Mathematical modelling of *Pseudomonas aeruginosa* biofilm growth and treatment in the cystic fibrosis lung

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Lung failure due to chronic bacterial infection is the leading cause of death for patients with cystic fibrosis (CF). It is thought that the chronic nature of these infections is, in part, due to the increased tolerance and recalcitrant behaviour of bacteria growing as biofilms. Inhalation of silver carbene complex (SCC) antimicrobial, either encased in polymeric biodegradable particles or in aqueous form, has been proposed as a treatment. Through a coordinated experimental and mathematical modelling effort, we examine this proposed treatment of lung biofilms. *Pseudomonas aeruginosa* biofilms grown in a flow-cell apparatus irrigated with an artificial CF sputum medium are analysed as an in vitro model of CF lung infection. A 2D mathematical model of biofilm growth within the flow-cell is developed. Numerical simulations demonstrate that SCC inactivation by the environment is critical in aqueous SCC, but not SCC-polymer, based treatments. Polymer particle degradation rate is shown to be an important parameter that can be chosen optimally, based on environmental conditions and bacterial susceptibility.

Keywords: mathematical modelling; biofilm; cystic fibrosis; *Pseudomonas aeruginosa*; silver carbene complex; nanoparticle.

1. Introduction

Cystic fibrosis (CF) is a common autosomal recessive disease, afflicting over 30,000 persons in the USA (Ramsey, 1996). The disorder is associated with defective ion transport across the lung epithelial layer (Boucher, 2004). The inability to balance ion concentrations in lung mucus results in dehydration of the lung mucus and a subsequent breakdown of mucociliary clearance mechanisms. Build-up of mucus

provides a nutrient rich site for bacterial infection (Singh *et al.*, 2000; Yoon *et al.*, 2002; Boucher, 2004; Harrison, 2007). Respiratory failure due to chronic infection is the leading cause of death in patients with CF (Lambiase *et al.*, 2006). While a wide variety of bacterial species are known to infect the lungs of CF patients, much attention has been focused on *Pseudomonas aeruginosa* in particular. This is largely due to findings suggesting that the establishment of *P. aeruginosa* infection marks a significant worsening of prognosis for the patient (Harrison, 2007).

Singh *et al.* (2000) reported evidence suggesting that *P. aeruginosa* may exist as a biofilm in the lungs of CF patients. Since bacteria in a biofilm exhibit higher antimicrobial tolerance than their planktonic counterparts (Vrany *et al.*, 1997; Hentzer *et al.*, 2001; Spoering & Lewis, 2001; Mulcahy *et al.*, 2008), this finding helped to explain some of the challenges encountered in treating chronic CF lung infection (Singh *et al.*, 2000). Bacterial biofilms, sessile communities encased in self-produced extracellular polymeric substances (EPSs), also frustrate the endocytosis of bacterial cells by neutrophils and other host immune responses (Costerton *et al.*, 1999). Thus, since 2000, there has been significant interest in understanding lung-associated biofilms, with a specific emphasis on treating such infections (Yu & Head, 2002; Häußler *et al.*, 2003; Carterson *et al.*, 2005; Moreau-Marquis *et al.*, 2008; Bjarnsholt *et al.*, 2009). *Pseudomonas aeruginosa* has been the model organism for much of this work, not only because *P. aeruginosa* is a model organism for biofilm formation (Harmsen *et al.*, 2010), but also because of its importance as a pathogen in the CF community (Harrison, 2007).

An important contributor to biofilm recalcitrance and antimicrobial tolerance is the existence of the persister population. The working definition of persister cells is the population of cells that survive antimicrobial challenge, despite the use of extremely high concentrations of antimicrobial (Bigger, 1944). Cultures grown from these surviving cells show the same fractional survival to repeated challenge with antimicrobial. Thus, these surviving cells have been termed persisters, to distinguish them from cells that develop resistance due to genetic mutation (Lewis, 2010). Persister cells are thought to represent a metabolically inactive stochastically expressed phenotype, in which tolerance is conferred by virtue of this dormant state (Lewis, 2010). Traditionally, the presence of persisters is demonstrated by antimicrobial challenge with high concentrations. Other methods for persister identification have been developed; see e.g. Shah *et al.* (2006). For a review of the mechanisms of persister formation, see Lewis (2010). The mechanism by which persisters revert to active bacteria is currently unknown (Lewis, 2010).

In planktonic cultures of *P. aeruginosa*, persister cells make up approximately 0.1% of the population. This proportion increases to 1% upon quorum-sensing activation (Möker *et al.*, 2010). Thus, it has been hypothesized that biofilms harbour large subpopulations of persisters which recolonize the biofilm following antimicrobial treatment. Further, the biofilm matrix acts as a safe haven for persisters, protecting them from host defences and thus allowing recolonization (Lewis, 2010). Repeated antimicrobial treatment also selects for mutants which are high in persister formation (*hip* mutants) (Lewis, 2010). This situation is exemplified in patients with CF, who are dosed heavily with antimicrobials over decades in an effort to control lung infections (Gibson *et al.*, 2003).

Antimicrobials such as tobramycin or colistin, inhaled through a nebulizer, are the standard of treatment for CF-associated *P. aeruginosa* lung infections (Gibson *et al.*, 2003; Anderson *et al.*, 2008; Moreau-Marquis *et al.*, 2009). This delivery mechanism is desirable because it allows the deposition of high concentrations of antimicrobial compounds at the site of infection. As a potential treatment for CF patients, novel antimicrobials based on silver carbene complexes (SCCs) (Youngs *et al.*, 2005; Panzner *et al.*, 2009) have been developed. Like other treatments, SCCs can be nebulized and inhaled in an aqueous form. In addition, SCCs have been encapsulated in L-tyrosine polyphosphate (LTP) nanoparticles (Ditto *et al.*, 2009; Hindi *et al.*, 2009). LTP offers several advantages for in vivo applications and for

nanoparticle formulation. We mention in particular that degradation products are not cytotoxic and do not alter the local pH (Gupta & Lopina, 2002; Ditto *et al.*, 2009; Hindi *et al.*, 2009). After being nebulized and inhaled, the enhanced survival percentage of mice infected with *P. aeruginosa* suggests that the LTP nanoparticles were deposited in the airways of the lungs and penetrated into biofilms (Hindi *et al.*, 2009; Leid *et al.*, 2012). This paper represents an investigation of both aqueous- and controlled-release SCC-polymer-based treatment of *P. aeruginosa* biofilms relevant to the CF lung.

Mathematical modelling has been used extensively in biofilm research to investigate topics ranging from multispecies interaction (Wanner & Gujer, 1986; Wanner & Reichert, 1996; Picioreanu *et al.*, 2004; Alpkvist & Klapper, 2007), to morphological development (Alpkvist *et al.*, 2004; Picioreanu *et al.*, 2007; Miller *et al.*, 2012), to mechanisms of antimicrobial tolerance (Dodds *et al.*, 2000; Cogan *et al.*, 2005b; Szomolay *et al.*, 2005; Anguige *et al.*, 2006; Chambless *et al.*, 2006) and others. These studies have used a wide range of modelling approaches; see Klapper & Dockery (2010) and Wang & Zhang (2010) for reviews of continuum models and Lardon *et al.* (2011) for a discussion of the current state of discrete models. Several groups have used continuum models similar to the model presented here to study the treatment of biofilms by antimicrobial (Stewart *et al.*, 1996; Dodds *et al.*, 2000; Roberts & Stewart, 2004; Cogan *et al.*, 2005a; Cogan, 2010; Shafahi & Vafai, 2010). Among these, the most relevant for our work are Cogan (2010), Cogan *et al.* (2005a) and Stewart *et al.* (1996), all of which model the treatment of a biofilm submerged in a bulk fluid. The modelling in these papers includes the transport of antimicrobial in the bulk fluid, and the treatment is described by specifying the concentration of antimicrobial at the inlet to the bulk fluid, similar to how we model treatment by aqueous SCCs. In Cogan (2010) and Stewart *et al.* (1996), the authors study the response of a biofilm to periodic dosing strategies similar to what we consider. We note that none of these papers model the release of antimicrobial from particles deposited within the biofilm.

