers on biofilms. This has now been achieved and has demonstrated that the particle size of inhaled dry powders can influence their anti-biofilm activity. These are the most significant aspects of these findings, as this tool can be used to test other antibiotics. Our system may be of particular relevance to the development of dry powder inhaled formulations of drugs

with a low aqueous solubility, as in this situation the differences between small and large particles may become more pronounced than for tobramycin (which is freely soluble in water). Investigation of this issue may therefore highlight additional ways to increase the effectiveness of poorly soluble inhaled drugs. Future research should also measure the dissolution rate of different antibiotic particle size fractions, to fully examine the potential relationship between anti-biofilm activity and particle dissolution.

5. Conclusion

Tobramycin dry powder inhaler is one of the most widely used inhaled antibiotics in the treatment of CF lung infections. Here we showed that small TIP particles (d_{ae}<2.82 μm) showed better efficacy as indicated by a 20% reduction in the viable count as compared to larger particles (d_{ae}<11.7 μm) at an incubation time of 3 h against *P. aeruginosa* biofilms; this reduction was statistically significant for two strains of the four strain, but the trend was observed in all strains. This short incubation time is important, as this is the same timeframe in which tobramycin is largely cleared from the lung. These initial findings highlight that particle size can affect TIP antibiofilm activity. Importantly, we have developed a system to test the effect of dry powder inhalers on bacterial biofilms, and we are planning to utilise this to test other antibiotics and as well as employing more advanced biofilm models.

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579 Table 1. The MICs (µg/mL) of tobramycin against *P. aeruginosa* clinical CF isolates and
580 PAO1 determined in triplicate by macro-dilution method in MH, MMM and ASM media.

581

***P. aeruginosa* strain**	**MICs in MH**	**MIC in MMM**	**MICs in ASM**
LMG 27649	0.5	1	4
LMG 27643	0.5	0.25	4
LMG 27648	1	0.5	4
PAO1	0.5	0.5	4

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588 **Figure legends**

589 **Figure 1.** Schematic of the colony biofilm. Cells were grown on a polycarbonate membrane to
590 form a biofilm, and on the third day a filter with tobramycin inhalation powder was placed on
591 top. After incubation for 3 or 24 hours, the cells were harvested and a viable count was
592 determined.

593 **Figure 2.** The *in vitro* activity of tobramycin against *P. aeruginosa* using the colony biofilm
594 assay. (A – C) show clinical CF isolates and (D) indicates laboratory strain PAO1. All *P.*
595 *aeruginosa* biofilms were grown for 48 h on MH agar, treated with 30 μg/disc tobramycin, and
596 incubated for 24 h at 37°C. The controls represent biofilms without tobramycin. The data
597 shown represent the standard error of the mean from three biological repeats and each
598 biological repeat consisted of at least two technical repeats. Statistics were analysed using
599 unpaired 2-tailed *t* test. Statistically significant differences between treated biofilms (***,
600 $p<0.001$; ****, $p<0.0001$) and the control are indicated.

601 **Figure 3.** Aerosol particle size distribution of tobramycin inhalation powder when aerosolised
602 for 10 seconds from the Podhaler® device into the NGI at 30 L/min (A) and 60 L/min (B).
603 Error bars represent the standard error of the mean from five independent experiments for each
604 flow rate. Stages 1-7 indicate the impactor stages, followed by their corresponding cut off
605 aerodynamic diameter in parentheses. MOC: micro-orifice collector.

606 **Figure 4.** Representative SEM micrographs of TIP particles from NGI stages at different flow
607 rates. Stage 2, at 30 L/min (A) and Stage 4, at 60 L/min (B). Pictures were taken at x4,000
608 magnification (scale bar = 5 μm).

609 **Figure 5.** Comparison of the cumulative geometric particle size distributions of tobramycin
610 inhalation powder particles collected at stage 2 (A) and 4 (B) of the NGI at 30 L/min and 60
611 L/min, respectively.

