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RESEARCH PAPER

Antibacterial Efficacy of Inhalable Levofloxacin-Loaded Polymeric Nanoparticles Against *E. coli* Biofilm Cells: The Effect of Antibiotic Release Profile

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ABSTRACT

Purpose To investigate the effect of the antibiotic release profiles of levofloxacin-loaded polymeric nanoparticles on their antibacterial efficacy against *E. coli* biofilm cells.

Methods Three distinct antibiotic release profiles are produced by encapsulating levofloxacin in PCL and PLGA nanoparticles by nanoprecipitation and emulsification-solvent-evaporation methods. The antibacterial efficacy is examined over six days by time-dependent biofilm susceptibility testing that takes into account the effects of the biofilm age, antibiotic exposure history, and simulated drug removal.

Results Biofilm cells that survive the initial antibiotic exposure exhibit a higher antibiotic tolerance than fresh biofilm cells, where the lower the initial exposure, the higher the tolerance of the surviving biofilm cells. The lower antibiotic susceptibility of the surviving biofilm cells is transferred to their planktonic cell progeny, which can subsequently form new biofilm colonies having a higher antibiotic tolerance, hence exacerbating the infections. A biphasic extended release profile at an appropriate dose can inhibit the biofilm growth for four days, therefore reducing the dosing frequency. The importance of a high initial antibiotic exposure renders a slow release profile ineffective despite the same dosing amount.

Conclusions The antibiotic release profile has an equally significant influence on the biofilm eradication rate as the antibiotic dose.

KEY WORDS antibiotics aerosols · biofilm · bronchiectasis · cystic fibrosis · persister cells

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ABBREVIATIONS

CFU	colony forming unit
COPD	chronic obstructive pulmonary disease
DCM	dichloromethane
DPI	dry powder inhaler
EPS	extracellular polymeric substance
ESE	emulsification-solvent-evaporation
LEV	levofloxacin
MBIC	minimum biofilm inhibitory concentration
MHB	mueller Hinton Broth
MIC	minimum inhibitory concentration
NP	nanoparticles
NPC	nanoprecipitation
OD	optical density
PBS	phosphate buffer saline
PCL	poly(caprolactone)
PLGA	poly(DL-lactide-co-glycolide)
PVA	poly (vinyl alcohol)

INTRODUCTION

Biofilm is a sessile community of bacterial cells that is enclosed by a self-secreted matrix composed of an extracellular polymeric substance (EPS). Biofilm is the predominant mode of growth of bacterial cells, as it renders them less susceptible (i.e. 10–1,000-fold) to antimicrobial agents compared to their planktonic cell counterparts (1). Because the phagocytic defense mechanism is incapable of removing the biofilm cells protected by the EPS matrix, the biofilm colonization causes persistent infections and inflammations in the airways that eventually lead to the deterioration of the lung function if they are not properly treated.

Individuals with an impaired mucociliary clearance in their respiratory systems are particularly vulnerable to

chronic biofilm infections by bacterial pathogens due to the consequential mucus build-up in their respiratory airways that provides a nutrient-rich environment ideal for the biofilm growth. The pulmonary disease that is typically associated with biofilm infections is bronchiectasis, which is an abnormal irreversible dilatation of bronchi and bronchioles due to the recurring infections and inflammations.

Unlike congenital bronchiectasis (e.g. cystic fibrosis, primary ciliary dyskinesia), whose occurrence is limited to a small population, acquired bronchiectasis is prevalent among individuals suffering from chronic obstructive pulmonary disease (COPD), which is the fourth leading cause of death globally (2), where 50% of adults with moderate to severe COPD are found to exhibit bronchiectasis symptoms (3). Unfortunately, the high biofilm tolerance towards antibiotics renders the current antibiotic therapy for bronchiectasis ineffective in curing the disease completely. Antibiotic therapies for moderate to severe bronchiectasis are therefore aimed primarily to alleviate the symptoms and to minimize the exacerbations (3).

The high tolerance of the biofilm cells towards antimicrobial agents, particularly antibiotics, has been attributed to various reasons, such as (i) the non-growing state of a majority of the biofilm cells rendering them less susceptible to certain antibiotics (e.g. β-lactam), (ii) the shielding of the biofilm cells from antibiotics by the EPS matrix allowing the bacterial cells to release their adaptive stress responses in time, and (iii) the existence of subpopulation persister cells that remain invulnerable regardless of the antibiotic concentration and its duration of exposure (4). In this regard, biofilm cells enclosed in a thin EPS matrix, nonetheless, have been found to remain invulnerable after an exposure to fluoroquinolone antibiotics, which are known to be capable of eradicating non-growing bacterial cells and effectively diffusing through the EPS matrix (5). For this reason, the existence of the persister cells is widely regarded as the universal reason, though not always the only reason, for the high antibiotic tolerance of the biofilm cells.

Because of the high biofilm tolerance towards antibiotics, a high therapeutic dose is needed in bronchiectasis treatments, which poses a high risk of systemic toxicity in the conventional antibiotic delivery routes by oral and intravenous administrations. Delivering the antibiotic directly to the lung by inhalation is therefore preferred to reduce the risk of systemic toxicity (6). Moreover, inhaled antibiotic delivery has been found to significantly increase the lung bioavailability of the antibiotic (i.e. twenty-fold greater), hence resulting in a higher antibacterial efficacy compared to that obtained by the conventional drug delivery routes (7,8).

Nebulization of aqueous solutions of antibiotics is by far the most widely used delivery platform for inhaled antibiotics, which is attributed to its extremely simple formulation that offsets its many drawbacks, such as low efficiency, lengthy treatment time, poor stability, and non-portability(6). Inhaled delivery of antibiotics in the nebulized free-drug solution form, however, is subjected to a rapid clearance in the lung that results in a short residence time and a low concentration of the antibiotic in the biofilm infection sites (9). Consequently, multiple doses need to be administered daily, resulting in poor patience compliance.

The residence time of the antibiotic in the biofilm infection sites can be prolonged by incorporating the antibiotic into carrier particles with a controlled release capability. In this regard, antibiotic-loaded nanoparticles have emerged as one of the most promising formulations in inhaled antibiotic therapy against biofilm infections. The small size of the nanoparticles enables them to effectively penetrate through the static mucus, surrounding the biofilm colonies in a matter of seconds (10–12). As a result, the antibiotic is released locally, therefore increasing its residence time and concentration in the biofilm infection sites, which leads to a higher antibacterial efficacy than that obtained by the free-drug formulation (11,13). In addition, nanoparticles have been found to exhibit a longer retention time in the lung compared to their micron-scale particle counterparts, as the lung macrophages are less effective in recognizing foreign particles smaller than 1–2 μm (14).

Furthermore, the bioavailability of poorly water-soluble drugs in the lung can be enhanced by formulating them into nanoparticles, whose high surface-area-to-volume ratio increases the drug dissolution rate. Attributed to the many attractive characteristics of the inhaled nanoparticle formulation, a wide range of therapeutic agents have been formulated into inhalable nanoparticles, which are typically delivered by nebulization of the aqueous nanoparticulate suspension, as the nebulization delivery platform does not require a further dosing formulation. In this regard, bronchodilators and corticosteroids have been formulated into nanoparticles for inhaled therapies of asthma and COPD (15,16). Antibiotics have also been encapsulated into polymeric nanoparticles for inhaled tuberculosis therapy (17,18). Lastly, inhaled nanoparticle formulations of antifungal agents, immunosuppressant agents, and therapeutic proteins, have also been investigated (19–21).

The biofilm susceptibility towards antibiotics is typically characterized by the minimum biofilm inhibitory concentration (MBIC), which is defined as the minimum antibiotic concentration needed to eradicate a majority of the non-persister cells and to inhibit the visible biofilm growth initiated by the surviving persister cells (22). The conventional wisdom for an effective antibiotic therapy is to expose the biofilm cells to antibiotic concentrations that are equal or above the MBIC value (11). In this regard, bacterial cells exposed to a sub-inhibitory antibiotic concentration have been found to ramp up their biofilm mode of growth as a

defensive response to the antibiotic (23). Furthermore, non-persister cells have been found to self-transform into persister cells upon an exposure to antibiotics as part of their adaptive defense mechanism (24).