There are several basic design challenges for SCC-based treatment of biofilms in the CF lung. These include understanding the distribution of inhaled particles within the lung, transport of SCCs through mucus and lung epithelium, the location and characteristics of CF-associated lung biofilms and biofilm response to SCC concentration. Here, we provide insights into some of these issues. Some work has already been conducted with regard to these issues. Miller *et al.* (2013) considered biofilm–particle interaction in a flow-cell environment. Stine *et al.* (2012) estimated what LTP particle concentrations one might have to achieve to reach a known minimal inhibitory concentration (MIC) value for the SCC drug. The authors assumed a no flux condition at the biofilm–fluid interface, which can be viewed as a best-case scenario for estimating dosing. Here, we consider a 2D biofilm with equality of flux and concentration at the interface, i.e. a perfectly leaky interface. A 2D model allows for the consideration of the fluid flow above the biofilm and its role in the transport of nutrients and antimicrobial. In addition, a 2D model can describe a non-flat interface and hence the surface area to volume ratio of the biofilm may be much larger than that described by a 1D model. These observations suggest that antimicrobial will readily diffuse out of the biofilm and be carried away by advective transport. Hence, our model may provide a worst-case scenario for dosing.

In this work, we use a combination of mathematical modelling and experimental research to further explore SCC treatment of flow-cell grown *P. aeruginosa* biofilms as a model for infection of the CF lung. We restrict our point of view to a local environment of a CF lung biofilm. Using experimental data to calibrate our mathematical model, we use modelling to examine the efficacy of various hypothetical treatment protocols. First, we consider twice daily dosage with aqueous SCCs. Our simulation results demonstrate that frequent dosing is necessary to compete with SCC inactivation. Second, we consider a single dose of SCC-loaded particles with uniform degradation time, demonstrating that local release reduces the impact of SCC inactivation. The optimal degradation time is shown to depend on

bacterial and environmental factors. Next, model behaviour demonstrates that dosing with particles of varying degradation times does not confer an advantage over a uniform degradation time. Further, we show that an SCC-polymer-based strategy can outperform comparable aqueous-based treatments. Finally, we demonstrate that little advantage is gained by using an aqueous and polymer combination strategy.

The remainder of the paper is organized as follows. Section 2 contains a description of the biological experiments and mathematical modelling that are coupled in this work. Section 3 describes the experimental and simulation results, and the discussion in Section 4 links the various facets of this work. Section 5 lists the primary assumptions we made, and the insights we gained.

2. Materials and methods

2.1 *Experimental methods*

Evidence provided by Worlitzsch *et al.* (2002) and Bjarnsholt *et al.* (2009) suggests that *P. aeruginosa* biofilms exist as spherical cellular aggregates in the mucus of CF lungs. Further, these aggregates/biofilms are thought to persist for extended periods of time (Høiby *et al.*, 2010). Methods for growing suspended aggregates over extended periods of time have not been fully developed. We therefore take the alternate approach of considering flow-cell-grown biofilms. Flow-cell grown biofilms have been shown to share many characteristics with cellular aggregates, including tolerance of antimicrobials (Alhede *et al.*, 2011), which suggests that this environment may be a reasonable first approach. In addition, the flow-cell environment balances many competing concerns. The simplified geometry and laminar flow are conducive to mathematical modelling. Flow-cells also provide the ability to observe and treat fully hydrated biofilms as well as measure particle transport and antimicrobial impact over extended time scales.

Biofilm surface attachment notwithstanding, the CF lung represents a unique nutritional environment which may affect bacterial physiology. We therefore seek to mimic this nutritional environment in vitro. Palmer *et al.* (2007) developed a synthetic CF sputum medium (SCFM). This medium was developed to mimic the nutritional characteristics of early stage CF. In SCFM, *P. aeruginosa* shows similar growth rate, growth phenotype, carbon usage preferences, gene expression and cell–cell signalling profiles to CF sputum (Palmer *et al.*, 2007). SCFM is a defined medium that does not contain mucin. Mucin is an important component of lung mucus, increasing viscosity, binding bacterial cells and possibly serving as a carbon source (Carnoy *et al.*, 1993; Palmer *et al.*, 2007). The choice of mucin may influence experimental results. We expect some differences between salivary, lung and gastric mucins. In addition, mucins from CF patients have been shown to have elevated binding affinity for *P. aeruginosa* as compared with that from non-CF individuals (Carnoy *et al.*, 1993). However, obtaining purified CF lung mucin in sufficient quantity is difficult. We therefore use SCFM augmented with porcine gastric mucin as a first approach to mimic the conditions of the CF lung.

Our primary in vitro model of biofilm infection of the CF lung is a flow-cell apparatus irrigated with SCFM and mucin. Using this model, we examine biofilm growth and SCC22 antimicrobial efficacy. The goal of these experiments is to build understanding and measure key parameter values for a mathematical modelling effort.

In addition, while previous reports have tested SCC22 against various pathogens grown to 10^5 – 10^6 CFU/ml (Panzner *et al.*, 2009; Leid *et al.*, 2012), we chose to test the antimicrobial efficacy of SCC22 against a relatively high planktonic cell density in the exponential growth phase (i.e. $\sim 10^8$ CFU/ml) due to recent reports suggesting that bacterial loads inside lungs of CF patients can range as high as 10^8 – 10^{10} CFU/ml (Singh *et al.*, 2000; Palmer *et al.*, 2005, 2007).

2.1.1 Planktonic culture growth and treatment. SCC22 was selected as the treatment agent in this study as it has been previously shown to possess superior water solubility/stability properties, as well as broad spectrum antimicrobial properties (Panzner *et al.*, 2009; Leid *et al.*, 2012). SCC22 challenge of exponential phase planktonic culture was investigated as follows. Overnight cultures of *P. aeruginosa* strains PAO1 (ATCC BAA-47), *gfp*-tagged PAO1, 7119 (CF clinical isolate), FRD1 (CF clinical isolate) and PDO300 (Δ mucA) in Luria-Bertani (LB), SCFM and SCFM augmented with 0.04% (wt/vol) porcine gastric mucin were diluted (1:100) in fresh corresponding media, and incubated at 37°C until an OD600 of 0.2 was reached (corresponding to $\sim 10^8$ CFU/ml as confirmed by plating; data not shown). Cultures were then divided into aliquots of 3.9 ml (treatment groups) and 4.0 ml (no treatment controls). SCC22 compound was prepared as described in Leid *et al.* (2012) and Panzner *et al.* (2009). Fresh batch stock of SCC22 solution (10 mg/ml) was prepared by vortexing 100 mg of SCC22 and 7.5 μ l of glacial acetic acid in 10 ml of sterile deionized (DI) water. The 10 mg/ml stock was diluted in DI water to working stocks corresponding to 40 $\times$ the final desired SCC22 concentrations. From these working stock aliquots, 100 μ l was added to the culture aliquots (treatment groups). The final SCC22 concentrations tested were: 0, 1, 2, 4, 6, 8, 12, 16, 24, 32, 64, 128 and 250 μ g/ml. Aliquots were incubated with concomitant agitation overnight at 37°C, 250 rpm for 20–24 h. MICs were determined by visual inspection the following day.

SCC22 challenge of exponential phase culture under anaerobic conditions was investigated. Anaerobic culture tubes were degassed with N₂ and sealed. Overnight culture of *gfp*-tagged *P. aeruginosa* PAO1 in SCFM was diluted in fresh medium and incubated until reaching an OD600 of 0.15. Anaerobic culture tubes were then inoculated with 3.9 ml of bacteria culture via injection. Tubes were incubated for 2 h at 37°C to allow residual O₂ to be consumed. A batch stock of SCC22 (10 mg/ml) was made and diluted (sterile DI water) into working concentration stocks as mentioned above. Following this, 100 μ l of respective working SCC22 concentration stocks was injected into culture tubes. The final SCC22 concentrations tested were: 0, 2, 4, 8, 16, 32, 64 and 128 μ g/ml. Cultures were incubated overnight at 37°C. The MIC was determined by visual inspection the following day.