Figure 6. The influence of differently size TIP particles on the eradication of *P. aeruginosa* biofilms. (A-C) show clinical isolates, and (D) indicates the laboratory strain PAO1. Bacterial cells were grown as colony biofilms for 48 h at 37°C and then were treated with different tobramycin particle size fractions of d_{ae}<11.7 µm and d_{ae}<2.82 µm for 3 h. The data shown represent the standard error of the mean from three biological repeats and each biological repeat consisted of at least two technical repeats. Statistics were analysed using an unpaired 2-tailed *t* test. Statistically significant differences between large and small particles are indicated (*, p<0.05).

Figure 7. The influence of differently sized TIP particles on the eradication of *P. aeruginosa* biofilms. (A-C) show clinical isolates, and (D) indicates the laboratory strain PAO1. Bacterial cells were grown as colony biofilms for 48 h at 37°C and then were treated with different tobramycin particle size fractions of d_{ae}<11.7 µm and d_{ae}<2.82 µm for 24 h. The data shown represent the standard error of the mean from three biological repeats and each biological repeat consisted of at least two technical repeats. Statistical significance was analysed using an unpaired 2-tailed *t* test.

628 Figures

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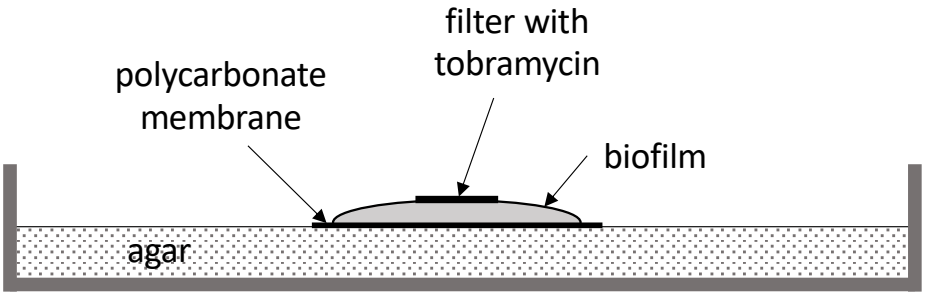
630 Figure 1