Therefore, the defensive response of the biofilm cells upon their exposure to antibiotics is highly dynamic, as the biofilm cells can adaptively react to promote their survival. Consequently, despite their similar overall dose, a constant antibiotic concentration over time above the MBIC value may not yield the same antibacterial efficacy as that obtained from a gradually decreasing or increasing antibiotic concentration that is also maintained above the MBIC value. For this reason, an investigation on the effect of the antibiotic release profile of the antibiotic-loaded nanoparticles on the biofilm susceptibility is warranted.

In this regard, several studies have reported that a sustained antibiotic release profile from carrier particles results in higher antibacterial efficacies against both planktonic and biofilm cells than those obtained from the faster release profile in the free-drug formulations (11,25–27). The superiority of the sustained release profile observed in these studies, however, is attributed more to the immediate clearance of the antibiotic in the free-drug formulations or, in the case of Misra *et al.* (27), the deactivation of the antibacterial activity of the free-drug formulation upon its administration that necessitates its encapsulation, rather than the effect of having a sustained antibiotic concentration on the bacterial cell defensive response.

Importantly, the influence of the immediate antibiotic clearance is less significant for the antibiotic-loaded nanoparticle formulation intended for biofilm infection therapy because the antibiotic is released locally inside the static mucus surrounding the biofilm colonies and also because of the stealth characteristics of nanoparticles towards the lung phagocytic defense mechanism. For this reason, the objective of the present work is to examine the effect of the antibiotic release profile on the antibacterial efficacy of the antibiotic-loaded nanoparticles in a testing condition in which the immediate antibiotic clearance is not a major determining factor.

Owing to the highly dynamic nature of the biofilm defensive response, the antibacterial efficacy of the antibiotic-loaded nanoparticles is examined in time-dependent biofilm susceptibility testing, where the effects of antibiotic exposure history, biofilm age, and *in-vitro* drug removal that simulates the *in-vivo* condition on the antibacterial efficacy results are taken into account. *Escherichia coli* (*E. coli*) biofilm, which is present in 7% of patients hospitalized for lower respiratory tract infections (28), is used as the biofilm model. Levofloxacin, a fluoroquinolone antibiotic, is used as the antibiotic model, as it is known to be effective against a wide range of dormant respiratory bacterial pathogens (29), and, unlike aminoglycoside antibiotics, it can diffuse through the EPS matrix and the surrounding mucus to reach embedded mature biofilm cells (30).

The antibiotic is encapsulated into biodegradable and biocompatible polymeric nanoparticles that are specifically engineered to produce different antibiotic release profiles (i.e. burst, intermediate, and slow). Specifically, poly (caprolactone) (PCL) and poly(DL-lactide-*co*-glycolide) (PLGA), which exhibit two distinct degradation rates between them, are used as the antibiotic carriers in order to facilitate the production of nanoparticles having different antibiotic release profiles. In this regard, the use of PLGA and PCL nanoparticles as antibiotic carriers has been investigated by Misra *et al.* (27) and Jeong *et al.* (26) using doxycycline and ciprofloxacin as the model antibiotics, respectively. Nevertheless, as opposed to the present work, which examines the antibiotic susceptibility of *E. coli* biofilm cells and biofilm-derived planktonic cells, Misra *et al.* and Jeong *et al.* examined the antibacterial efficacy of their nanoparticle formulations against *E. coli* planktonic cells produced from an inoculum.

The biofilm susceptibility testing was conducted over a six-day period, with daily dosings at different antibiotic concentrations corresponding to the antibiotic release profile being examined. In addition, the antibiotic susceptibility testing is conducted for floating planktonic cells, which are steadily shed from the biofilm matrix as part of the biofilm mode of growth. These biofilm-derived planktonic cells are of significant importance, as they have been found to be the main cause of exacerbations by forming new biofilm colonies elsewhere (31).

MATERIALS AND METHODS

Materials

The polymers used to prepare the antibiotic-loaded nanoparticles were PCL (MW = 80,000), purchased from Sigma-Aldrich (USA), and PLGA (Purasorb 5004A), received as a gift from PURAC Biomaterials (Netherlands). Analytical/HPLC grade chemicals used in the nanoparticle synthesis were dichloromethane (DCM), acetone, poly (vinyl alcohol) (PVA, MW = 23,000), Pluronic F-68, and levofloxacin (LEV) antibiotic, purchased from Sigma-Aldrich (USA). The phosphate buffer saline solution (PBS, pH = 7.4) and the dialysis membrane with a molecular weight cut-off size of 12,400 g/mol used in the *in-vitro* drug release study were purchased from 1st Base (Singapore) and Sigma-Aldrich (USA), respectively. The *E. coli* K-12 (W3110) strain was obtained from Coli Genetic Stock Center, Yale University (USA).

Methods

Preparation of LEV-Loaded Nanoparticles by Nanoprecipitation (NPC)

A nanoprecipitation method of Govender *et al.* (1999) (32) is employed to obtain LEV-loaded nanoparticles with the burst release profile. The polymer and the highly water-soluble LEV (≈ 0.1 g/mL) are dissolved in water-miscible acetone. Upon addition of the polymer/LEV solution into water, the acetone rapidly diffuses into the aqueous phase, resulting in the formation of polymeric nanoparticles in which LEV is predominantly adsorbed on the nanoparticle surface, resulting in the burst release profile.

Briefly, 8 mg of LEV and 100 mg of PLGA are dissolved in 5 mL of acetone, after which the solution is poured into 10 mL aqueous solution of 0.1% (*w/v*) Pluronic F-68. The resulting nanoparticulate suspension is stirred overnight at room temperature to evaporate off the acetone present in the aqueous phase, which is followed by two centrifugations at 14,000 RPM to remove the non-encapsulated LEV from the nanoparticulate suspension. The LEV-loaded PCL nanoparticles are prepared following the same procedures but using 75 mg of PCL instead. Blank nanoparticles are prepared using the same procedures but without incorporating LEV.

Preparation of LEV-Loaded Nanoparticles by Emulsification-Solvent-Evaporation (ESE)

An emulsification-solvent-evaporation method of Sung *et al.* (2009) (17) is employed to prepare LEV-loaded nanoparticles with the biphasic extended release profile. The polymer and LEV are dissolved in a volatile and water-immiscible solvent (i.e. DCM) where, upon addition of the polymer/LEV solution into water, an oil-in-water nano-emulsion is formed by ultrasonication. Next, the DCM is slowly evaporated to transform the nano-emulsion into a nanoparticulate suspension in which LEV is encapsulated inside the polymer matrix, resulting in the extended release profile.

Briefly, 8 mg of LEV and 80 mg of PLGA are dissolved in 2 mL of DCM after which the solution is poured into 6 mL aqueous solution of 1.0% (*w/v*) PVA. The resulting solution is emulsified for 60 s using a Vibra-Cell probe sonicator (VC 5040, Sonics and Materials, USA). The nano-emulsion is next added to 10 mL aqueous solution of 0.1% (*w/v*) PVA and is stirred overnight at room temperature to evaporate off the DCM, resulting in the nanoparticle production. The resulting nanoparticulate suspension is then centrifuged twice at 11,000 RPM to remove the non-encapsulated LEV. The same procedures are used to prepare the blank nanoparticles and the LEV-loaded PCL nanoparticles.

Physical Characterizations of LEV-Loaded Nanoparticles

The nanoparticle size is measured by photon correlation spectroscopy (PCS) using a Brookhaven 90Plus Nanoparticle Size Analyzer (Brookhaven Instruments Corporation, USA). The drug encapsulation efficiency (i.e. % encapsulation) is determined from the ratio of the amount of the encapsulated LEV to the amount of LEV initially added. The amount of the encapsulated LEV is determined by subtracting the LEV amount present in the supernatant after the first centrifugation from the amount of LEV initially added. The LEV concentration in the supernatant is measured using UV-VIS spectrophotometer (UV Mini-1240, Shimadzu, Japan) at the LEV absorbance wavelength of 254 nm. The drug loading is determined from the ratio of the amount of the encapsulated LEV to the total amount of the nanoparticles produced.

In-Vitro Drug Release Study

The LEV release rate from the nanoparticles is determined using the dialysis-bag method under a sink condition. The dialysis bag containing 2 mL of the LEV-loaded nanoparticulate suspension is placed in an opaque bottle filled with 6 mL of PBS as the release medium. The dissolution test is conducted at 37°C in a water bath under gentle stirring for six days. The opaque bottle is used, as fluoroquinolone antibiotics in their aqueous solution form are known to be photodegradable (33). Every 24 h, 4 mL of the release medium is withdrawn, and the amount of LEV released is immediately measured by the UV-VIS spectrophotometer.