The response of stationary phase planktonic culture to SCC22 was also investigated. An overnight culture of *gfp*-tagged *P. aeruginosa* PAO1 grown in SCFM was divided into aliquots ranging from 1.872 to 2 ml. Between 0 and 128 μ l of a 2 mg/ml preparation of SCC22 (10 mg of SCC22 compound dissolved in 5 ml of sterile DI water) was added to each aliquot. The final SCC22 concentrations tested were 0, 2, 4, 8, 16, 32, 64 and 128 μ g/ml. Aliquots were incubated for 2 h at 37°C, after which cultures were plated on tryptic soy agar (TSA) plates and the CFU/ml was measured. Dilution of cultures caused by the addition of the SCC22 was corrected for in the final calculations.

2.1.2 Biofilm culture growth and treatment. Biofilms were grown in continuous flow-cell apparatuses consisting of medium and waste reservoirs, bubble trap, flow-cell and a peristaltic pump (Tolker-Nielsen & Sternberg, 2011). A polycarbonate cover-slip-based flow-cell by BioCentrum-DTU at the Technical University of Denmark (DTU) Center for Systems Microbiology was used for biofilm visualization. The chambers of this flow-cell measure 40 mm long, 4 mm wide and 1 mm deep. The polycarbonate and #1 coverslip design allows for in situ visualization of biofilm growth. The FC 271 coupon-based flow-cell manufactured by BioSurface Technologies Corp. was used in the measurement of viable cell population within biofilm cultures. The FC 271 (two-well configuration) accepts two coupons with diameter 12.7 mm and thickness 3.8 mm (0.5 inch $\times$ 0.15 inch). The chambers of this flow-cell measure 39 mm long, 13 mm wide and 0.35 mm deep. All experiments using this apparatus used borosilicate glass coupons (BioSurface Technologies Corp.). The gasket and coupon design of this system allows for aseptic access to biofilm cultures.

Medium, consisting of 10% (vol/vol) SCFM augmented with 0.04% (wt/vol) mucin, was pumped through the flow-cell systems in a once-through fashion at a rate of 3 ml/h during the biofilm growth phase (18–22 h). This corresponds to a very low wall shear stress of 0.00865 dyn/cm² in the DTU flow-cell and 0.0217 dyn/cm² in the FC 271. The shear stresses were estimated assuming a parabolic fluid velocity and using the dynamic viscosity of water at 37°C. The flow-cell apparatuses were inoculated with 300 µl of a 1:100 dilution of an overnight culture of *gfp*-tagged *P. aeruginosa* PAO1. Cells were introduced into the flow-cell with a syringe and incubated for 1 h at 37°C to allow for initial attachment. During the inoculation process, the medium flow was stopped and the tubing was clamped both up- and downstream of the flow-cell. Following this, tubes were unclamped and the medium flow was restored. The flow-cell was incubated for 18–24 h at 37°C.

Measurement of the efficacy of SCC22 on biofilms was performed using the FC 271 flow-cell. Biofilms were grown overnight in 10% SCFM augmented with 0.04% mucin prior to treatment. For treatment with aqueous SCC22, the irrigation of biofilm cultures was switched to culture medium containing SCC22 at 0, 34, 170 and 340 µg/ml. Antimicrobial-containing medium was passed through the apparatus for 2 h, after which viable cell counts were measured. For treatment with SCC22-LTP particles, irrigation of biofilm cultures was switched to medium containing SCC22-LTP at 0.5 mg/ml. The particle-medium suspension was passed through the system for 10 min, after which irrigation was switched back to particle-free growth medium. Irrigation was continued, either at the customary 3 ml/h or at a reduced rate of 3.5 µl/h, for 2 h. A flow rate of 3 ml/h results in an average advective velocity of 75 cm/h which is on the same order as 22 cm/h, the advective velocity of mucus within a healthy lung (Boucher, 2004). After the 2-h treatment, viable cell counts were measured as follows. Borosilicate glass coupons were aseptically removed from the flow-cell apparatus and placed into individual tubes containing 5 ml of sterile DI water. Biofilms were disrupted by sonication of the tubes for 5 min using a bath sonicator (E/MC Corp., Model 250) followed by vortexing for 30 s. Tube solutions were then plated on TSA plates and incubated, after which the CFU/ml was measured.

The spatial localization of the mucin incorporated into the biofilm was determined by staining with wheat germ agglutinin (WGA) conjugated to Alexa Fluor® 633 (Invitrogen), a lectin stain that binds sialic acid and N-acetylglucosaminyl residues. The working solution of WGA Alex Fluor® 633 conjugate was prepared by diluting stock solution to a concentration of 1.0 µg/ml in water. Using a 1 ml syringe, 0.5 ml of solution was injected into the flow-cell after stopping the peristaltic pump. The tubing was clamped up- and downstream of the flow-cell and the puncture sealed with silicone. The flow-cell apparatus was incubated for 10–20 min at room temperature before the flow was restarted.

2.1.3 Microscopy methods. Biofilms were visualized using confocal microscopy. Biofilm morphology was quantified using COMSTAT, a biofilm image analysis program for MATLAB (MathWorks, Natick, MA, USA) (Heydorn *et al.*, 2000a,b). Images were captured with a Zeiss 510 Meta confocal microscope, using a 100× objective with a vertical step size of 1 µm for vertical sectioning (z-stack). Samples were excited with a wavelength of 488 nm and/or 633 nm to induce fluorescence of the *gfp*-tagged bacteria and Alex Fluor® 633-conjugated WGA. Emission wavelengths between 505 nm and 530 nm were assigned the colour green indicating Gfp, and those above 650 were assigned the colour pink or blue indicating the Alex Fluor® 633. The biofilm water volume fraction, an important parameter in the mathematical models of biofilm growth presented below, was defined as the total biofilm volume divided by the total biomass volume. This metric was calculated using COMSTAT.

FIG. 1. A 2D biofilm model domain.

 TABLE 1 *Model variables*

Variable	Description
H	Biofilm–fluid interface (cm)
$\vec{v}^I$	Advective flow velocity for fluid in region I (cm h^{-1})
$\vec{v}$	Advective flow velocity for biomass in biofilm in region II (cm h^{-1})
P^I	Pressure in fluid in region I ($\text{g cm}^{-1} \text{h}^{-1}$)
P	Pressure within biofilm in region II ($\text{g cm}^{-1} \text{h}^{-1}$)
S^I	Nutrient concentration in region I (g cm^{-3})
S	Nutrient concentration in region II (g cm^{-3})
C^I	SCC22 concentration in region I (g cm^{-3})
C	SCC22 concentration in region II (g cm^{-3})
B	Density of active bacteria (g cm^{-3})
B_i	Density of inert bacteria (g cm^{-3})
B_p	Density of persister bacteria (g cm^{-3})
E	Density of EPSs (g cm^{-3})
C_u	Upstream SCC22 concentration (g cm^{-3})

2.2 Model development

We develop a continuum model of bacterial biofilms in the vein of [Wanner & Gujer \(1986\)](#) and [Wanner & Reichert \(1996\)](#). We treat the flow-cell environment as the fluid flow between parallel plates. Further, we assume symmetry in the transverse direction and across the centre line $y = r$. Thus, we reduce the biofilm to a 2D model as depicted in Fig. 1. The model domain is divided into two separate regions; region I: the bulk fluid above the biofilm, and region II: the bacteria- and EPS-containing biofilm. The boundary between these two regions is the fluid–biofilm interface described by $H(x, t)$. In region I, we model fluid flow and the advective–diffusive transport of soluble species (e.g. nutrients and SCC22). In region II, we model the evolution of biofilm constituents and biofilm morphology. Summaries of model variables and parameters are given in Tables 1 and 2, respectively.

