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Aggregatibacter actinomycetemcomitans biofilm killing by a targeted ciprofloxacin prodrug

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A pH-sensitive ciprofloxacin prodrug was synthesized and targeted against biofilms of the periodontal pathogen *Aggregatibacter actinomycetemcomitans* (Aa). The dose required to reduce the viability of a mature biofilm of Aa by ~80% was in the range of ng cm^{-2} of colonized area (mean biofilm density 2.33×10^9 cells cm^{-2}). A mathematical model was formulated that predicts the temporal change in the concentration of ciprofloxacin in the Aa biofilm as the drug is released and diffuses into the bulk medium. The predictions of the model were consistent with the extent of killing obtained. The results demonstrate the feasibility of the strategy to induce mortality, and together with the mathematical model, provide the basis for design of targeted antimicrobial prodrugs for the topical treatment of oral infections such as periodontitis. The targeted prodrug approach offers the possibility of optimizing the dose of available antimicrobials in order to kill a chosen pathogen while leaving the commensal microbiota relatively undisturbed.

Keywords: *Aggregatibacter actinomycetemcomitans*; ciprofloxacin; prodrug; biofilm; periodontal disease

Introduction

One objective of controlled drug release is to expose pathogens to a sustained lethal dose of an antimicrobial, while minimizing the amount of drug released into the body (Southard & Godowski 1998). The chances of both bacterial resistance and adverse drug reactions are thereby reduced compared to systemic administration (Schwach-Abdellaoui et al. 2000; Walker & Karpinia 2002). The localized community structure that endows biofilms with phenotypic drug resistance can be exploited by targeting them with a prodrug that can be triggered to release the free drug. This essentially renders the biofilm itself into a miniature controlled release device. Since delivery is localized with micron scale precision, the total quantity of drug released into the environment is minimized using this approach.

Controlled release is especially relevant as an alternative treatment of periodontitis, a chronic inflammatory condition induced by oral biofilms (Southard & Godowski 1998; Schwach-Abdellaoui et al. 2000). *Aggregatibacter actinomycetemcomitans* (Aa) is a major risk factor for localized aggressive periodontitis (Fine et al. 2007) and is used to induce the symptomatic chronic inflammatory condition in animal models (Schreiner et al. 2003; Garlet et al. 2005, 2007; Trombone et al. 2009; Li et al. 2010). Aa is particularly susceptible to the DNA gyrase inhibitor, ciprofloxacin (Hoogkamp-Korstanje & Roelofs-Willems 2000; Muller et al. 2002). Sustained

exposure of oral tissues to lethal doses of ciprofloxacin can be achieved by systemic application but at the cost of exposure of the body to gram quantities over the dosing period (Conway et al. 2000; Tozum et al. 2004). Controlled release devices can minimize the dose, but the typical dose level for controlled release devices is still between 1 and 12 mg (Raj & Dentino 2005). In this study, biofilms of Aa were targeted with a ciprofloxacin prodrug that could be triggered to release the free drug. Sustained release of the cidal dose resulted in significant killing of Aa biofilms consisting of considerable biomass, while releasing ng quantities into the *in vitro* environment.

Materials and methods

Culturing of biofilms

Aa strain D7S, a rough colony clinical isolate obtained from the central incisor of an African American female patient with generalized aggressive periodontitis was provided by Casey Chen, University of Southern California. The medium for culturing Aa consisted of modified tryptic soy broth (MTSB; Suci et al. 2010). Frozen stocks were maintained at -80°C in 20% glycerol, 80%MTSB. All culturing was performed at 37°C in 5%CO₂. Biofilms were cultured in 96 well polystyrene microtiter plates (Falcon OptiluxTM, Fisher Scientific) in MTSB using a published protocol (Suci & Young 2011).

Synthesis of ciprofloxacin prodrug, biotinylated prodrug and biotinylated fluorescent analogue

The synthesis of the ciprofloxacin prodrug was based on two previous publications (Weber et al. 1990; Hennard et al. 2001). The biotinylated prodrug and biotinylated fluorescent analogue were synthesized by coupling a commercially available biotinylation reagent to maleimides of either the prodrug or fluorescein *via* glutathione. Details of the syntheses are in the Supplementary material [Supplementary material is available *via* a multimedia link on the online article webpage].