The withdrawn sample is replaced with 4 mL of fresh PBS; hence, the drug concentration in the release medium is diluted two-fold every 24 h to simulate the *in-vivo* drug removal into the systemic circulation. The LEV solutions collected from the six days (i.e. LEV1, LEV2, and …) are stored in opaque bottles to be subsequently used, after a ten-fold dilution, in the time-dependent biofilm susceptibility testing. The *in-vitro* release study is also conducted using 6 mL of MHB as the release medium for comparison.

Antibiotic Susceptibility Testing

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) is defined as the lowest antibiotic concentration that inhibits a visible planktonic bacterial growth after an overnight incubation at 37°C. An optical density measurement at 600 nm (OD_{600}) is typically used to examine the visible bacterial growth in which OD_{600} <0.1 indicates a zero bacterial growth. Briefly, the MIC is determined by broth micro-dilution

using a 96-well microplate (F96 MicroWell, Nunc, Denmark). An *E. coli* bacterial suspension to be used as the inoculum is adjusted to a 0.5 McFarland standard in Mueller Hinton broth (MHB) after which it is further diluted by 100-fold to produce a bacterial cell suspension having 1.0×10^6 colony forming units (CFU)/mL.

Next, 100 μL of LEV solution in MHB is added to 100 μL of the bacterial cell suspension in each well of the microplate to yield a final cell concentration of 5.0×10^5 CFU/mL. The range of LEV concentrations investigated is between 0.0037 μg/mL and 7.5 μg/mL. After a 24-hour incubation at 37°C, OD_{600} of the cell suspension in each well is measured using a microplate reader (Synergy HT, Biotek, USA). The lowest LEV concentration that yields OD_{600} <0.1 is determined as the MIC.

Minimum Biofilm Inhibitory Concentration (MBIC) Determination

The minimum biofilm inhibitory concentration (MBIC) is defined as the lowest antibiotic concentration that inhibits a visible biofilm cell growth. The biofilm cultivation method and MBIC determination follow the methods of Harrison *et al.* (2006) (34) and Ceri *et al.* (1999) (35), respectively. Briefly, a bacterial cell suspension to be used as the inoculum is adjusted to 1.0 McFarland standard and is diluted by thirty-fold in MHB to produce 1.0×10^7 CFU/mL. Next, 150 μL of the inoculum is transferred to each well of a 96-well microplate, and a 96-peg lid is fitted on top of the microplate to provide a surface for the biofilm growth. After 24-hour incubation in a shaking incubator at 150 RPM and 37°C, the peg lid is lifted and the biofilm formed on the pegs is rinsed with PBS to remove loosely attached planktonic cells.

Next, two pegs are broken off to enumerate the biofilm cells formed. The biofilm formed in the remaining 94 pegs is exposed in a new microplate to 200 μL of LEV solution in MHB. After a 24-hour incubation at 37°C, the peg lid is lifted and the pegs are rinsed with PBS to remove the dead biofilm cells. Next, the non-eradicated biofilm cells are detached from the pegs into a recovery plate containing 200 μL of fresh MHB by 5-minute sonication. After a 24-hour incubation at 37°C, OD_{600} in each well of the recovery plate is measured using the microplate reader. The lowest LEV concentration that results OD_{600} <0.1 is determined as the MBIC. The range of LEV concentration investigated is the same as the one used in the MIC determination. The MBIC value obtained from the OD_{600} measurement is verified by viable cell counting, where the biofilm cells in the recovery plate are serially diluted ten-fold and plated onto agar plates. The viable CFU is determined after an overnight incubation at 37°C. Both the MIC and MBIC determinations are repeated three times on three different days using three replicates on each day.

Blank Nanoparticle Susceptibility Testing

To investigate whether the PLGA and PCL nanoparticles exhibit any antibacterial activities, biofilm cells are cultivated on a 96-peg lid following the same procedures used to determine the MBIC. Afterwards, the biofilm formed on the pegs is exposed in a microplate to a suspension containing 40 μL of the blank nanoparticulate suspension (1.5 % *w/v*) and 160 μL of MHB. Biofilm cells exposed to 40 μL of PBS and 160 μL of MHB are used as the experimental controls. After 6, 12, and 24 h of incubation at 37°C, the biofilm formed on the pegs is rinsed with PBS and is later detached from the pegs into a new microplate by sonication.

The recovered biofilm cells are serially diluted ten-fold, plated onto agar plates and are incubated overnight at 37°C to determine the viable CFU. The biofilm-derived planktonic cells, which are present in the wells of the microplate after the blank nanoparticle exposure, are enumerated after 24 h. The experiment is conducted in two replicates. The viable CFU counts of the biofilm-derived planktonic cells and the biofilm cells, which have been exposed to the blank nanoparticles, are compared with those of the experimental controls.

Time-Dependent Biofilm Susceptibility Testing

For the six-day time-dependent biofilm susceptibility testing (i.e. time-kill), the biofilm is cultivated on six 96-peg lids following the same procedures used to determine the MBIC. On the first day of the time-kill, the biofilm cells from the six peg lids are exposed to an antibiotic solution in a microplate, which contains 20 μL of the aforementioned LEV1 solution obtained from the *in-vitro* drug release study and 180 μL of MHB. A solution containing 20 μL of PBS and 180 μL of MHB is used as the positive control. The fresh MHB must be supplied daily to sustain the biofilm existence over the six-day time-kill period. After a 24-hour incubation at 37°C (i.e. second day of the time-kill), one of the six peg lids is rinsed with PBS and is sonicated to recover the non-eradicated biofilm cells.

Next, the viable CFU is determined using two replicates following the same procedures used in the blank nanoparticle susceptibility testing. In addition, OD_{600} of the biofilm-derived planktonic cell suspension present in the wells of the first microplate is measured. The remaining five peg lids are exposed to an antibiotic solution containing 20 μL of the LEV2 solution and 180 μL of MHB. These procedures are repeated daily for five consecutive days using LEV3, LEV4, LEV5, and LEV6 antibiotic solutions in sequence as

illustrated in Fig. 1. The biofilm susceptibility testing is repeated in a pseudo-time-kill study using one-day-old biofilm cells to examine the effect of the antibiotic exposure history on the biofilm susceptibility.

RESULTS

LEV-Loaded Nanoparticles

A summary of the LEV-loaded nanoparticles produced is presented in Table I. The experimental uncertainties in the % encapsulation and the drug loading measurements based on three replicates are between 4−7% and 5%, respectively. The results indicate that the NPC method produces smaller nanoparticles (≈ 100 nm) compared to those produced by the ESE method (≈ 200 nm) for both the PCL and PLGA. Importantly, nanoparticles in this size range have been found to be ideal for the sputum penetration (10). The high aqueous solubility of LEV results in a majority of LEV to diffuse out into the aqueous phase during the preparation steps, resulting in low % encapsulation (≤ 16%) and, consequently, relatively low drug loadings (0.30–1.10% *w*/*w*). Significantly, PLGA nanoparticles exhibit higher % encapsulation and drug loadings than their PCL nanoparticle counterparts.