Several quantities such as advective velocity, nutrient concentration and SCC22 concentration are tracked in both region I and region II. To maintain uniform notation, all quantities in region I that are also tracked in region II are noted with a superscript I. The region II quantities lack a superscript. Thus, the advective velocity and pressure in the bulk fluid are given as $\vec{v}^I$ and P^I , and the advective velocity

TABLE 2 *Model parameters and values*

Parameter	Description	Value
w	Flow-cell chamber width (cm)	1.3
$2r$	Flow-cell chamber height (cm)	0.035
L	Flow-cell chamber length (cm)	3.9
$2wQ$	Volumetric flow rate ($\text{cm}^3 \text{h}^{-1}$)	3.0
μ	Fluid dynamic viscosity ($\text{g cm}^{-1} \text{h}^{-1}$)	24.894
D_S^I	Diffusion coefficient of nutrient in region I ($\text{cm}^2 \text{h}^{-1}$)	0.10692
D_S	Diffusion coefficient of nutrient in region II ($\text{cm}^2 \text{h}^{-1}$)	$0.1D_S^I$
D_C^I	Diffusion coefficient of SCC22 in region I ($\text{cm}^2 \text{h}^{-1}$)	0.036
D_C	Diffusion coefficient of SCC22 in region II ($\text{cm}^2 \text{h}^{-1}$)	$0.1D_C^I$
k_C	Degradation rate of SCC22 (h^{-1})	0.2325
ρ_B	Mass density of bacteria (g cm^{-3})	0.2 (Xavier <i>et al.</i> , 2005)
ρ_E	Mass density of EPSs (g cm^{-3})	0.033 (Xavier <i>et al.</i> , 2005)
κ_g	Metabolic rate to the biomass production rate conversion factor (unitless)	$0.21(K_S + S_u)/(\mu_S S_u)$ (Beyenal <i>et al.</i> , 2003)
κ_e	Metabolic rate to the EPS production rate conversion factor (unitless)	κ_g
μ_S	Maximal nutrient consumption rate (h^{-1})	0.4 (Beyenal <i>et al.</i> , 2003)
K_S	Nutrient consumption half-saturation constant (g cm^{-3})	$0.65 \cdot 10^{-6}$ (Beyenal <i>et al.</i> , 2003)
k_F	Rate of persister formation (h^{-1})	$0.003\kappa_g$
k_R	Rate of persister reversion (h^{-1})	$0.1\kappa_g$
κ	SCC22 effectiveness rate ($\text{cm}^3 \text{g}^{-1}$)	$1.9292 \cdot 10^5$
α_1	Stationary SCC22 effectiveness rate ($\text{cm}^3 \text{g}^{-1} \text{h}^{-1}$)	$(4.88 \cdot 10^4)\kappa_g$
α_2	Stationary SCC22 minimal concentration (h^{-1})	$(32 \cdot 10^{-6})\alpha_1$
b	Active bacteria endogenous death rate (h^{-1})	$0.05\kappa_g$
ϕ	Water volume fraction within the biofilm (unitless)	0.5
M	Number of SCC22(aq) doses (doses)	0, 3, 5
N	SCC22-polymer particle number within the biofilm (particles)	$(6.35 \cdot 10^9) \iint_{\mathbb{D}} 1 \text{ dA}$
R	SCC22-polymer particle radius (cm)	$9.698 \cdot 10^{-5}$
C_L	SCC22-polymer particle loading (g cm^{-3})	0.135
γ_P	Polymer particle degradation time (days)	1, 2.34, 4 or 7
k	SCC22-polymer particle degradation constant ($\text{g cm}^{-2} \text{h}^{-1}$)	$C_L R / (24 * \gamma_P)$
λ_1	Horizontal spreading coefficient ($\text{cm}^3 \text{h g}^{-1}$)	$1 \cdot 10^{-14}$
λ_2	Vertical spreading coefficient ($\text{cm}^3 \text{h g}^{-1}$)	$10\lambda_1$
S_u	Upstream nutrient concentration (g cm^{-3})	$1 \cdot 10^{-6}$
C_d	SCC22(aq) dose concentration (g cm^{-3})	Varies
t^*	Time at which treatment initiates (h)	24
t_i^d	Time at which SCC22(aq) dose i is delivered (h)	Varies

and pressure within the biofilm are given as $\vec{v}$ and P , respectively. It should be noted that advective velocity, while describing a similar quantity, differs in interpretation between region I and region II. The region I advective velocity $\vec{v}^I$ describes the motion of bulk fluid while $\vec{v}$ describes the motion of biofilm

constituents. Thus, region I and region II quantities are described by different governing equations. We will begin by describing the equations that govern transport in the bulk and follow with a description of the biofilm region.

The advective velocity of the medium passing over the biofilm in the flow-cell apparatus is modelled as an incompressible fluid satisfying

$$\rho(\vec{v}_t^I + \vec{v}^I \cdot \nabla \vec{v}^I) = -\nabla P^I + \mu \nabla^2 \vec{v}^I, \quad (2.1)$$

$$\nabla \cdot \vec{v}^I = 0. \quad (2.2)$$

The vector $\vec{v}^I = \langle u^I, v^I \rangle$ represents the fluid velocity, P^I the pressure and μ the viscosity. Continuity of pressure is assumed at the fluid–biofilm interface. Since the height of the biofilm is small compared with the full domain height, the fluid velocity near the biofilm is very small compared with the channel mean velocity. Therefore, following Luna *et al.* (2004), we make the approximation of zero flow velocity at the fluid–biofilm interface. In addition, we require symmetry at the top of the domain. Thus, $u_y^I = v^I = 0$ at $y = r$. Further, the volumetric flow rate is fixed as $2w \int_H^r u^I dy = 2wQ$, where w is the chamber width. The constant Q represents the characteristic flux through the domain.

We assume that the bacterial metabolic rate and the biomass production rate are functions of a single growth rate-limiting nutrient (e.g. oxygen) S^I . This nutrient is transported by the bulk fluid along with the SCC22 antimicrobial, which has concentration C^I , according to

$$S_t^I + \vec{v}^I \cdot \nabla S^I = D_S^I \nabla^2 S^I, \quad (2.3)$$

$$C_t^I + \vec{v}^I \cdot \nabla C^I = D_C^I \nabla^2 C^I - k_C C^I. \quad (2.4)$$

The loss term $k_C C^I$ denotes deactivation of the antimicrobial due to interaction of SCC22 with the environment. Continuity of concentration and flux are enforced across the fluid–biofilm interface.

We assume well-mixed conditions upstream of the biofilm. We therefore fix the upstream nutrient concentration at S_u . The upstream SCC22 concentration satisfies

$$\frac{d}{dt} C_u(t) = -k_C C_u(t) + \sum_{i=1}^M \delta(t - t_i^d) C_d, \quad (2.5)$$

where the constant C_d represents the dose concentration administered at time t_i^d , for $i = 1, \dots, M$, and $\delta(t - t_i^d)$ is the Dirac delta function.

Within the biofilm, the bacterial population consists of active B , inert B_i and persister B_p bacteria. Active bacteria are those that are actively consuming nutrients and producing biomass and EPS E . Inert (i.e. dead) bacteria are those that are no longer capable of producing biomass or participating actively in any way, but still occupy space. Persister bacteria are those that are in a temporary dormant state that renders them highly tolerant of antimicrobials. The population density of the active subpopulation evolves according to

$$B_t + \nabla \cdot (\vec{v}B) = \kappa_g \frac{\mu_s S}{K_S + S} B - \kappa C \frac{\mu_s S}{K_S + S} B - \max\{\alpha_1 C - \alpha_2, 0\} B - bB - k_F B + k_R B_p. \quad (2.6)$$

The monod term $\mu_s S/(K_S + S)$ represents the metabolic rate of the active bacteria. With this in mind, the right-hand side of (2.6) can be thought of as the sum of the following processes: biomass production with rate κ_g , death due to antimicrobial with the rate determined by the metabolic rate of the bacteria,

death due to antimicrobial activity in the absence of nutrient, natural death with rate b , formation of persister cells with rate k_F and reversion from persister to active with rate k_R .