Measurement of kinetics of prodrug cleavage

A 1 μ l aliquot of a 20 mg ml⁻¹ (29 mM) solution of the prodrug in dimethylformamide (DMF) or a 3 μ l aliquot of a 6.7 mg ml⁻¹ (9.7 mM) solution of the biotinylated prodrug in DMF was added to 100 μ l of buffer preheated to 37°C. The buffer was either 50 mM TRIS (pH 7, 7.5 or 8) or 50 mM MES (pH 6 or 6.5). Aliquots were vortexed and maintained at 37°C for 5, 10, 30 or 60 min (prodrug), or 120, 240 or 480 min (biotinylated prodrug), and then 20 μ l were added to 0.1% trifluoroacetic acid (TFA) HPLC running buffer. The free ciprofloxacin produced by the base catalyzed cleavage was identified as a distinct band with an absorbance at 325 nm measured using reverse phase HPLC. Details of the instrumentation and running parameters are in the Supplementary material. Data were least-squares fit to a first order rate equation using SigmaPlot 2.0 (Jandel Scientific, San Rafael, CA, USA).

Visualization of the biofilm using confocal microscopy

Confocal laser scanning microscopy (CLSM) images were acquired with a Leica TCS-SP2-AOBS (Leica Microsystems, Buffalo Grove, IL, USA). Biofilm microcolonies were visualized through the underside of the wells using the protocols of Suci et al. (2010) and Suci and Young (2011). Images were collected with a 63X 0.9 NA HCX APO LU-V-I water immersion objective. Fluorescence emissions from different fluorophores were discriminated using acousto-optical tunable filters (Suci et al. 2010; Suci & Young 2011). Images were acquired at 2.0 μ m intervals throughout the depth of the biofilms. Stacks were combined in Imaris software (Bitplane AG, Zürich, Switzerland) to yield final images. The biovolume of *Aa* colonies were determined using the Imaris volumetric analysis software add-on (Periasamy & Kolenbrander 2009; Suci & Young 2011).

Kinetics of Aa-specific antibody binding to the biofilm

The *Aa*-specific targeting reagent was a monoclonal antibody (*Aa*-mAb) 325AA2 (Gmür & Thumheer 1996) against *Aa*, isotype mIgG2b, produced in mice, donated by Rudolf Gmür, University of Zürich and purified as

described previously (Suci et al. 2010). The *Aa*-mAb 325AA2 epitope was shown to be highly specific for *Aa* (Gmür & Thumheer 1996). For measurement of the kinetics of binding of the *Aa*-mAb to the biofilm, the biofilm was first exposed to 1% bovine serum albumin (BSA) in 10 mM phosphate buffered saline (PBS; pH 7.0, 100 mM NaCl) for 5 min to block non-specific binding. This was followed by a rinse with PBS. Biofilms cultured in different wells were exposed to *Aa*-mAb for 5, 10, 30 or 60 min, rinsed with PBS and exposed to a fluorescently tagged secondary anti-mouse antibody (Ab; Alexa Fluor 488 rabbit anti-mouse IgG; Life Technologies, Grand Island, NY, USA) for 60 min. Biofilms were subsequently stained with a nucleic acid stain (SYTO 59; Invitrogen, Carlsbad, CA, USA) before visualization using CLSM. Green and red pixel intensities were obtained from images consisting of the sum of the composite sections using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA).

Targeting of the biofilm with biotinylated prodrug and biotinylated fluorescent analogue

The biotinylated prodrug and the biotinylated fluorescent analogue were both used to target 24 h old biofilms of *Aa* using a multistep procedure. The biofilm was first exposed to 1% BSA in PBS for 5 min. This was followed by a series of exposures to the following reagents with a rinse with PBS between each step: (1) *Aa*-mAb, 50 min; (2) biotinylated secondary Ab (biotin-rabbit anti mouse IgG; Invitrogen), 50 min; (3) streptavidin (Invitrogen), 30 min; and (4) either the biotinylated fluorescent analogue or the biotinylated prodrug, 30 min. The primary and secondary Ab and the streptavidin were dissolved in PBS (pH 7.0) at 50 μ g ml⁻¹. The biotinylated fluorescent analogue was dissolved in PBS (pH 7.0) at 5 μ M. The biotinylated prodrug was stored in DMF until minutes before exposure and dissolved in PBS (pH 6.3) at 5 μ M for reaction with the biofilm. Fluorescein labelled streptavidin (Invitrogen) with a mean labelling ratio of 1.6 fluorescein per streptavidin was used in step 3) as a standard of comparison.