In-Vitro LEV Release Profiles

The *in-vitro* LEV release profiles of the different nanoparticle formulations in PBS examined over a six-day period are presented in Fig. 2. The experimental uncertainties in the *in-vitro* LEV release profiles based on three replicates are equal to approximately 5%. The effect of changing the release medium to MHB is found to be negligible. Both LEV-loaded PCL and PLGA nanoparticles prepared by the NPC method (i.e. PCL/PLGA NPC) exhibit monophasic burst release profiles in which ≈ 80% of the loaded drug is released within the first hour, and the entire drug is released after 6 h. The *in-vitro* release profiles

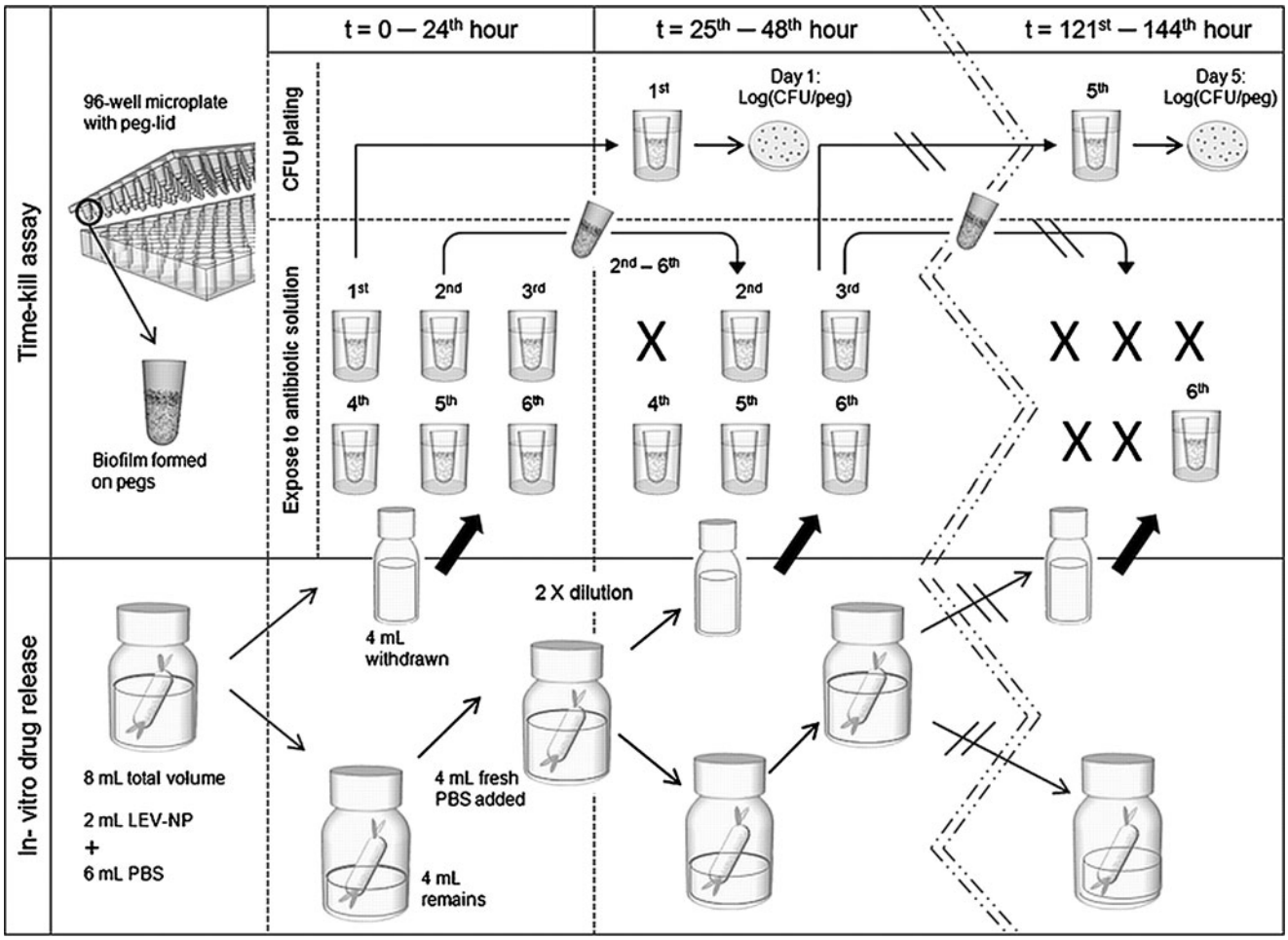

Fig. 1 An illustration of the time-dependent biofilm susceptibility testing using the LEV solution from the *in-vitro* drug release study (LEV-NP: LEV-loaded nanoparticles).

Table I Physical Characteristics of the Nanoparticles Produced

Nanoparticle (NP)	Preparation Method	Size (nm)	% Encapsulation	Drug Loading (% *w/w*)
PLGA	NPC	80 ± 30	15	0.65
	ESE	190 ± 50	16	1.10
PCL	NPC	110 ± 40	5	0.40
	ESE	230 ± 80	4	0.30

of the PCL and PLGA NPC nanoparticles are found to be fairly similar. Importantly, as the burst release period is significantly longer than the typical sputum penetration time of the nanoparticles reported in the literature, a majority of the antibiotic is to be released inside the static mucus surrounding the biofilm colonies.

On the other hand, nanoparticles produced by the ESE method (i.e. PCL/PLGA ESE) exhibit biphasic extended release profiles. For the PLGA ESE nanoparticles, ≈ 80% of the loaded drug is released after one day, which represents the initial fast release that is followed by a slower release resulting in the entire drug being released only after the sixth day. Attributed to the considerably lower drug loading and the slowly degrading nature of PCL, the PCL ESE nanoparticles exhibit a slower drug release profile than the PLGA ESE nanoparticles, where only ≈ 40% of the loaded drug is released after one day and only ≈ 75% of the drug is released after six days.

Blank Nanoparticle Susceptibility

After 6, 12, and 24-hour exposures to the blank nanoparticles, the viable CFU of the exposed biofilm cells are found to remain closely similar to the viable CFU of the experimental control as shown in Fig. 3A, which indicates that the four blank nanoparticles (i.e. PCL/PLGA NPC and PCL/PLGA ESE) do not possess any antibacterial activities towards the biofilm cells. The experimental uncertainties in the viable CFU based on the two replicates are approximately equal to ± 0.2 log (CFU/peg). In addition, the presence of the blank nanoparticles does not alter the biofilm growth either, as the viable CFU of the biofilm-derived planktonic cells, presented in Fig. 3B are also equal to the viable planktonic CFU of the experimental control.

The antibacterial efficacy of the LEV-loaded nanoparticles can therefore be attributed exclusively to the antibiotic exposure and are not caused by the nanoparticle inclusion or by the trace of chemicals used in the nanoparticle preparation. As the presence of the nanoparticles does not have any effects on the biofilm cells, the biofilm susceptibility testing is conducted by using only the LEV solution obtained from the *in-vitro* release study without physically including the nanoparticles in the testing.

MIC and MBIC

The MIC and MBIC values of LEV against *E. coli* planktonic and biofilm cells are approximately equal to

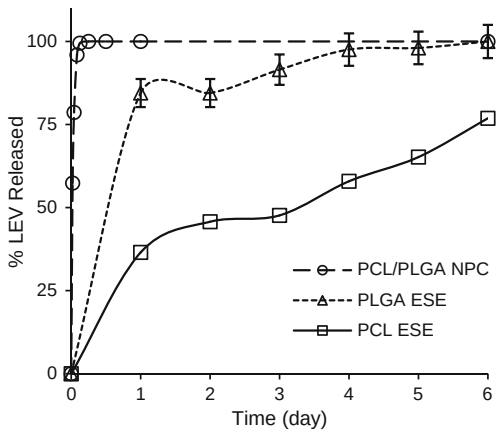

Fig. 2 *In-vitro* antibiotic release profiles of the different nanoparticle formulations.

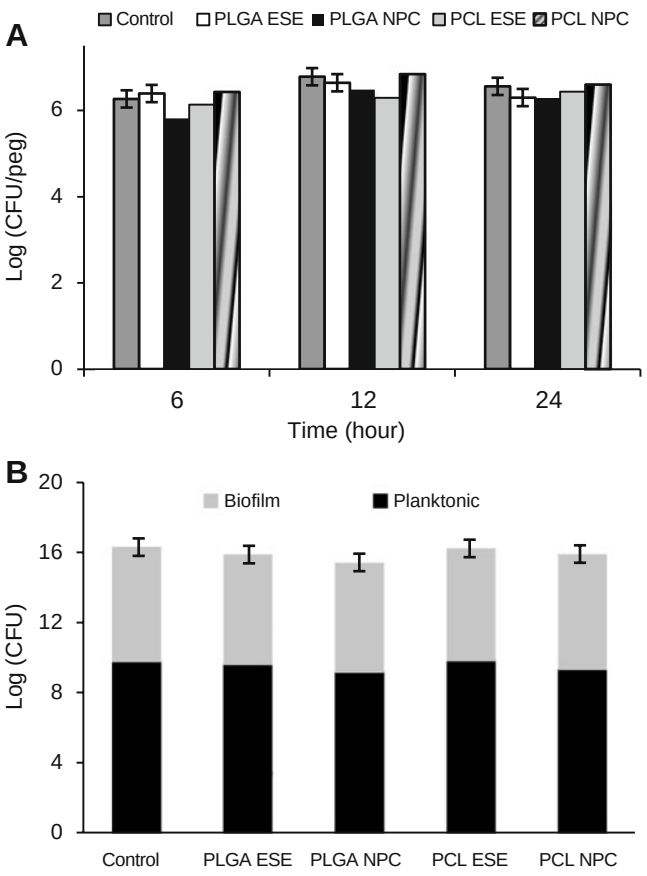

Fig. 3 Viable bacterial cell counts from the blank nanoparticle susceptibility testing **A** biofilm cells, **B** biofilm + planktonic cells after 24-hour-incubation.