SCCs function as a carrier for silver ions, slowly releasing Ag^+ which has antimicrobial activity (Kascatan-Nebioglu *et al.*, 2007; Panzner *et al.*, 2009). The mechanism by which silver ions result in bacterial death, however, is not fully understood. Proposed modes of action include interference with electron transport, DNA binding and cell membrane interaction (Thurman & Gerba, 1989). The following argument for the form of the corresponding terms in (2.6) is meant to provide a qualitative explanation for model components that are ultimately empirical. We assume, broadly speaking, that silver ions damage cellular components. Thus, in our model, silver leads to cell death when damaged components are called upon to provide necessary functions, but cannot due to their damaged state. This happens more frequently when a cell is metabolically more active. Thus, the first component of death due to antimicrobial is modulated by the monod term representing the metabolic rate. It is not the case, however, that under low-nutrient conditions, cells are nearly immune to the effect of silver ions. This is demonstrated here by treatment of stationary phase culture, discussed in the experimental results. Therefore, we include a second component, $\max\{\alpha_1 C - \alpha_2, 0\}B$. The rationale behind this component is that under low-nutrient, high cell-density conditions, bacteria may upregulate damage repair genes to cope with these stresses. In order for death by antimicrobial to occur, enough damage must be done so as to overwhelm these repair mechanisms. Thus, we argue that under these conditions the rate of death by silver is zero up to some critical concentration α_2/α_1 , after which it is linear in C . Naturally, we restrict $\alpha_1 > 0$ and $\alpha_2 > 0$. Further, in the presence of nutrient, both the $\kappa C(\mu_s S/(K_S + S))B$ and $\max\{\alpha_1 C - \alpha_2, 0\}B$ terms contribute to the killing efficacy of C . However, in the absence of nutrient, only the latter term contributes. Thus, under low-nutrient conditions, higher values of C are required to achieve the same rate of killing as that achieved under high levels of nutrient. These assumptions are similar in spirit to the work of Cogan (2006), albeit different in implementation.

The remaining biofilm constituents evolve according to

$$(B_i)_t + \nabla \cdot (\vec{v} B_i) = \kappa C \frac{\mu_s S}{K_S + S} B + \max\{\alpha_1 C - \alpha_2, 0\} B + bB, \quad (2.7)$$

$$(B_p)_t + \nabla \cdot (\vec{v} B_p) = k_F B - k_R B_p, \quad (2.8)$$

$$E_t + \nabla \cdot (\vec{v} E) = \kappa_E \frac{\mu_s S}{K_S + S} B. \quad (2.9)$$

Equations (2.7) and (2.8) contain terms complementary to those found in (2.6). Equation (2.9) describes the production of EPSs.

Within the biofilm, soluble species are transported in the fluid-filled space between bacterial cells and EPSs. This water volume fraction is denoted as ϕ , and is taken to be constant throughout the biofilm. The water volume fraction satisfies

$$1 = \phi + \frac{B}{\rho_B} + \frac{B_i}{\rho_B} + \frac{B_p}{\rho_B} + \frac{E}{\rho_E}, \quad (2.10)$$

where ρ_B and ρ_E represent the density of bacterial cells and EPSs, respectively. Within this space, nutrient and antimicrobial concentrations satisfy

$$\phi S_t + (\vec{v} \cdot \nabla) S = \phi D_S \nabla^2 S - \frac{\mu_s S}{K_S + S} B, \quad (2.11)$$

$$\phi C_t + (\vec{v} \cdot \nabla)C = \phi D_C \nabla^2 C - k_C C + C_{\text{Source}}, \quad (2.12)$$

$$C_{\text{Source}} = \sum_{i=1}^N \delta(\vec{x} - \vec{x}_i) 4\pi R^2 k \left(1 - \frac{kt}{C_L R}\right)^2. \quad (2.13)$$

These transport equations resemble those in region I, with the addition of the volume fraction ϕ and source terms on the right-hand sides. Nutrient is consumed by active bacteria according to $\mu_s S / (K_S + S)$. Antimicrobial is released from polymer particles at positions $\vec{x}_i$, $i = 1, \dots, N$, according to (2.13). This release rate is derived from Hopfenberg (1976), assuming each particle is uniform and spherical with radius R . The constants k and C_L represent the decay rate and particle loading, respectively. Our previous work (Miller *et al.*, 2013) demonstrated that LTP particles distribute uniformly throughout the biofilm. We therefore take particle positions $\vec{x}_i$ to be uniformly distributed.

Advective velocity of biofilm constituents $\vec{v}$ is determined by

$$\vec{v} = -\lambda \nabla P, \quad (2.14)$$

$$\nabla \cdot \vec{v} = \frac{1}{1 - \phi} \left[\frac{\kappa_g}{\rho_B} + \frac{\kappa_e}{\rho_E} \right] \frac{\mu_s S}{K_S + S} B. \quad (2.15)$$

Equation (2.14) approximates the force balance within the biofilm (Klapper, 2004; Klapper & Dockery, 2010). The matrix $\lambda = \text{diag}(\lambda_1, \lambda_2)$ represents the frictional coefficients in the x and y directions, which may differ due to biofilm–mucin interaction as described in the experimental results. Equation (2.15) represents conservation of volume, and follows from (2.10) and the sum of (2.6–2.8) divided by ρ_B and (2.9) divided by ρ_E .

Evolution of the fluid–biofilm interface is described by

$$\frac{\partial}{\partial t} H = \vec{v}|_H \cdot \vec{n}, \quad (2.16)$$

where $\vec{n}$ is the outward-facing normal.

The governing equations do not describe the processes of bacterial attachment/detachment from the interface. We assume these processes balance and thus ignore them. In addition, due to the slow flow rate and subsequent small shear forces (see Section 2.1.2), we ignore the effect of shear forces and viscosity. We approximate solutions to these equations using a combination of numerical and analytical techniques. The following is a brief outline of our solution methodology. First, we take advantage of multiple time scales in biofilm development. Transport of soluble species occurs on a time scale of seconds, whereas biomass production occurs on a time scale of hours (Klapper & Dockery, 2010). We therefore seek quasi-steady state solutions for the concentrations of soluble species and fluid flow velocities. Our solution approach is to solve these equations in region I using scaling and asymptotic methods, taking advantage of the disparity in length scales in the flow-cell. Equations in region II are solved numerically making use of standard numerical techniques, modified to handle the non-linearities and irregular domain. Advective velocity of biofilm constituents $\vec{v}$ is determined by substituting (2.14) into (2.15), and solving the resulting elliptic equation for P . No flux conditions are imposed on the bottom of the domain and continuity of pressure, nutrient and SCC22 is enforced at the biofilm–fluid interface. Periodic conditions on the sides of the domain are a natural choice when simulating a portion of the biofilm. These conditions are inconsistent with the transport problem, however, as nutrient and SCC22 levels exhibit a disparity between the left and right sides of the domain. We, therefore, apply no flux conditions as an alternative. For a detailed treatment, see Miller (2012).

FIG. 2. *gfp*-tagged *P. aeruginosa* PAO1 biofilm in 10% SCFM with 0.04% mucin at 0 (a), 2 (b), 4 (c), 6 (d) and 24 h (e).

3. Results

3.1 Experimental results

A tower-like biofilm morphology was observed after overnight incubation of *P. aeruginosa* PAO1 in 10% SCFM with 0.04% mucin. Figure 2 shows confocal images of *gfp*-tagged *P. aeruginosa* PAO1 biofilms at 0, 2, 4, 6 and 24 h after inoculation. After inoculation, the biofilm consists of isolated cell aggregates in combination with a layer of single cells. Over time, the cell aggregates grow in size. Concurrently, the individual, surface-associated, cells appear to increase in density, but maintain a monolayer until approximately hour 6. After 24 h, a thin layer of cells is present at the base of the biofilm, approximately 4–6 μm deep. Scattered amongst this base are tower-like formations, reaching sizes of 30 μm in diameter and 50 μm in height. Tower-like structures were between 20 and 60 μm from the closest neighbour structures. An effort was made to take images at similar locations over time; however, time-series images are not from the same focal field. The aspect ratio of the tower-like formations is near 1 (height/diameter) early in biofilm development. Not until after 6 h was the aspect ratio observed to increase, reaching values of approximately 1.67 at 24 h. Biofilm water volume fraction was found to be approximately 0.5. In addition, mucin concentration was varied between 0.04 and 2%. The tower size was observed to be impacted by mucin concentration, with higher concentrations resulting in larger tower morphologies (not shown). Figure 3 shows confocal images of a 24-h-old biofilm stained using a WGA Alex Fluor® 633 conjugate. The bacterial towers (green) show a spatially localized Alex Fluor® 633 signal (pink).

Table 3 shows results for MIC measurement in various culture media for planktonic culture in exponential phase. The MIC for *gfp*-tagged *P. aeruginosa* PAO1 ranges from 4.0 to 12 $\mu\text{g}/\text{ml}$ depending on

FIG. 3. *gfp*-tagged *P. aeruginosa* PAO1 biofilm (green) in 10% SCFM with 0.04% mucin at 24 h stained with WGA Alexa Fluor® 633 conjugate (pink).