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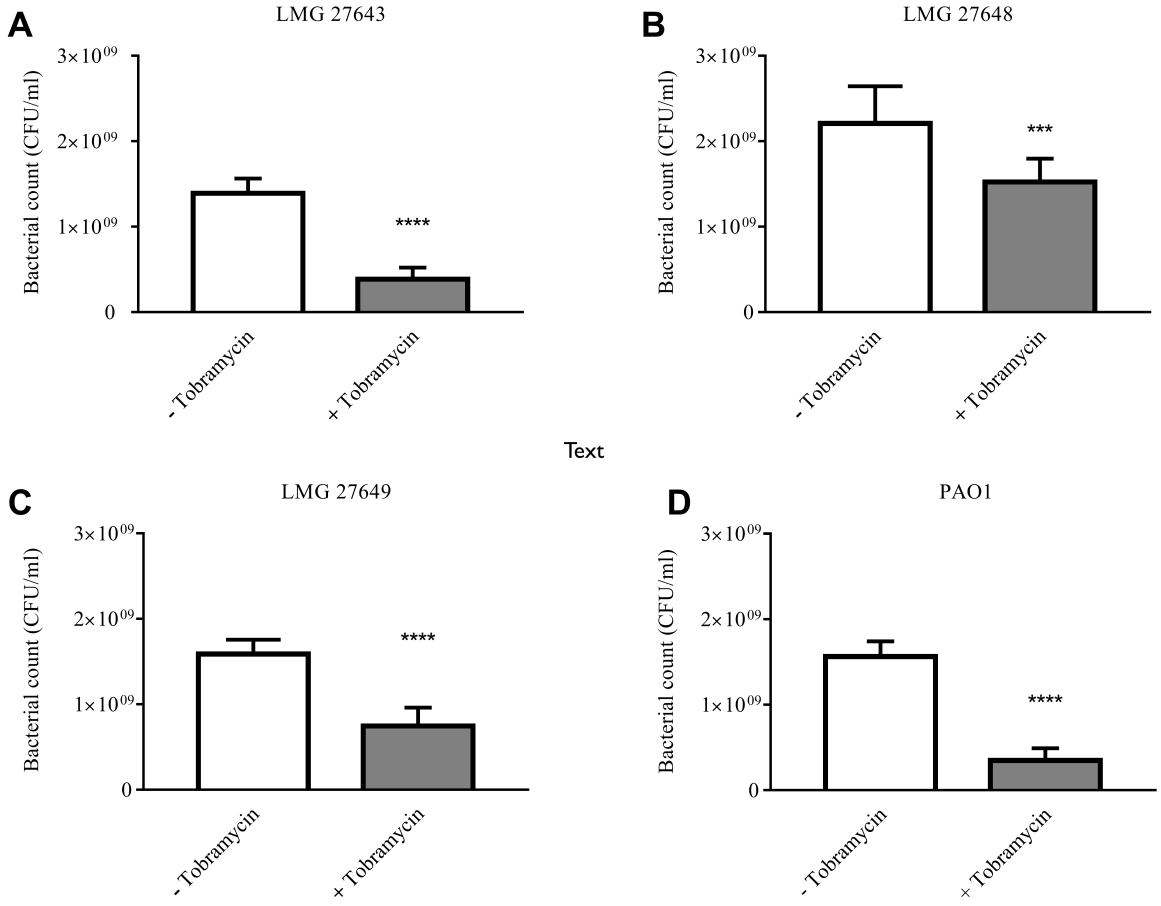
633 Figure 2



Text

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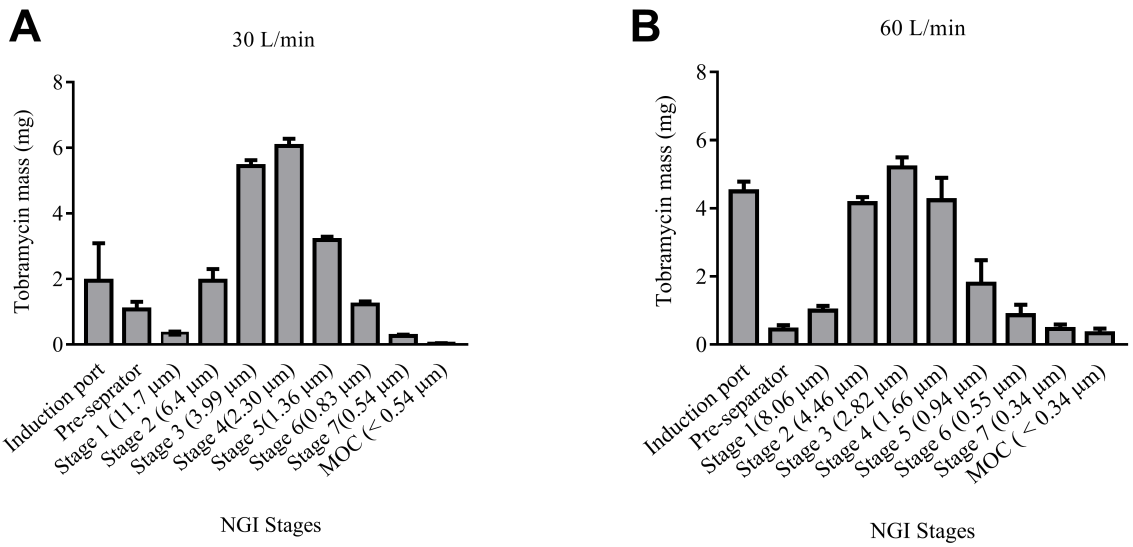
636 Figure 3