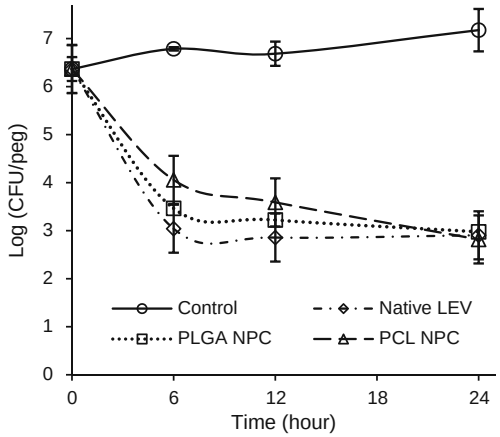

Fig. 4 Effect of encapsulation on the LEV antibacterial activity.

0.03 and 0.15 μg/mL, respectively, indicating that the biofilm mode of growth of *E. coli* bacterial cells results in a five times higher tolerance towards LEV than the planktonic mode. The MIC and MBIC values obtained from the OD_{600} measurement have been verified by a viable cell counting method.

Effect of LEV Encapsulation on its Antibacterial Activity

To investigate whether the antibacterial activity of LEV is altered by its encapsulation into the nanoparticles, the antibacterial efficacies of the native LEV (i.e. free drug) and the LEV released from the PCL and PLGA NPC nanoparticles are examined against one-day-old biofilm cells at an equal antibiotic concentration (i.e. 0.45 μg/mL). After taking into account the experimental uncertainties in the viable CFU of the two replicates, which is approximately equal to ± 0.5 log (CFU/peg), the results in Fig. 4 indicate that the eradication rates of the encapsulated LEV after 6, 12, and 24 h of antibiotic exposures are relatively similar to those of the native LEV, where up to three log (CFU/peg) (i.e. 99.9%) of the biofilm cells are eradicated. A similar result is found for the LEV released from the PCL and PLGA ESE nanoparticles, but the data are not shown here for brevity. Hence, LEV can be safely encapsulated into the polymeric nanoparticles without affecting its antibacterial activity.

In-Vitro Dilution Rate to Simulate the *In-Vivo* Drug Removal

In the biofilm susceptibility testing, the *in-vivo* drug removal by various clearance mechanisms in the lung (e.g. absorption, phagocytosis, enzymatic degradation) is simulated by a two-fold dilution of the LEV solution at a fixed time interval. This static dilution approach represents a simpler alternative to simulate the *in-vivo* drug removal than the more conventional approach using a chemostat. This approach is nevertheless limited to biofilm studies in which the impact of the interaction between the drug-bearing particles and the biofilm cells on the biofilm mode of growth is minimal, as is the case with the present study.

In this regard, the typical half-life of intravenously administered LEV in the lung is approximately equal to 8 h (36). In the present work, a longer half-life is simulated, as LEV has been incorporated into nanoparticulate carriers, hence prolonging the residence time of LEV in the biofilm infection sites. To determine the appropriate dilution rate, the effect of the two-fold dilution at two different time intervals (i.e. 12 and 24 h) on the LEV concentration variations during the six-day time-kill period is examined in Fig. 5 by using the LEV-concentrated solution released from the PLGA NPC and PLGA ESE nanoparticles.

The total amount of LEV used in the *in-vitro* release study to determine the appropriate dilution rate is set at 0.16 mg for both nanoparticles yielding a maximum LEV concentration of ≈ 2.3 μg/mL. This concentration range is in the same order of magnitude as the maximum serum concentration (≈ 5.5 μg/mL) of 500 mg orally administered LEV that is typically used in antibiotic therapies against

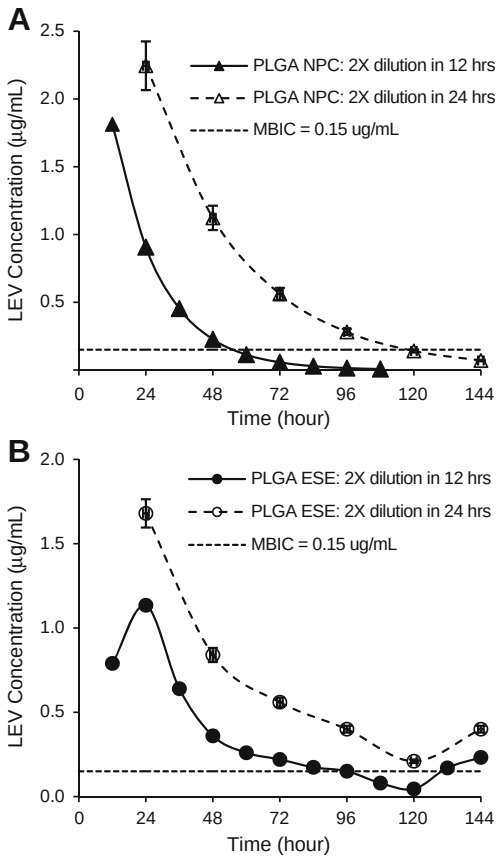

Fig. 5 Effect of the two-fold dilution rate on the day-to-day variations of the LEV concentrations for **A** PLGA NPC and **B** PLGA ESE nanoparticles.

common respiratory pathogens (37). Importantly, the maximum LEV concentration is equal to ≈ 80 times the MIC value and ≈ 15 times the MBIC value of the *E. coli* bacterial cells.

For the PLGA NPC nanoparticles having a burst release profile, a two-fold dilution every 12 h in Fig. 5A causes the LEV concentrations to drop below the MBIC value after 2 days before reaching a practically zero value after 3.5 days. As a result, the biofilm susceptibility testing from Day 2 onwards will not likely reveal any useful information other than an uninhibited biofilm growth. In contrast, a two-fold dilution every 24 h keeps the LEV concentrations above the MBIC value for 5 days with a half-life of 24 h.

For the PLGA ESE nanoparticles having a slower release profile, a two-fold dilution every 12 h in Fig. 5B maintains the LEV concentrations above the MBIC value up to 4 days. Nonetheless, the LEV concentrations from Day 3 onwards are not much higher than the MBIC value; hence, they are unlikely to be effective. On the contrary, a two-fold dilution every 24 h results in LEV concentrations that are at least 1.5 times above the MBIC value throughout the six days with a half-life of 24 h. For this reason, a twice dilution at 24-hour intervals is selected as the *in-vitro* dilution rate to simulate the *in-vivo* drug removal at the LEV dose being investigated in the present work (i.e. 0.16 mg).

Dilution rates at shorter or longer time intervals, nevertheless, can be selected when higher or lower LEV doses are used; thereby, the LEV concentrations are maintained above the MBIC value throughout a majority of the biofilm susceptibility testing period, such that meaningful biofilm susceptibility testing results can be obtained. However, as experimental data of the half-life of LEV-encapsulated nanoparticles in the lung are not yet available, the simulated *in-vivo* LEV removal cannot be straightforwardly validated in the present work. In this regard, the lung half-life of amikacin antibiotic, which exhibits a relatively similar aqueous solubility as LEV (i.e. 180 mg/mL for amikacin *versus* 100 mg/mL for LEV), has been shown by Meers *et al.* (11) *in-vivo* to be prolonged to >24 h when the amikacin is encapsulated into nanoparticles, which is significantly longer than the half-life of the free-drug formulation (i.e. ≈ 2 h). Therefore, the 24-hour half-life simulated in the present work is thought to represent a reasonable approximation of the *in-vivo* LEV removal.