Exposure of biofilms of Aa to ciprofloxacin in solution

A 20 mg ml⁻¹ ciprofloxacin solution was made from ciprofloxacin hydrochloride in sterile water (ICN Biomedicals Inc., Irvine, CA, USA). Appropriate volumes of the 20 mg ml⁻¹ ciprofloxacin solution were added to culture medium to obtain 0.01 and 0.05 μ g ml⁻¹ working solutions. Biofilms were exposed to ciprofloxacin by filling wells with the working solutions and incubating under the culturing conditions (see above). A reduction in the colony forming units (cfu) produced by exposure to ciprofloxacin was computed as the fraction of cfu obtained with dosing *cf* a no dose control.

Assessment of biofilm killing

Biofilms, which had been cultured for 24 h were targeted with the biotinylated ciprofloxacin prodrug as described above, rinsed with PBS ($\times 2$) before 200 μl of medium were added to the wells. The medium used was MTSB with 50 mM TRIS titrated to pH 8 with NaOH with or without the addition of 10 mg ml^{-1} carboxymethylcellulose (CMC; Sigma Chemical Co., St Louis, MO, USA). Biofilms were cultured for an additional 18 h and then assayed for viable cells. For cfu analysis, wells were first rinsed ($\times 2$) with 200 μl of PBS and then 120 μl of PBS were added to each well. Biofilm was displaced from the surface by scraping with a 20 μl pipet tip in a circular motion for 20 repetitions followed by drawing 20 μl of the solution up into the pipet tip and injecting back into the well. This procedure was repeated $\times 20$ for each well. A 20 μl aliquot of the PBS cell suspension was used to make dilutions for cfu analysis and measurements of the optical density of 80 μl of the suspension were acquired at 600 nm (OD_{600}) using a UV-vis plate reader (HTS-

XT; Bruker Optics Inc., Billerica, MA, USA). The cell suspension consisted of a mixture of single cells and cell aggregates. A possible consequence of the presence of aggregated cells is an underestimation of the extent of killing, since it would only require one viable cell in an aggregate to act as a cfu. Preliminary studies indicated that the extent of aggregation was the same for biofilms exposed to ciprofloxacin and not exposed to ciprofloxacin (data not shown).

Measurement of the targeted biotinylated fluorescent analogue

The amount of biotinylated fluorescent analogue bound to the biofilms was measured by a depletion assay, ie as the difference between the mass of the biotinylated fluorescent analogue depleted from the bulk medium for targeted and non-targeted (NT) conditions, where the targeted condition included all the targeting reagents and the non-targeting condition was identical except for omission of the *Aa*-mAb. This was converted to moles