TABLE 3 MIC values ($\mu\text{g/ml}$) for planktonic cultures of different strains of *P. aeruginosa* in different media during the exponential phase. Values given with standard error

Strain	SCFM	SCFM + 0.04% mucin	LB
PAO1	9.3 ± 2.7	16 ± 0.00	24 ± 0.00
<i>gfp</i> -tagged PAO1	4.0 ± 0.00	6.7 ± 0.67	12 ± 0.00
7119	4.7 ± 0.67	6.0 ± 1.2	15 ± 1.3
FRD1	5.3 ± 0.67	5.3 ± 0.67	24 ± 0.00
PDO300	12 ± 0.00	21 ± 2.7	12 ± 0.00

medium type. The MIC for *gfp*-tagged *P. aeruginosa* PAO1 under anaerobic conditions was measured to be $13.33 \pm 2.66 \mu\text{g/ml}$. Our model, calibrated using the aqueous SCC22 treatment data below, suggests an MIC of $4.54 \mu\text{g/ml}$ for *P. aeruginosa* PAO1 growing in a 24 h old biofilm. Table 4 shows the treatment efficacy of aqueous SCC22 on stationary (overnight) planktonic culture. No effect is observed until SCC22 concentration exceeds $32 \mu\text{g/ml}$. Table 5 represents treatment of overnight biofilms using either aqueous SCC22 or SCC22-LTP nanoparticles. No significant effect of SCC22-LTP on bacterial survivability was observed in this system.

3.2 Simulation results

Numerical simulations were run for the purposes of demonstrating model characteristics, comparing with experimental results and analysing treatment scenarios. All simulations were run using the following initial conditions. We assume at early stages little EPS has been produced by the biofilm. We therefore take the initial EPS concentration to be one-tenth of the steady-state value $E_0 = 0.1(1 - \phi)\kappa_e(\kappa_g/\rho_B + \kappa_e/\rho_E)^{-1}$. The remainder of the biofilm is assumed to consist of active bacteria; $B_0 = (1 - \phi - E_0/\rho_E)\rho_B$, $B_{i0} = 0$, $B_{p0} = 0$. The initial biofilm profile is a single layer of cells with occasional mounds spaced $30 \mu\text{m}$ apart. This initial condition corresponds to our observations of *P. aeruginosa* biofilms in SCFM with 0.04% mucin shortly after inoculation.

3.2.1 Biofilm morphology. Figure 4 represents evolution of the simulated biofilm from time $t = 0$ to $t^* = 24 \text{ h}$. Disparate expansion rates presumed to result from a mucin–biofilm interaction cause the

TABLE 4 *Survivability for stationary phase *P. aeruginosa* PAO1 challenged with SCC22(aq) for 2 h, with standard error*

SCC22 concentration ($\mu\text{g/ml}$)	CFU/ml ($\times 10^8$)	Post- to pretreatment ratio (%)
Pretreatment	66.0 ± 9.01	n/a
0	61.3 ± 5.17	92.9
2	71.3 ± 3.53	108
4	70.3 ± 5.78	106
8	77.6 ± 4.47	117
16	71.5 ± 1.69	108
32	66.9 ± 1.81	101
64	4.90 ± 4.65	7.42
128	0.0899 ± 0.06	0.136

TABLE 5 *Comparison of simulation results and experimental data for 2-h challenge of *gfp*-tagged *P. aeruginosa* PAO1 biofilm with SCC22. The survivability given with standard error*

Treatment	Survivability (%)	Simulation result
SCC22(aq) 34 $\mu\text{g/ml}$, flow rate 3 ml/h	17.34 ± 7.52	15.40
SCC22(aq) 170 $\mu\text{g/ml}$, flow rate 3 ml/h	1.94 ± 1.63	0.84
SCC22(aq) 340 $\mu\text{g/ml}$, flow rate 3 ml/h	1.98 ± 1.08	0.82
SCC22-LTP 0.5 mg/ml, flow rate 3 ml/h	102.08 ± 3.98	99.86
Blank-LTP 0.5 mg/ml, flow rate 3.5 $\mu\text{l/h}$	76.69 ± 16.75	83.27
SCC22-LTP 0.5 mg/ml, flow rate 3.5 $\mu\text{l/h}$	76.83 ± 22.27	82.81

FIG. 4. The biofilm–fluid interface $H(x, t)$ for $t = 0$ (light grey) to $t = t^* = 24$ h (black), by 4-h steps.

development of a tower-like morphology. Simulation results for $t = 24$ h (Fig. 4, black line) show tower-like structures measuring $36 \mu\text{m}$ tall, approximately $20 \mu\text{m}$ in diameter and spaced $30 \mu\text{m}$ apart. This implies that the simulated biofilm has an aspect ratio of 1.8 at 24 h. The simulation results differ somewhat from the experimental results in the shape of the tower formations. The tower structures in Fig. 2 show a more rounded top, whereas simulation results show a more pointed top. This difference may indicate that assuming disparate frictional coefficients in the x and y directions does not fully describe the momentum balance of the biofilm. We note that surface tension of the EPS has been ignored, and that inclusion of the relevant equations may produce better simulation results. For a discussion of the impact of surface tension, the reader is referred to Cogan (2004).

FIG. 5. Total viable, active and persister, bacterial population under a constant SCC22 concentration.

3.2.2 Aqueous SCC22 treatment. SCC22 effectiveness parameters κ , α_1 and α_2 were chosen to fit the experimental data for 2-h treatments with aqueous SCC22 at 34, 170 and 340 $\mu\text{g/ml}$ and survivability data for stationary phase planktonic culture. Fit was achieved by minimizing the sum of squared differences between the relative viable cell population predicted by the model and the relative viable population measured by experiment. Relative viable cell population is defined as the number of viable cells after treatment divided by the number of viable cells under the control conditions. These data for 2-h treatment with aqueous SCC22 and treatment with SCC22-LTP, along with model simulation results, are represented in Table 5. In addition, k_C , which describes the SCC22 deactivation rate in 10% SCFM with 0.04% mucin was fit using data from long-term (2 and 24 h) treatment of flow-cell-grown *gfp*-tagged *P. aeruginosa* biofilms with aqueous SCC22 (data not shown).

Simulations were conducted to examine various treatment scenarios. Under constant concentration, antimicrobial treatment demonstrates a biphasic killing phenomenon, represented in Fig. 5. The active bacterial population is killed quickly in a concentration-dependent manner. The persister population, however, must revert to active bacteria before they can be killed. The rate of reversion k_R , assumed to be constant here, is slower than the death rate of the active bacteria for large concentrations of SCC22. This results in an apparent change in the rate of population decrease once the active population has been reduced to near zero. Note that the reduced killing rate is independent of the SCC22 concentration.

Figure 6(a) represents treating a biofilm twice per day with aqueous SCC22 at 8, 16 and 64 $\mu\text{g/ml}$. Figure 6(b) shows the SCC22 concentrations over time for these treatments. While all of these dosage concentrations are above the MIC, the concentration within the flow-cell drops due to the SCC22 decay rate k_C . Population recalcitrance is evident between doses. Daily treatment with 8 $\mu\text{g/ml}$ is unable to eradicate the biofilm, but a dosage of 16 $\mu\text{g/ml}$ is high enough that the bacterial population is unable to recover fully before the next dose. Thus, 16 $\mu\text{g/ml}$ is able to overcome the biofilm over several twice-daily doses.

3.2.3 SCC22-particle treatment. Our previous work (Miller *et al.*, 2013) has shown that LTP particles reach a uniform distribution within the biofilm within a few hours. We therefore assume a uniform distribution of particles within the simulated biofilm. The concentration achieved by SCC22-loaded particle degradation results from the competition between the particle release rate and the rate at which the SCC22 diffuses out of the biofilm and is removed by the advective flow above the biofilm. The removal rate is influenced by the volumetric flow rate $2wQ$ as well as the morphology of the biofilm. Figure 7 shows the peak SCC22 concentration resulting from polymer particle delivery as a function of

FIG. 6. Twice per day aqueous SCC22 treatment simulation results for various values of C_d . Curves end if the biofilm grows larger than the computational domain.