Effects of Antibiotic Release Profile on the Biofilm Susceptibility

Three distinct antibiotic release profiles (i.e. burst release by PCL/PLGA NPC, intermediate release by PLGA ESE, and slow release by PCL ESE) are employed to examine their effects on the antibacterial efficacy of the LEV-loaded nanoparticles. The antibacterial efficacies produced by the burst and the slow release profiles are examined first. The total amount of LEV used is set at 0.16 mg for both nanoparticles. The day-to-day variations in the LEV concentrations of the two release profiles after a two-fold dilution every 24 h are presented in Fig. 6A, whereas the time-kill results are presented in Fig. 6B. The experimental uncertainties in the viable CFU in the time-kill study are approximately equal to ± 0.5 log (CFU/peg) on average.

The LEV concentrations from the burst release profile decrease with time following first-order kinetics and fall below the MBIC value on the sixth day. In contrast, the LEV concentrations from the slow release profile remain at least 3 times above the MBIC value throughout the six days. The main difference between the two release profiles lies in their LEV concentrations on Day 1. The PCL/PLGA NPC nanoparticles release 2.25 μg/mL of LEV (i.e. 15 × MBIC) on Day 1, whereas the PCL ESE nanoparticles release only1.05 μg/mL of LEV (i.e. 7 × MBIC). Nevertheless, both concentrations likely can eradicate a majority of the young biofilm cells on Day 1 without difficulty, as they are both high above the MBIC value. The

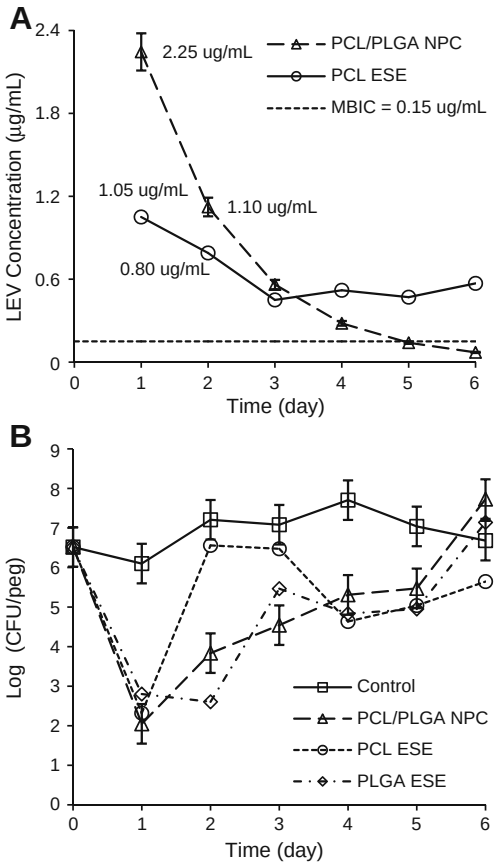

Fig. 6 **A** LEV concentration variations and **B** time-kill results of the different antibiotic release profiles.

eradication rates on the subsequent days after the biofilm cell defensive response, however, remain to be investigated.

The viable CFU of the positive control in Fig. 6B remain relatively constant between six and seven log (CFU/peg) throughout the six days, which denotes a sustained existence of the biofilm cells at a steady state, where the rate of the planktonic cells shed from the biofilm is equal to the adhering rate of new bacterial cells onto the biofilm. Therefore, the variations in the viable CFU observed in the biofilm susceptibility testing results can be attributed solely to the antibiotic exposure and are not influenced by the biofilm-growing protocols. Importantly, the sustained existence of the positive control throughout the six days indicates that the technique employed in the present work to uphold the biofilm existence over a prolonged period is equally competent as the one conducted using a chemostat.

For both the burst and the slow release profiles, the LEV concentrations released on Day 1 as expected eradicate about four log (CFU/peg) (i.e. 99.99%) of the biofilm cells as shown in Fig. 6B. Importantly, even though the antibacterial efficacy of fluoroquinolone antibiotics is known to be concentration dependent, the higher initial LEV concentration in the burst release profile (i.e. 2.25 μg/mL) does not result in a higher eradication rate on Day 1 than that of the slow release profile (i.e. 1.05 μg/mL).

The presence of a plateau in the eradication rate as the antibiotic concentration is increased typically signifies the existence of surviving persister cells (5). For this reason, the antibiotic susceptibility of one-day-old biofilm cells is examined in a dose-kill study at LEV concentrations ranging from 0.15 to 3.10 μg/mL. The results in Fig. 7 indicate that the variations in the viable CFU after exposures to a wide range of LEV concentrations are statistically insignificant, as they fall within the experimental uncertainties. The viable CFU is found on average to be approximately equal to two log (CFU/peg), indicating the survival of 0.01% subpopulation persister cells.

Even though the biofilm eradication rates on Day 1 are equal between the burst and the slow release profiles, their eradication rates on Day 2 are vastly different. For the slow release profile, the surviving persister cells can freely multiply, producing new biofilm cells on Day 2 despite their exposure to 0.80 μg/mL of LEV, which is more than five times above the MBIC value. As a result, a similar number of biofilm colonies as the ones existing prior to the antibiotic exposure are formed on Day 2. For the burst release profile, the biofilm growth on Day 2 is inhibited to a significantly larger extent due to the higher LEV concentration present at 1.10 μg/mL.

From Day 3 onwards, the biofilm growth in the burst release profile continues at a relatively constant pace as the LEV concentration decreases exponentially with time. The LEV concentration eventually drops below the MBIC value

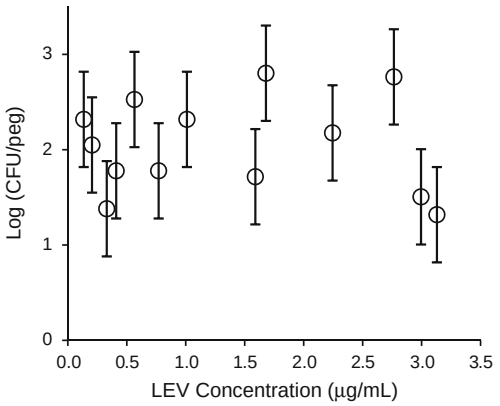

Fig. 7 Viable bacterial cell counts of one-day-old biofilm cells exposed to a wide range of LEV concentrations.

on Day 6, resulting in a sharp increase in the biofilm growth. The time needed for the biofilm to recover to its initial state upon exposure to the burst release profile is therefore around five days. On the other hand, the LEV concentrations in the slow release profile are maintained at ≈ 0.50 μg/mL from Day 3 onwards. As a result, the biofilm growth is slightly subdued on the subsequent days. Nevertheless, it is not significant enough to offset the rapid biofilm growth on Day 2. Hence, in terms of the speed of the biofilm formation recurrence, the burst release profile is found to be superior to the slow release profile, even though the LEV concentrations in the latter are already maintained above the MBIC value throughout the six days.

A similar trend in the biofilm growth is observed for the biofilm cells exposed to the intermediate release profile from the PLGA ESE nanoparticles. The result is not unexpected, as the LEV concentrations from the intermediate release profile after the two-fold dilution, which is shown earlier in Fig. 5B, fall within the same range as that of the burst release profile throughout the six days. Fittingly, similar biofilm eradication rates are observed in Fig. 6B between the burst and the intermediate release profiles, where the variations in the viable CFU between the two release profiles still fall within the experimental uncertainties.

Antibiotic Susceptibility of Biofilm-Derived Planktonic Cells

Two separate time-kill studies (i.e. real and pseudo time-kills) are conducted in which the LEV concentration is varied between 0.14 and 1.80 μg/mL. In the pseudo time-kill, the susceptibility testing is conducted against planktonic cells shed from one-day-old biofilm cells that have not been exposed to antibiotics. In the real time-kill, the testing is conducted against planktonic cells shed from biofilm cells that have been exposed to antibiotics for a period of one to

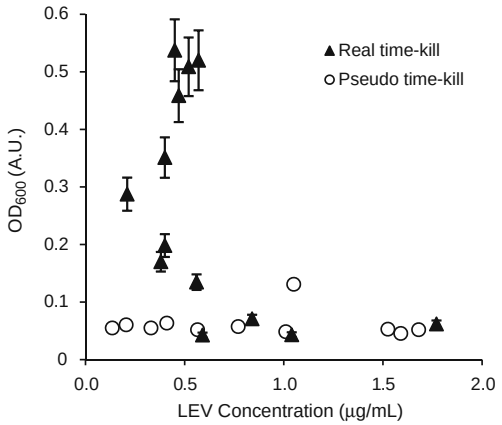

Fig. 8 Antibiotic susceptibility testing of biofilm-derived planktonic cells.

six days. For brevity, the biofilm-derived planktonic cells used in the pseudo- and real time-kill studies are referred from this point onwards as fresh and exposed biofilm-derived planktonic cells, respectively.