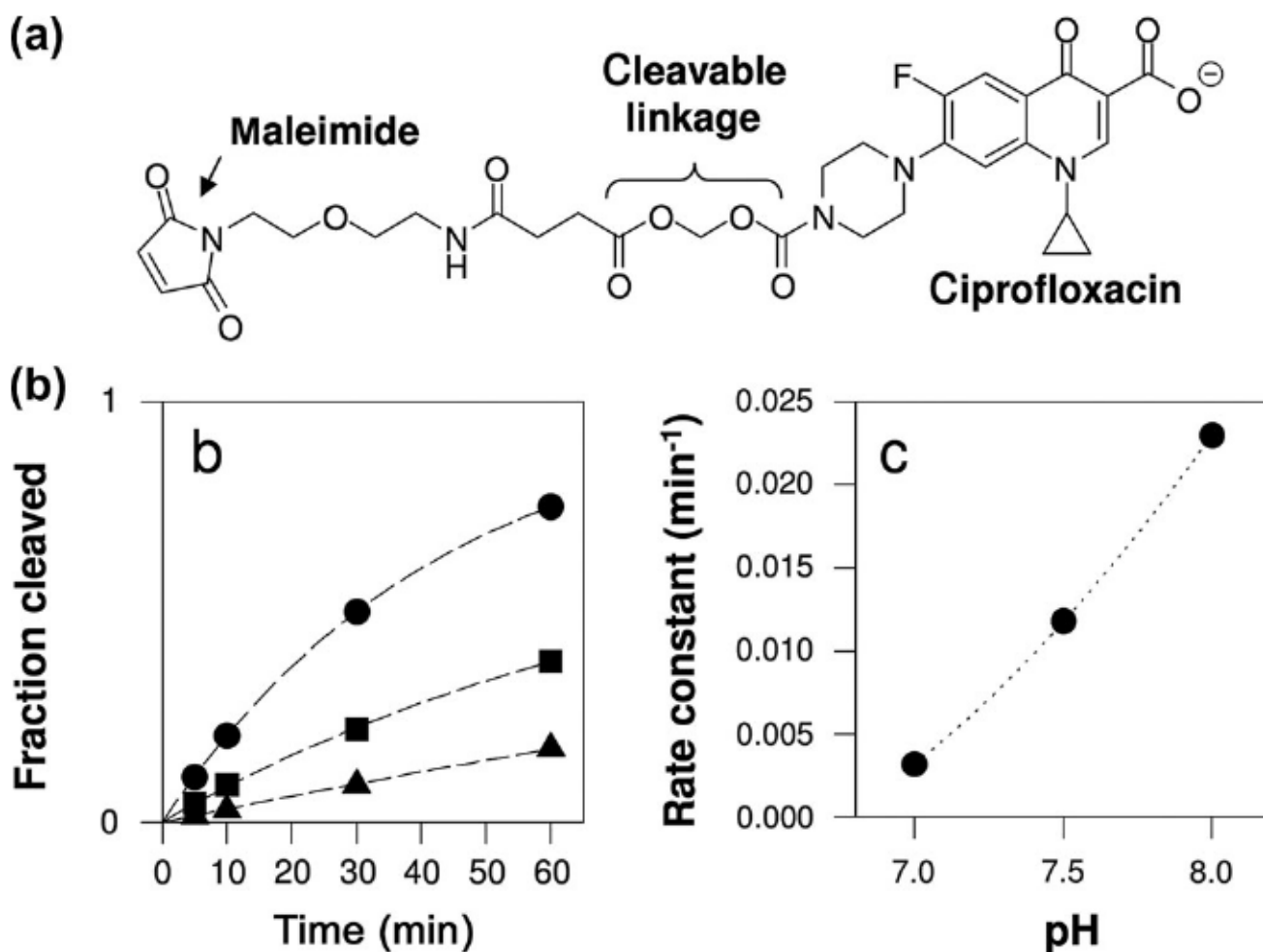


Figure 1. The structure of the prodrug and the dependence of the cleavage rate on pH. (a) Structure. (b) Cleavage kinetics for pH 7 (triangles), pH 7.5 (squares) and pH 8 (circles). Dotted lines are the fits to a first order rate. (c) First order rate constants. The dotted line is a quadratic fit to the data to emphasize the trend.

and used as an estimate of the amount of targeted pro-drug. The concentration of the biotinylated fluorescent analogue in the bulk medium was determined by the absorbance of fluorescein at 480 nm. In order to obtain a sufficient volume for accurate determination of the difference in fluorescein absorbance for targeted and NT biofilms, exposure to the biotinylated fluorescent analogue in the last targeting step was in 25 μ l volumes and solutions from eight wells were combined for each of the three experiments. Biofilm volumes were computed from CSLM images of the biofilms used for the depletion assay and used to estimate prodrug concentration in the biofilm. The mean volumes were estimated from biofilms in three separate wells.

Model of time dependence of ciprofloxacin concentration in the biofilm

Temporal change in drug concentration in the biofilm was approximated using a one dimensional model. The modelling was implemented with the program AQUASIM, 2.0 (Peter Reichert, Computer Systems Sciences Department, EAWAG, Switzerland). Drug concentration is increased in the biofilm as the prodrug is cleaved, and is decreased in the biofilm by diffusion into the bulk medium and by non-specific adsorption of the drug to the biofilm. Diffusion into the bulk medium was modelled by:

$$dC(x;t)/dt = D \, d^2C(x;t)/dx^2 \quad (1)$$

where C is the concentration of ciprofloxacin ($\mu\text{g ml}^{-1}$), x is the distance into the bulk medium from the plane of the biofilm (μm), t is time (min) and D is the diffusion coefficient of ciprofloxacin ($\mu\text{m}^2 \text{min}^{-1}$). Production of ciprofloxacin by prodrug cleavage and loss by non-specific adsorption in the biofilm were modelled by:

$$dC(t)/dt = r_p + r_A \quad (2)$$

The rate of production (r_p) is given by:

$$r_p = k_D(C_T - C_F(t)) \quad (3)$$

coupled to:

$$dC_F(t)/dt = k_D(C_T - C_F(t)) \quad (4)$$

where C_T is the concentration of ciprofloxacin targeted to the biofilm ($\mu\text{g ml}^{-1}$) and k_D (min^{-1}) is the first order rate constant for prodrug cleavage. C_T and k_D were both measured empirically. The time dependent variable, $C_F(t)$, is free ciprofloxacin in the biofilm produced by cleavage of the prodrug (omitting losses due to diffusion or adsorption processes). Expressions for the non-specific adsorption rate (r_A) are described in the Supplementary material.

The Stokes-Einstein relation was used to adjust the diffusion coefficient for viscosity.

$$D = kT/6\pi\eta r \quad (5)$$

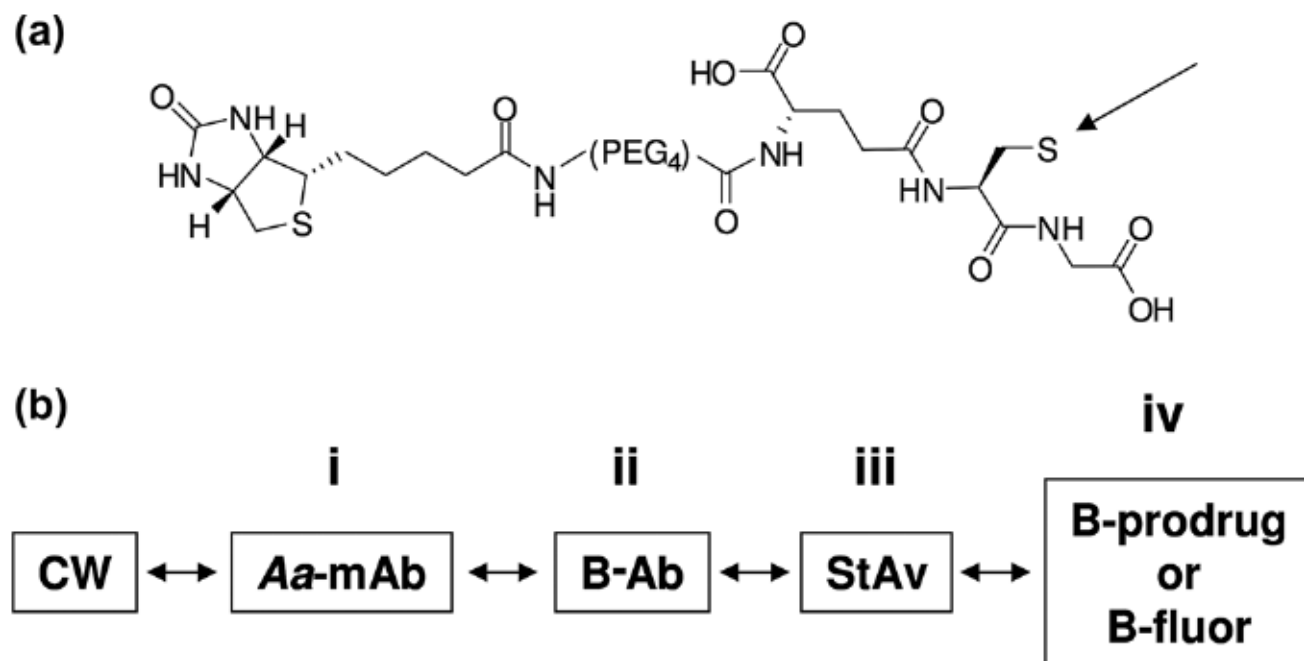


Figure 2. Targeting scheme. (a) Maleimides of the prodrug or fluorescein were linked to a biotinylation reagent via a glutathione coupler with conjugation at the sulfhydryl (arrow). (b) Biotinylated compounds were targeted to the cell wall (CW) of biofilm cells via streptavidin (StAv) in a sequence of reactions. B-Av=biotinylated secondary Ab; B-prodrug=biotinylated prodrug; B-fluor=fluorescent analogue.