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642 Figure 4

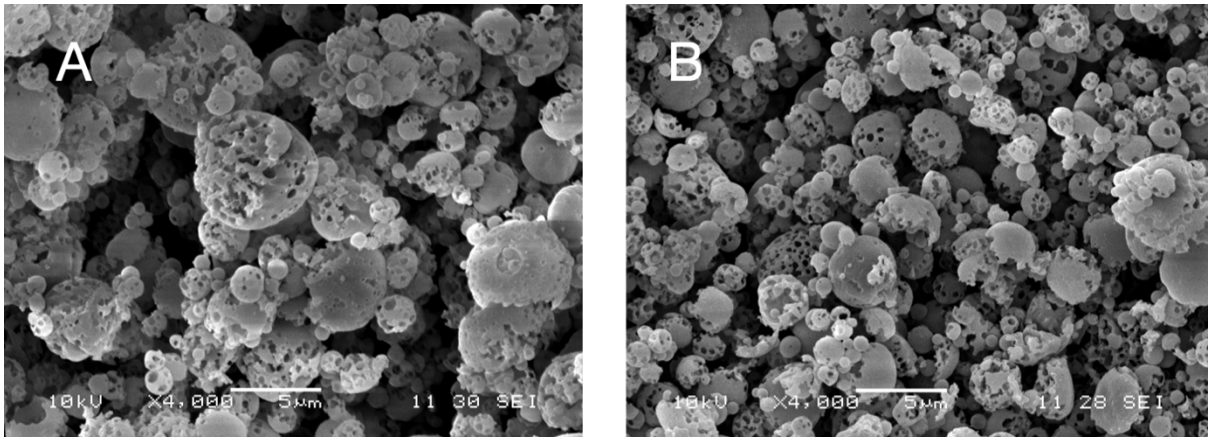
643 

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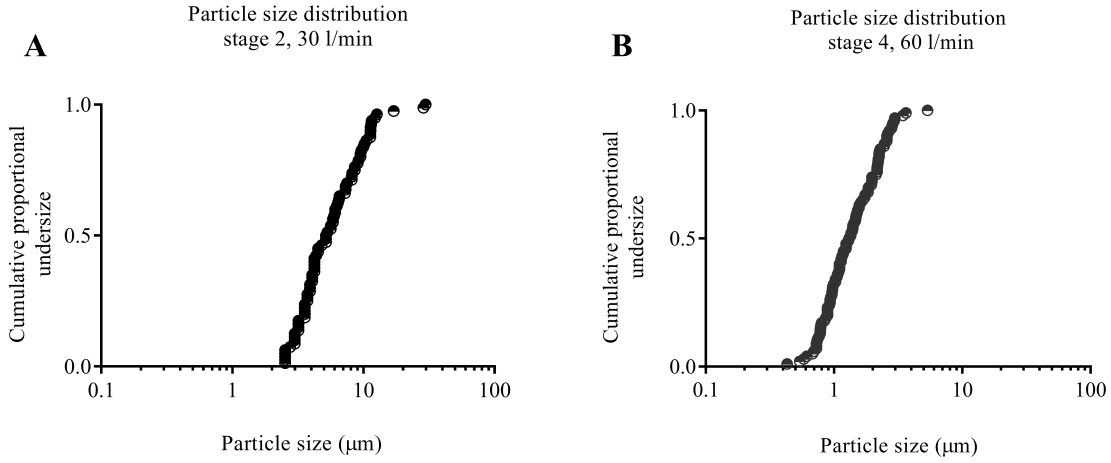
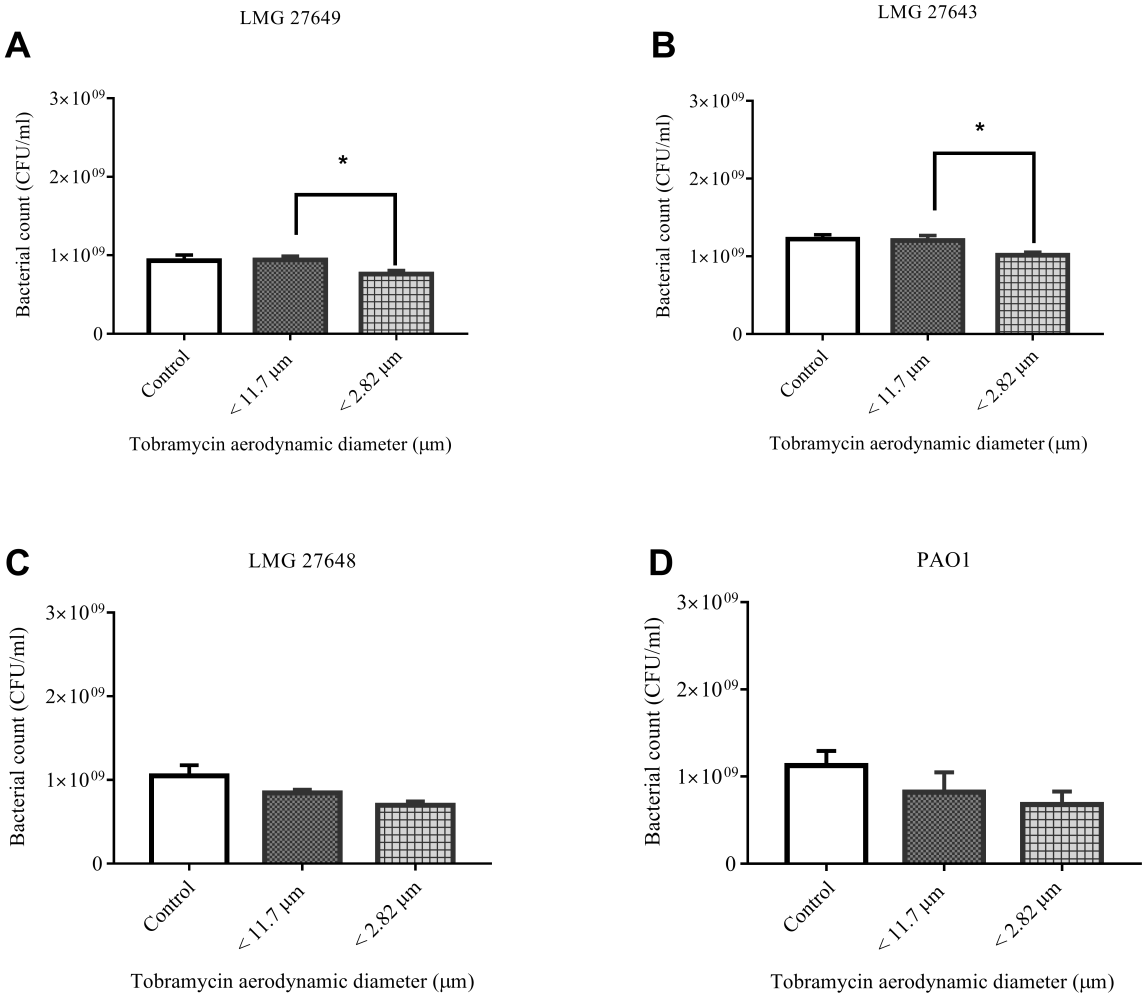
Figure 5


A
Particle size distribution
stage 2, 30 l/min
Cumulative proportional undersize
1.0
0.5
0.0
0.1
1
10
100
Particle size (µm)
B
Particle size distribution
stage 4, 60 l/min
Cumulative proportional undersize
1.0
0.5
0.0
0.1
1
10
100
Particle size (µm)