The results of the antibiotic susceptibility testing in Fig. 8 indicate that the growth of the fresh biofilm-derived planktonic cells is successfully inhibited in the range of the LEV concentrations investigated, as reflected in $OD_{600} \leq 0.1$ that is observed for the most part. The results are well expected, as the lowest LEV concentration investigated is close to five times the MIC value. On the contrary, the exposed biofilm-derived planktonic cells exhibit a significantly lower antibiotic susceptibility than the fresh ones, where a minimum of 0.60 μg/mL of LEV (≈ 20 × MIC) is needed to inhibit the planktonic cell growth.

DISCUSSION

The time-kill results from the three antibiotic release profiles indicate that LEV concentration that is significantly higher than the MBIC value (i.e. >1.10 μg/mL) is needed to inhibit the biofilm growth caused by the multiplications of the surviving persister cells. The results hence signify the importance of the antibiotic exposure history because the surviving persister cells, as part of their defensive response, acquire a higher antibiotic tolerance after the initial antibiotic exposure. Nevertheless, the higher antibiotic tolerance acquired by the surviving persister cells after the initial dosing is often overlooked in many biofilm susceptibility studies because of the daily, or even more frequent, administrations of a new dose in these studies, which mask the higher antibiotic tolerance of the surviving biofilm cells. Another reason for the overlook is because of the short duration of the antibiotic exposure examined in these studies.

Simply maintaining the LEV concentrations slightly above the MBIC value is therefore inadequate to inhibit the biofilm growth, as the MBIC value is determined from biofilm cells that have no previous antibiotic exposure. At the current LEV dose, the burst and the intermediate release profiles are more effective than the slow release profile in terms of slowing down the biofilm formation recurrence because of their higher initial antibiotic concentrations. Nevertheless, neither of them is effective in inhibiting the growth of the surviving persister cells over the six days unless a new dose is administered daily.

The question that remains is whether the higher antibiotic tolerance acquired by the surviving persister cells is dependent on their antibiotic exposure on Day 1. The effect of increasing the LEV dose on the biofilm eradication rate is therefore investigated, where the total amount of LEV used is increased from 0.16 mg to 0.30 mg. The PLGA ESE nanoparticles are employed, as their intermediate release profile allows the LEV concentration to remain above 1.10 μg/mL for a prolonged period, while at the same time providing a high initial antibiotic concentration needed for the purpose of this study.

The day-to-day variations of the LEV concentrations and the corresponding time-kill results are presented in Fig. 9. Owed to the higher dose, the LEV concentration on Day 1 is equal to 3.20 μg/mL, and the LEV concentrations do not fall below 1.10 μg/mL for three days. On Day 1, ≈ 99.99% of the biofilm cells are eradicated, indicating the survival of the 0.01% subpopulation persister cells. On Days 2 and 3, the LEV concentrations (≥ 1.10 μg/mL) can sufficiently inhibit the growth of the surviving persister cells. As the LEV concentrations fall below 1.00 μg/mL from Day 4 onwards, the biofilm growth picks up its pace, resulting in a complete recovery of the biofilm cells on Day 5.

The results from Days 2 and 3 indicate that the new MBIC value of the surviving persister cells is approximately equal to 1.10 μg/mL, which is more than seven times above

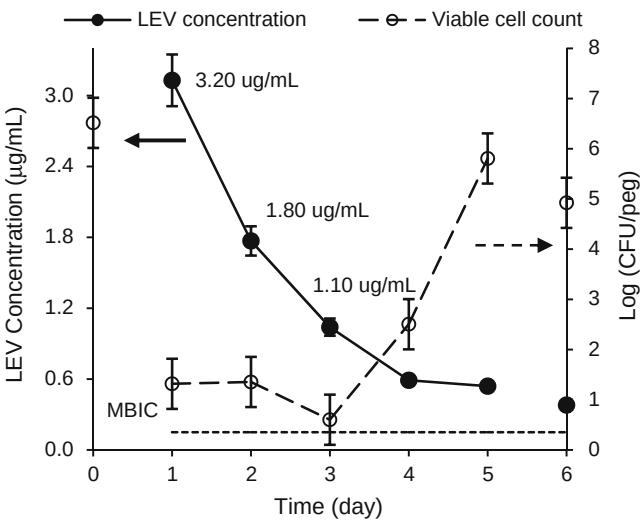

Fig. 9 LEV concentration variations and time-kill results at a higher LEV dose.

the MBIC value prior to the antibiotic exposure. Interestingly, even though the LEV concentration drops to 0.60 μg/mL on Day 4, the biofilm growth is relatively benign, as only approximately two log (CFU/peg) new biofilm colonies are formed. The intermediate release of the higher LEV dose hence eliminates the need for daily dosings, as the biofilm growth is suppressed for up to four days.

The time-kill results at a lower LEV dose presented earlier in Fig. 6B, however, suggest that 1.10 μg/mL of LEV on Day 2 is insufficient to inhibit the biofilm growth of the surviving persister cells, despite a similar number of viable biofilm colonies present initially. The main difference between the two studies lies in their LEV concentration on Day 1, where the one conducted at the lower dose is at 2.25 μg/mL, and the one conducted at the higher dose is at 3.20 μg/mL. The results therefore suggest that the higher antibiotic tolerance of the surviving persister cells is dependent on their initial antibiotic exposure, where the higher the LEV concentration on Day 1, the lower the increase in the antibiotic tolerance of the surviving persister cells.

The importance of a high initial antibiotic exposure on the subsequent biofilm eradication rates denotes the preference of having a biphasic intermediate release profile, where the fast release in the beginning ensures the high initial antibiotic concentration, which is followed by the slower release that maintains the antibiotic concentration above the new MBIC value of the surviving persister cells for a prolonged period. In this regard, a similar impact can be achieved using the burst release profile provided that the initial dose is sufficiently high, but remains below the toxicity limit, to sustain the antibiotic concentrations above the new MBIC value of the surviving persister cells between dosings. On the contrary, to obtain a high initial antibiotic exposure in the slow release profile is not practical, as it requires a significantly larger amount of nanoparticles due to the lower drug loading, hence limiting their feasibility to be delivered by inhalation.

The antibiotic susceptibility of the biofilm-derived planktonic cells is investigated to examine whether they respond in the same manner as the surviving biofilm persister cells in terms of acquiring a higher antibiotic tolerance upon their exposure to antibiotics. The results indicate that the surviving persister biofilm cells transfer their lower antibiotic susceptibility to their progeny. The exposed biofilm-derived planktonic cells, however, do not necessarily become invulnerable towards antibiotics like the persister cell, as the persister cells have been found to not transfer their invulnerability towards antibiotics to their progeny (38).

The lower antibiotic susceptibility of the exposed biofilm-derived planktonic cells, however, can also be possibly caused by the presence of floating planktonic cells that originate from the non-eradicated mature biofilm cells, which are not present in the fresh biofilm-derived planktonic cell population. In this regard, mature biofilm cells are known to exhibit a higher antibiotic tolerance than the young biofilm cells because their cells are less permeable and less metabolically active (39). Nevertheless, mature biofilm cells are typically embedded deeply in the biofilm matrix, such that the ones shed into the planktonic state predominantly consist of young biofilm cells that occupy the periphery fs. Therefore, the role of the mature biofilm cells in the lower antibiotic susceptibility of the exposed biofilm-derived planktonic cells is likely to be minimal, though it cannot be ruled out completely.

CONCLUSION

The results of the biofilm susceptibility testing of the antibiotic-loaded nanoparticles signify the importance of the initial antibiotic exposure on the biofilm eradication rates, where the surviving biofilm cells exhibit a higher antibiotic tolerance than the fresh biofilm cells depending on the initial antibiotic concentration. In this regard, the lower the initial antibiotic exposure, the higher the increase in the antibiotic tolerance of the surviving biofilm cells. Furthermore, the surviving biofilm cells transfer their lower antibiotic susceptibility to their biofilm-derived planktonic cell progeny, hence promoting the exacerbations. A successful inhaled antibiotic therapy against biofilm infections requires a biphasic extended release profile, where the fast antibiotic release in the beginning ensures a high initial antibiotic concentration and the slower extended release sustains a sufficiently high antibiotic concentration to inhibit the biofilm growth and to minimize the exacerbation.