The relationship predicts that D is inversely proportional to absolute viscosity (μ) and proportional to temperature (K). The Boltzmann constant (k) and the hydrated radius (r) were not used in the calculations.

The diffusion coefficient of ciprofloxacin (D) was estimated previously to be $4.9 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ ($2.94 \times 10^4 \mu\text{m}^2 \text{ min}^{-1}$) in aqueous medium at 37°C using a published formula (Vrany et al. 1997). The Stokes-Einstein relationship (Equation 5) was used to determine the reduction in D due to the addition of CMC, and also to adjust D to account for the reduced viscosity of water at 37°C (0.705 centipoise (cP); Lemmon et al. 2011) which was overlooked in the previous calculation.

Statistics

A Student's t test was used to calculate p values.

Results

Ciprofloxacin prodrug base catalyzed cleavage

The structure of the ciprofloxacin prodrug is shown in Figure 1a. The methylene-O-diester cleaves at an appreciable rate under mild basic conditions (Figure 1b) with ~ 1 st order kinetics that can be used to obtain rate constants (Figure 1c). Although this bond has been incorporated into prodrugs previously (Hennard et al. 2001; Noel et al. 2011) the pH dependence of the cleavage was not, to the authors' knowledge, reported.

Targeting of Aa biofilm

Ciprofloxacin prodrug was biotinylated *via* an intervening glutathione linker for targeting biofilms of *Aa* (Figure 2a). A stable biotinylated fluorescent analogue was also synthesized in order to enable visualization of targeting by confocal microscopy and to facilitate