FIG. 7. The peak SCC22 concentration within the biofilm achieved by release from SCC22-polymer particles as a function of the volumetric flow rate.

$2wQ$. Therapeutic concentrations are unable to reach biologically relevant levels, those above the MIC, at any but the lowest flow rates. Under low flow rate conditions, slow-release polymer particles are able to keep the concentration of antimicrobial above the MIC for extended periods of time, as shown in Fig. 8(b).

As illustrated in Table 5, LTP particle-based treatment does not reduce the bacteria population at high flow rates. The model suggests that while the particles continue to release SCC22, the antimicrobial is rapidly removed by the advective fluid flow. This does not allow the antimicrobial concentration to reach sufficient levels. This difficulty is rectified at low flow rates. At low flow rates, however, the model suggests that nutrient concentration S drops so that the term $-bB$ dominates the right-hand side of (2.6). The resulting loss of viable bacteria matches experimental results empirically, shown in Table 5. This statement is qualified by the fact that we cannot claim this population change results from natural death alone, as detachment and other processes may play a role. Regardless, the experimental and simulation results agree that the flow-cell environment, even at low flow rates, may be insufficient to investigate the behaviour of polymer particle-based treatments. We therefore modify model parameter values to investigate expected treatment behaviour for the case in which a low flow rate allows relevant concentrations of SCC22 released from particles to accumulate and a sufficiently high nutrient level prevents the term

FIG. 8. SCC22-polymer particle treatment of the biofilm for various particle degradation times for $r = 0.07$ and $2wQ = 0.007$. Treatment is initiated at time $t^* = 24$ h.

$-bB$ from dominating the right-hand side of (2.6). This may be appropriate as nutrient availability is likely dependent on epithelial cell behaviour and less so on fluid transport. These modifications allow for meaningful, albeit theoretical, investigation of SCC22-polymer treatment behaviour. We therefore emphasize the caveat that the following numerical results may not directly correspond to an in vitro or in vivo system. We double the flow-cell chamber height r , which reduces advective flow velocities in region I, and consider a biofilm grown for 24 h at a flow rate of 3 ml/h. The flow rate is reduced for SCC22-polymer particle treatment to a value of $7 \mu\text{l/h}$. Finally, S_0 is increased so that the average nutrient value at the time of treatment for $2wQ = 0.007$ is equal to that when $2wQ = 3 \text{ ml/h}$. The following simulation results are presented using these modified parameter values.

Figure 8(a) shows treatment simulations for SCC22-polymer particles with various degradation times. Particles were assumed to deposit at a density of 6.35×10^9 particles/ml of biofilm (Miller *et al.*, 2013). The degradation time of 2.34 days was determined to minimize the minimal viable bacteria population over the duration of the simulation. In addition, the 2.34-day degradation time out-performed scenarios in which deposition of particles with multiple degradation times were considered. We investigated whether an improvement in the killing efficacy, as measured by the minimal viable bacteria population, could be gained by first achieving a high concentration of SCC22 for a short time, and then maintaining a low level slightly above the MIC for a long duration. We assumed the total number of particles to be fixed. Under this constraint and the simulation parameters outlined above, the advantage to such a scheme is outweighed by the costs of dividing the particles into distinct degradation rates. That is, though a higher concentration of SCC22 can be reached by including particles that degrade in 1 day, the ability to maintain the concentration above the MIC suffers because fewer particles are responsible for this task. These results are context-specific, however, as the length of time that the concentration of SCC22 can be maintained above the MIC by polymer particles depends on the number of particles, the MIC and the mass transfer characteristics of the system (medium flow rate, etc.).

For 6.35×10^9 particles/ml of biofilm (Miller *et al.*, 2013), the SCC22 concentration can be kept above the MIC for nearly 30 h using a degradation time of 2.34 days. This represents a difference in character between SCC22-polymer particle-based treatment and treatment with aqueous SCC22. Under this latter treatment paradigm, the SCC22 concentration quickly falls to zero after each dose. This behaviour does not depend on the flow rate, since SCC22 degradation occurs in the bulk and growth medium reservoir. This differs from the SCC22-polymer case because the particles function as

FIG. 9. Comparison between twice-daily SCC22(aq) treatment and a single SCC22-polymer treatment with degradation time 2.34 days for $r = 0.07$ and $2wQ = 0.007$. Treatment is initiated at time $t^* = 24$ h.

FIG. 10. Optimal particle degradation time as a function of the volumetric flow rate for $r = 0.07$. Optimal particle degradation time is defined as the time in which polymer particles are set to degrade that minimizes the minimal total-viable-cell population over a 60-h treatment time frame.

a reservoir of antimicrobial that shelters the antimicrobial from deactivation by reaction with the environment. Figure 9 illustrates a comparison between an aqueous-based treatment strategy and a polymer particle-based strategy. Both scenarios are considered under the modified parameter values and the aqueous SCC concentration is chosen to be that reached by the polymer particles at the outset of treatment. The ability of the particles to maintain concentrations above the MIC for extended periods of time gives this treatment strategy a significant advantage over an aqueous-based strategy.

Using this model, we analysed the optimal particle degradation time for a range of volumetric flow rates. We define optimal degradation time to mean the particle degradation time that minimizes the minimal total viable cell population ($B + B_p$) over the 60-h treatment simulation. The optimal degradation time, restricted to the interval of 1–7 days, is shown in Fig. 10. As the volumetric flow rate increases, the SCC22 concentration achieved by the particles decreases. This reduces the amount of time a particular particle formulation can keep the concentration above the MIC. In addition, lower concentrations result in slower killing. The balance between the need to achieve a high concentration and the need to sustain the concentration above the MIC for long periods shifts towards achieving higher concentrations. Thus, as the flow rate increases, the optimal degradation time decreases.

In addition, we investigated the utility of an aqueous SCC22 and SCC22-polymer combination therapy. We consider the impact of a single aqueous SCC22 dose delivered with a single SCC22-polymer dose. The optimal particle degradation time increases as the concentration of the initial aqueous SCC22 dose increases. Under an initial dose of 32 $\mu\text{g/ml}$, the optimal particle degradation time is increased by 30% and the minimal viable cell population is reduced by 20% of that with no initial aqueous dose. In addition, the time at which this minimum occurred changed 1.5 h, indicating that an initial aqueous dose has only a small impact on particle-based therapy.

4. Discussion

The treatment of biofilm-based infections in the CF lung represents a complex temporal–spatial problem in which bacterial susceptibility and persistence as well as drug delivery and transport are key factors. We seek to better understand these issues by extending information gained from in vitro experimentation through the use of mathematical modelling. We developed a mathematical model of biofilm infection in the CF environment and treatment with SCC22 based on the flow-cell apparatus. This model describes various treatment strategies and the effect of a persister subpopulation.

Tower formation demonstrated in flow-cell-grown biofilms suggests a disparity in vertical and horizontal expansion rates. Here, we assume different values for the coefficients of friction λ_1 and λ_2 in (2.14). WGA Alexa Fluor® 633 conjugate staining localized to the exterior of towers is used to support this modelling assumption. We point out, however, that this evidence is inconclusive. It is tempting to claim that Alexa Fluor® 633 signal represents mucin localization, which is what we suspect. In our lab, however, we have observed that WGA-conjugated Alexa Fluor® 633 bound to both mucin in the absence of bacterial cells and *P. aeruginosa* cells in the absence of mucin. Both of these interactions have been reported elsewhere (Strathmann *et al.*, 2002; Gabor *et al.*, 2004). In addition, the WGA conjugate is a large molecule and may suffer from restricted penetration into the biofilm. Regardless, model behaviour is only slightly influenced by perturbations in biofilm morphology and the assumption of disparate friction coefficients produces a morphology similar to that observed in the experimental system.

Other modelling approaches may provide additional resolution of the mechanical forces at play in the evolution of the biofilm interface. In particular, Zhang *et al.* (Zhang *et al.*, 2008a,b; Wang & Zhang, 2012) have developed a phase field theory model of biofilm growth, building on the work of Cogan (2004). This approach demonstrates the ability to capture complex fluid–biofilm interaction, including shedding and sloughing detachment events (Zhang *et al.*, 2008a,b). We note, however, that the experimental set-up presented here is characterized by a slow flow rate resulting in low shear. As a result, these interactions may not contribute significantly to our system of interest. In addition, the CF lung environment is characterized by sticky, viscous mucus. Hence, sloughing may not be relevant in such an environment.