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REFERENCES

1. Davies D. Understanding biofilm resistance to antibacterial agents. Nat Rev Drug Discov. 2003;2(2):114–22.
2. Pauwels PRA, Rabe KF. Burden and clinical features of chronic obstructive pulmonary disease (COPD). Lancet. 2004;364 (9434):613–20.
3. O'Donnell AE. Bronchiectasis. Chest. 2008;134(4):815–23.
4. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. Int J Med Microbiol. 2002;292(2):107–13.

5. Spoering AL, Lewis K. Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J Bacteriol. 2001;183(23):6746–51.
6. Traini D, Young PM. Delivery of antibiotics to the respiratory tract: an update. Expert Opin Drug Deliv. 2009;6:897–905.
7. Labiris NRC, Holbrook AM, Chrystyn H, Macleod SM, Newhouse MT. Dry powder *versus* intravenous and nebulized gentamicin in cystic fibrosis and bronchiectasis—A pilot study. Am J Resp Crit Care. 1999;160(5):1711–6.
8. de Jesus Valle MJ, Lopez FG, Hurle AD-G, Navarro AS. Pulmonary *versus* Systemic Delivery of Antibiotics: Comparison of Vancomycin Dispositions in the Isolated Rat Lung. Antimicrob Agents Chemother. 2007;51(10):3771–4.
9. Dudley MN, Loutit J, Griffith DC. Aerosol antibiotics: considerations in pharmacological and clinical evaluation. Curr Opin Biotech. 2008;19(6):637–43.
10. Suk JS, Lai SK, Wang YY, Ensign LM, Zeitlin PL, Boyle MP *et al.* The penetration of fresh undiluted sputum expectorated by cystic fibrosis patients by non-adhesive polymer nanoparticles. Biomaterials. 2009;30(13):2591–7.
11. Meers P, Neville M, Malinin V, Scotto AW, Sardaryan G, Kurumunda R *et al.* Biofilm penetration, triggered release and *in vivo* activity of inhaled liposomal amikacin in chronic Pseudomonas aeruginosa lung infections. J Antimicrob Chemoth. 2008;61(4):859–68.
12. Sanders NN, De Smedt SC, Van Rompaey E, Simoens P, De Baets F, Demeester J. Cystic fibrosis sputum—A barrier to the transport of nanospheres. Am J Resp Crit Care. 2000;162(5):1905–11.
13. Halwani M, Hebert S, Suntres ZE, Lafrenie RM, Azghani AO, Omri A. Bismuth-thiol incorporation enhances biological activities of liposomal tobramycin against bacterial biofilm and quorum sensing molecules production by Pseudomonas aeruginosa. Int J Pharm. 2009;373(1–2):141–6.
14. Chono S, Tanino T, Seki T, Morimoto K. Influence of particle size on drug delivery to rat alveolar macrophages following pulmonary administration of ciprofloxacin incorporated into liposomes. J Drug Target. 2006;14(8):557–66.
15. Jacobs C, Muller RH. Production and characterization of a budesonide nanosuspension for pulmonary administration. Pharm Res. 2002;19(2):189–94.
16. Ostrander KD, Bosch HW, Bondanza DM. An *in-vitro* assessment of a NanoCrystal (TM) beclomethasone dipropionate colloidal dispersion via ultrasonic nebulization. Eur J Pharm Biopharm. 1999;48(3):207–15.
17. Sung J, Padilla D, Garcia-Contreras L, VerBerkmoes J, Durbin D, Peloquin C *et al.* Formulation and Pharmacokinetics of Self-Assembled Rifampicin Nanoparticle Systems for Pulmonary Delivery. Pharm Res. 2009;26(8):1847–55.
18. Pandey R, Sharma A, Zahoor A, Sharma S, Khuller GK, Prasad B. Poly (DL-lactide-co-glycolide) nanoparticle-based inhalable sustained drug delivery system for experimental tuberculosis. J Antimicrob Chemoth. 2003;52(6):981–6.
19. Tam JM, McConville JT, Williams RO, Johnston KP. Amorphous Cyclosporin Nanodispersions for Enhanced Pulmonary Deposition and Dissolution. J Pharm Sci. 2008;97(11):4915–33.
20. Hoeben BJ, Burgess DS, McConville JT, Najvar LK, Talbert RL, Peters JI *et al. In vivo* efficacy of aerosolized nanostructured itraconazole formulations for prevention of invasive pulmonary aspergillosis. Antimicrob Agents Ch. 2006;50(4):1552–4.
21. Steckel H, Eskandar F, Witthohn K. The effect of formulation variables on the stability of nebulized aviscumine. Int J Pharm. 2003;257(1–2):181–94.
22. Moskowitz SM, Foster JM, Emerson J, Burns JL. Clinically Feasible Biofilm Susceptibility Assay for Isolates of Pseudomonas aeruginosa from Patients with Cystic Fibrosis. J Clin Microbiol. 2004;42(5):1915–22.
23. Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature. 2005;436(7054):1171–5.
24. Dorr T, Lewis K, Vulic M. SOS Response Induces Persistence to Fluoroquinolones in Escherichia coli. PLoS Genet. 2009;5(12):1–9.
25. Owusu-Ababio G, Rogers J, Anwar H. Effectiveness of ciprofloxacin microspheres in eradicating bacterial biofilm. J Control Release. 1999;57(2):151–9.
26. Jeong Y-I, Na H-S, Seo D-H, Kim D-G, Lee H-C, Jang M-K *et al.* Ciprofloxacin-encapsulated poly(dl-lactide-co-glycolide) nanoparticles and its antibacterial activity. Int J Pharm. 2008;352(1–2):317–23.
27. Misra R, Acharya S, Dilnawaz F, Sahoo SK. Sustained antibacterial activity of doxycycline-loaded poly(D, L-lactide-co-glycolide) and poly(Îµ-caprolactone) nanoparticles. Nanomedicine. 2009;4(5):519–30.
28. Fluit AC, Verhoef J, Schmitz F-J. Sentry Participants Group. Antimicrobial resistance among isolates cultured from patients hospitalized with lower respiratory tract infection in Europe. Int J Infect Dis. 2002;6(2):144–6.
29. Eng RH, Padberg FT, Smith SM, Tan EN, Cherubin CE. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. Antimicrob Agents Ch. 1991;35(9):1824–8.
30. Kumon H, Tomochika KI, Matunaga T, Ogawa M, Ohmori H. A sandwich cup method for the penetration assay of antimicrobial agents through Pseudomonas exopolysaccharides. Microbiol Immunol. 1994;38(8):615–9.
31. Kobayashi P. Airway biofilm disease. Int J Antimicrob Ag. 2001;17(5):351–6.
32. Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: Drug loading and release studies of a water soluble drug. J Control Release. 1999;57(2):171–85.
33. Tiefenbacher EM, Haen E, Przybilla B, Kurz H. Photodegradation of some quinolones used as antimicrobial therapeutics. J Pharm Sci. 1994;83(4):463–7.
34. Harrison J, Ceri H, Yerly J, Stremick C, Hu Y, Martinuzzi R *et al.* The use of microscopy and three-dimensional visualization to evaluate the structure of microbial biofilms cultivated in the calgary biofilm device. Biol Proced Online. 2006;8(1):194–215.
35. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. J Clin Microbiol. 1999;37(6):1771–6.
36. Conte JJE, Golden JA, McIver M, Zurlinden E. Intrapulmonary pharmacokinetics and pharmacodynamics of high-dose levofloxacin in healthy volunteer subjects. Int J Antimicrob Ag. 2006;28(2):114–21.
37. Rebuck JA, Fish DN, Abraham E. Pharmacokinetics of intravenous and oral levofloxacin in critically ill adults in a medical intensive care unit. Pharmacotherapy. 2002;22(10):1216–25.
38. Keren I, Kaldalu N, Spoering A, Wang YP, Lewis K. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett. 2004;230(1):13–8.
39. Anwar H, Strap JL, Costerton JW. Establishment of aging biofilms: possible mechanism of bacterial resistance to antimicrobial therapy. Antimicrob Agents Ch. 1992;36(7):1347–51.