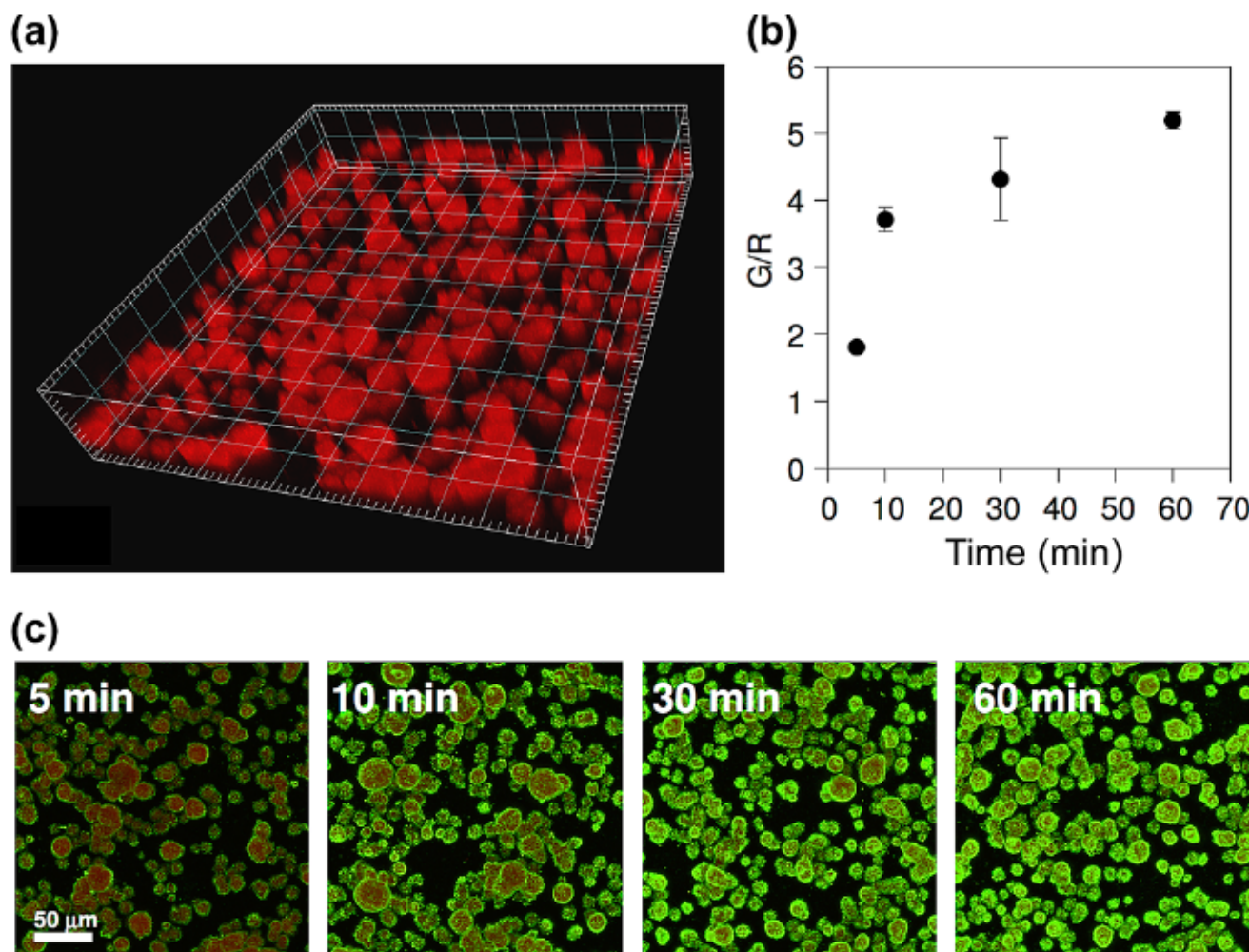


Figure 3. Transport of *Aa*-mAb into biofilm microcolonies. (a) *Aa* biofilm (3D confocal image, increments on the scale bar are $4 \mu\text{m}$ apart). (b) Transport of *Aa*-mAb into the microcolonies of the *Aa* biofilm based on the green to red ratio of fluorescence (G/R), where the *Aa*-mAb is tagged with a green fluorophore and total cells are stained red. Error bars are SD, $n=3$. (c) Representative images used to acquire data in (b).

determination of the amount of ciprofloxacin prodrug delivered to the biofilms *via* the biotin-streptavidin couple. The targeting scheme that is described in the methods section is summarized in Figure 2b.

The size of biomolecules, such as IgG Ab, has been shown to significantly hinder transport into oral biofilms (Thurnheer et al. 2003). Biofilms of *Aa* consist of dense isolated microcolonies (Kaplan et al. 2003; Figure 3a). The kinetics of binding of the primary Ab to biofilms of *Aa* is shown in Figure 3b and c. The images in Figure 3c show that the periphery of the microcolonies become labelled early, followed by labelling of more central locations. This indicates that the rate limiting step in the process of binding to biofilms of *Aa* was the rate of diffusion of the relatively large *Aa*-mAb into the microcolonies, rather than the kinetics of *Aa*-mAb binding to the cell wall epitopes. Fifty minute exposure times were chosen for the primary and secondary Ab as a compromise between optimizing targeting density and minimizing the time required to complete the sequence of labelling reactions.

In order to confirm that the biotinylated ciprofloxacin prodrug and the biotinylated fluorescent analogue were binding *via* the biotin functional group, specifically to streptavidin in the sequence of reactions, a biofilm of *Aa* that had been reacted with the primary and secondary Ab and then streptavidin was reacted with either biotin in solution or the biotinylated ciprofloxacin prodrug. Both biotin and the biotinylated ciprofloxacin blocked

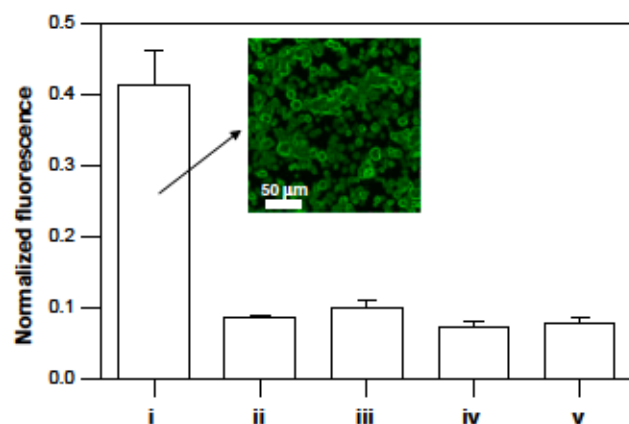


Figure 4. Specific binding of the biotinylated fluorescent analogue and the biotinylated ciprofloxacin prodrug to the biofilm of *Aa* *via* the streptavidin/biotin couple. The fluorescence was normalized to that of fluorescently tagged streptavidin added in step iii of the sequence of targeting reactions (see Figure 2). Abscissa labels: i=targeted biotinylated fluorescent analogue, ii=blocked with biotin before step iv, iii=blocked with biotinylated prodrug before step iv, iv=targeted biotinylated fluorescent analogue with no *Aa*-mAb added in step i, v=biofilm with PBS. Error bars are SD, $n=3$; ii and iii are not significantly different at the 0.05 level. The insert shows a confocal image of the fluorescence from the targeted fluorescent analogue.