652 Figure 6



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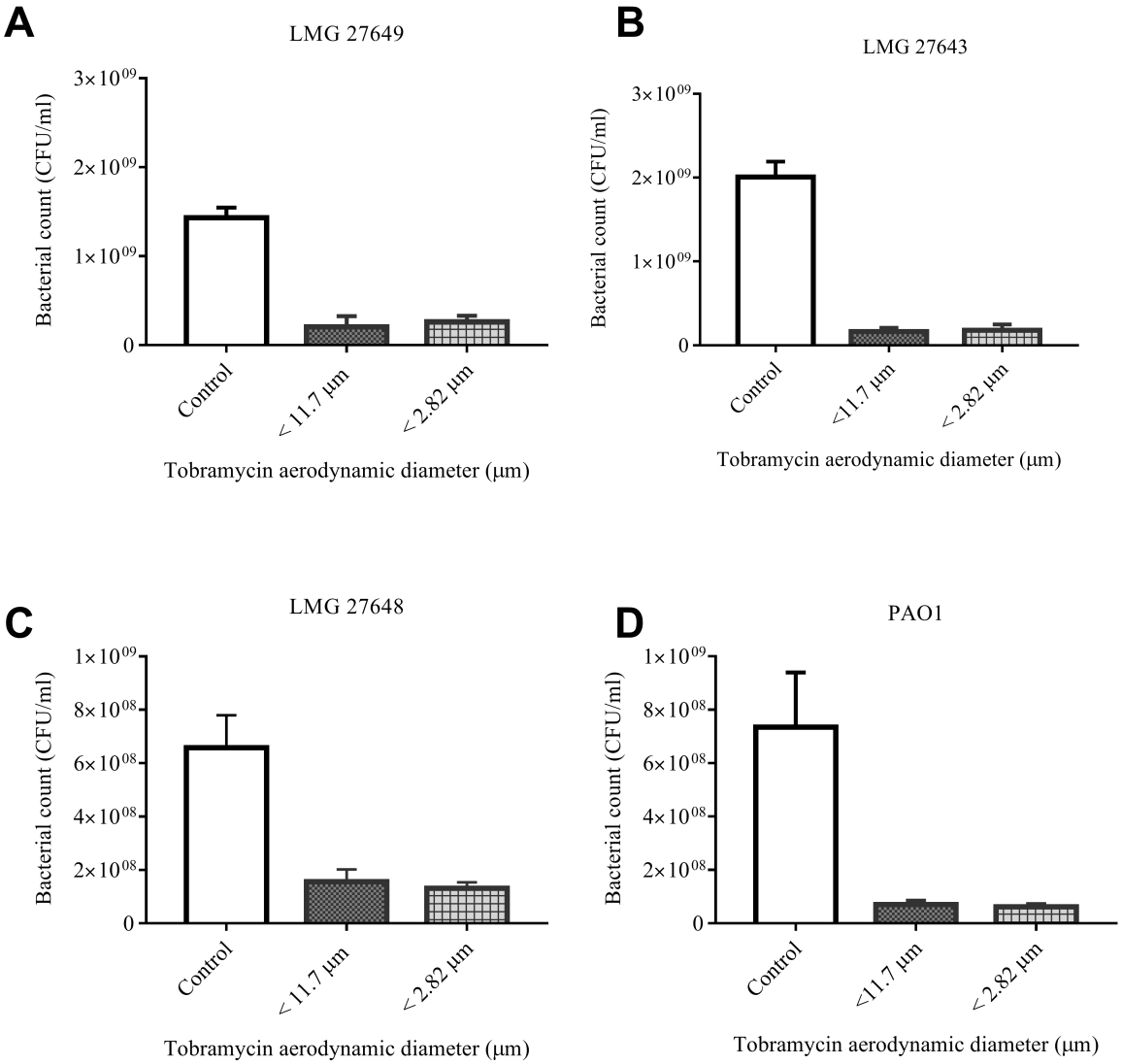
654 Figure 7



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