We demonstrate that SCC22 is effective against flow-cell-grown *P. aeruginosa* biofilms in a synthetic CF mucus medium. In addition, we demonstrate that SCC22 is effective against anaerobic *P. aeruginosa* cultures. This is relevant since the CF lung environment may be oxygen depleted (Boucher, 2004). Our modelling effort indicates an MIC for biofilm growth that is closer to that of planktonic cultures in SCFM without mucin. We expect antimicrobial tolerance for cells in a biofilm state to be closer to that of stationary phase cultures (Spoering & Lewis, 2001). We hypothesize that the disparity between our observations and expectations may be due to the observed biofilm morphology. Simulation results suggest that nutrient concentrations in the interior of the tower formations are approximately half of the concentration at peak. Thus, the size and morphology of the observed biofilms

may not result in significant nutrient starvation, which in turn may not lead to increased antimicrobial tolerance.

Our model demonstrates that the existence of a persister population results in biphasic killing in which an initial fast killing phase is preceded by a slow killing phase. Further, if the rate at which bacteria in the persistent state revert to a susceptible state is independent of the environment, as we assume here, the rate of killing in the slow killing phase is constant. This implies that temporally limited treatment strategies may be frustrated by biofilm recalcitrance despite the use of aggressively high concentrations of antimicrobial. In the CF context, all treatment strategies are temporally limited to one degree or another. Inhalation of nebulized antimicrobial in an aqueous phase may be capable of delivering high concentrations directly to the site of infection, but high concentrations may not be sustainable as the drug interacts with the environment or is absorbed into the body. Further, with respect to the quality of life, the number of times per day a patient can be expected to be dosed in this manner is limited. Slow-release polymers provide utility in this situation by maintaining antimicrobial concentrations over long periods of time.

Our experimental results demonstrate that the flow-cell environment is not well suited for an investigation of slow-release polymers. The number of particles that embed themselves in the biofilm following a 10-min perfusion of 0.5 mg/ml SCC22-LTP particles does not provide a sufficiently strong source to counter the SCC removal by advective–diffusive processes under high flow conditions. Under low flow conditions, delivery of nutrients to the biofilm appears to be hampered. Despite the model prediction that biologically relevant levels of SCC22 are reached under these conditions, low nutrient levels may frustrate killing by antimicrobial activity. Using the model, we were able to investigate expected SCC22-polymer treatment behaviours by modifying model parameters so that reasonable simulation results could be generated. Since our model parameters reflect the flow-cell environment as opposed to the lung environment, we do not think this modification is unreasonable. Application of our simulation results to the CF lung environment would require an investigation into proper model parameter values. In particular, the volumetric flow rate represents an important parameter controlling the SCC22 concentration that results from particle release. While these fluid dynamics are not present in the lungs, other loss mechanisms may be present. The loss due to fluid flow above the biofilm in our model may approximate these loss mechanisms for an appropriately chosen flow rate.

In any case, our simulation results suggest that polymer particles can effectively treat bacteria under conditions in which mass transfer does not dominate SCC release. This may be the case in the lung environment. Little SCC may be lost through the lung epithelial layer or air–mucus interface. In addition, in the lung, particles may distribute throughout the lung mucus and biofilm after deposition on the air–mucus interface, which could significantly increase the number of particles trapped in the environment. For a review of particle penetration into mucus, the reader is referred to the work of Hanes *et al.* (Suh *et al.*, 2007; Suk *et al.*, 2009; Tang *et al.*, 2009; Wang *et al.*, 2008).

Our results also indicate that an optimal particle degradation time exists that minimizes the viable bacterial population over the course of a single dose. The existence of this optimum represents the trade-off between the need to achieve high concentrations and the need to maintain these concentrations over an extended period of time. The optimal degradation time depends on the bacterial growth rate, persister population dynamics, SCC efficacy and volumetric flow rate. For the parameters describing bacterial population dynamics used here and for a particular range of volumetric flow rates, the particle degradation time that minimizes the viable bacterial population occurs between 1 and 7 days. This range of flow rates is narrow, however, and represents a low rate of SCC loss. This suggests that, provided SCC loss rates are not large in vivo, there is room for optimizing the use of polymer particles as a drug delivery mechanism in treating *P. aeruginosa* infection in the lung. In this way, our work suggests one

future line of research is the determination of the optimal polymer degradation time in the conditions of the lung, and the development of polymers with the associate behaviour.

Simulations suggest that particles with an optimal degradation time, under the parameter values used here, are able to maintain the concentration of SCCs high enough to decrease the bacterial population for 30 h, whereas aqueous treatment effectively reduces the bacterial population for up to 8 h per dose. In this 30-h period, particles that degrade in 2.34 days are able to achieve a reduction in bacterial population greater than 2 orders of magnitude. Twice daily dosing with a comparable concentration of aqueous antimicrobial requires more than 40 h to achieve a 2 order of magnitude reduction in the viable bacteria population. In addition, while a combination dosing strategy does increase the efficacy of treatment, a single aqueous SCC22 dose has only a small impact on SCC22-polymer treatment dynamics. Under these conditions, patients treated once every 24–48 h with SCC22-polymer could clear infections more rapidly and with fewer dosing administrations than under a twice-daily aqueous SCC22 treatment strategy. This is only conjecture, however, as under the mathematical model described here, the bacterial population can never be pushed all the way to zero and will thus always repopulate after the treatment terminates. The host immune system may be able to clear the remaining infection once treatment has decreased the bacterial population to some critical value. However, this value is unknown and likely patient dependent. Conditions like CF impair the host immune response as well (Gibson *et al.*, 2003). These issues make using mathematical modelling to determine the patient outcome from various treatment options difficult.

The addition of fluid flow over the biofilm as a mechanism for nutrient and SCC transport represents an extension of Stine *et al.* (2012). Stine *et al.* (2012) showed that 10^9 particles per ml of biofilm is able to surpass the MIC in a *Bacillus anthracis* biofilm. This particle density is similar to that used in the model presented here, but is shown to effectively treat a biofilm with an MIC 10 times higher than that of *P. aeruginosa* used here. Stine *et al.* (2012) represents an investigation of the ideal case. That is, no flux conditions result in all of the SCCs released from polymer particles remaining within the biofilm. This differs from the model presented here, in which the antimicrobial is washed away by advective flow. Within the lung, water, salt and mucus transport occurs at the epithelial interface (Boucher, 2004) and polymer particles are likely to distribute throughout the mucus layer. The mucus layer itself moves slowly or not at all. Despite this, we expect a certain amount of antimicrobial loss. We can, therefore, think of the work presented here for $2wQ$ large as a worse case scenario, representing a large rate of antimicrobial loss. When $2wQ$ is small, this model may represent a reasonable approximation.

5. Summary

With the understanding that the biofilm state represents a challenge to therapy, we analyse the capabilities of SCC22 as a novel antimicrobial and SCC22-polymer drug delivery systems using a combination of experiments and mathematical modelling. In this work, the use of SCFM and mucin in a flow-cell environment represents a novel in vitro model of CF lung biofilms. We confirm the effectiveness of SCC22 on 24-h *P. aeruginosa* biofilms (Panzner *et al.*, 2009). We demonstrate the ability of mathematical models to inform dosage strategy by taking into account underlying fluid flow and antimicrobial loss mechanisms.

A number of simplifying assumptions were made in the development of the mathematical model. These include the following:

- the concentration of a single rate limiting nutrient is sufficient to describe bacterial metabolic behaviour;
- persister conversion and reversion rates are independent of the environment;

- the rate of SCC/Ag⁺ loss due to reaction with bacteria is small enough to be ignored.

With these assumptions in mind, we are able to provide the following insights regarding SCC-based treatment of CF lung biofilms:

- persister population behaviour is important in the determination of the optimal treatment strategy;
- SCC22 losses due to reaction with the environment necessitate frequent dosing under the aqueous strategy;
- under low loss conditions, SCC22-polymer particle-based treatments are able to outperform aqueous-based treatments that reach comparable SCC concentrations;
- particle degradation rate is an important system parameter impacting treatment efficacy.

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