binding of the biotinylated fluorescent analogue (Figure 4), indicating that both the biotinylated fluorescent analogue and the biotinylated ciprofloxacin prodrug utilized the biotin-streptavidin specific couple. The insert in Figure 4 shows a confocal image of the fluorescence from the biotinylated fluorescent analogue targeted to the biofilm using the scheme outlined in Figure 2b. Similar to the images in Figure 3c, the periphery of the *Aa* microcolonies are brighter than the interior, suggesting that targeting is incomplete due to limitations of transporting the relatively large Ab.

The biotinylated fluorescent analogue was used to estimate the amount of ciprofloxacin prodrug targeted to biofilms of *Aa* *via* the biotin-streptavidin couple using a depletion assay as described in the methods section (Table 1). The estimated total amount of ciprofloxacin targeted to the biofilm was in the ng cm^{-2} range for biofilms of *Aa* cultured on three separate days. Based on the volumes of the biofilms the mean density was $2.33 \times 10^9 \text{ cells cm}^{-2}$ (Suci & Young 2011).

Ciprofloxacin loading on the *Aa* biofilm could potentially be increased by conjugating the prodrug directly to the *Aa*-mAb. The fluorescence scale in Figure 4 has been normalized to the fluorescence from fluorescently tagged streptavidin targeted to the biofilm in step iii of the scheme (Figure 2b). Based on the known ratio of the labelling of the fluorescently tagged streptavidin, and subtracting the intrinsic background fluorescence associated with the (unlabelled) *Aa* biofilm (Figure 4, v on the abscissa) yields an estimate of the stoichiometry of binding of the fluorescent analogue of ~ 0.6 biotinylated fluorescent analogues per streptavidin. This suggests that ciprofloxacin loading could be increased by a factor of ~ 2 by conjugation of the prodrug directly to the *Aa*-mAb.

Killing of *Aa* biofilm by targeted prodrug

Biofilm killing by the targeted prodrug was assessed in two experiments performed on biofilms cultured on two separate occasions each in triplicate for biofilms cultured in separate wells (Figure 5). For the NT condition biofilms were exposed to PBS without the addition of *Aa*-mAb in the first step. The reduction in cfu produced by dosing with ciprofloxacin in the bulk medium at 0.01 and $0.05 \mu\text{g ml}^{-1}$ for an 18 h time period was measured ($n=3$). The mean reduction in cfu, expressed as a frac-

Table 1. Estimated prodrug targeted to biofilms of *Aa*.

Targeted prodrug per biofilm volume ($\mu\text{g ml}^{-1}$)	Targeted prodrug per biofilm area (ng cm^{-2})
10.6	8.2
14.0	10.9
9.6	8.7
11.4 (2.3)*	9.3 (1.4)

*Mean (standard deviation) of three measurements.

tion, was multiplied by the cfu for the NT condition and is presented in Figure 5 for comparison (broken lines in each graph). The proportionate reduction in cfu after exposure to ciprofloxacin in the bulk medium for 18 h was 0.065 and 0.08 for the 0.01 and 0.05 $\mu\text{g ml}^{-1}$ doses, respectively.

The addition of CMC decreases the diffusion coefficient of ciprofloxacin and increases the level of killing in biofilms of *Aa*. Mouthwash with 0.1% CMC has a viscosity of 5.7 cP at 37 °C which is similar to the viscosity of saliva at low shear rates (Preetha & Banerjee 2005),

(the viscosity of saliva is substantially greater at high shear rates). The level of mortality obtained by the targeted prodrug in medium without the addition of CMC (Figure 5a and c) is close to the level of mortality obtained by dosing with ciprofloxacin in the bulk medium at 0.01 $\mu\text{g ml}^{-1}$ for an 18 h time period. The level of mortality obtained by the targeted prodrug in medium with the addition of CMC (Figure 5b and d) is slightly less than the level of mortality obtained by dosing with ciprofloxacin in the bulk medium at 0.05 $\mu\text{g ml}^{-1}$ for an 18 h time period.

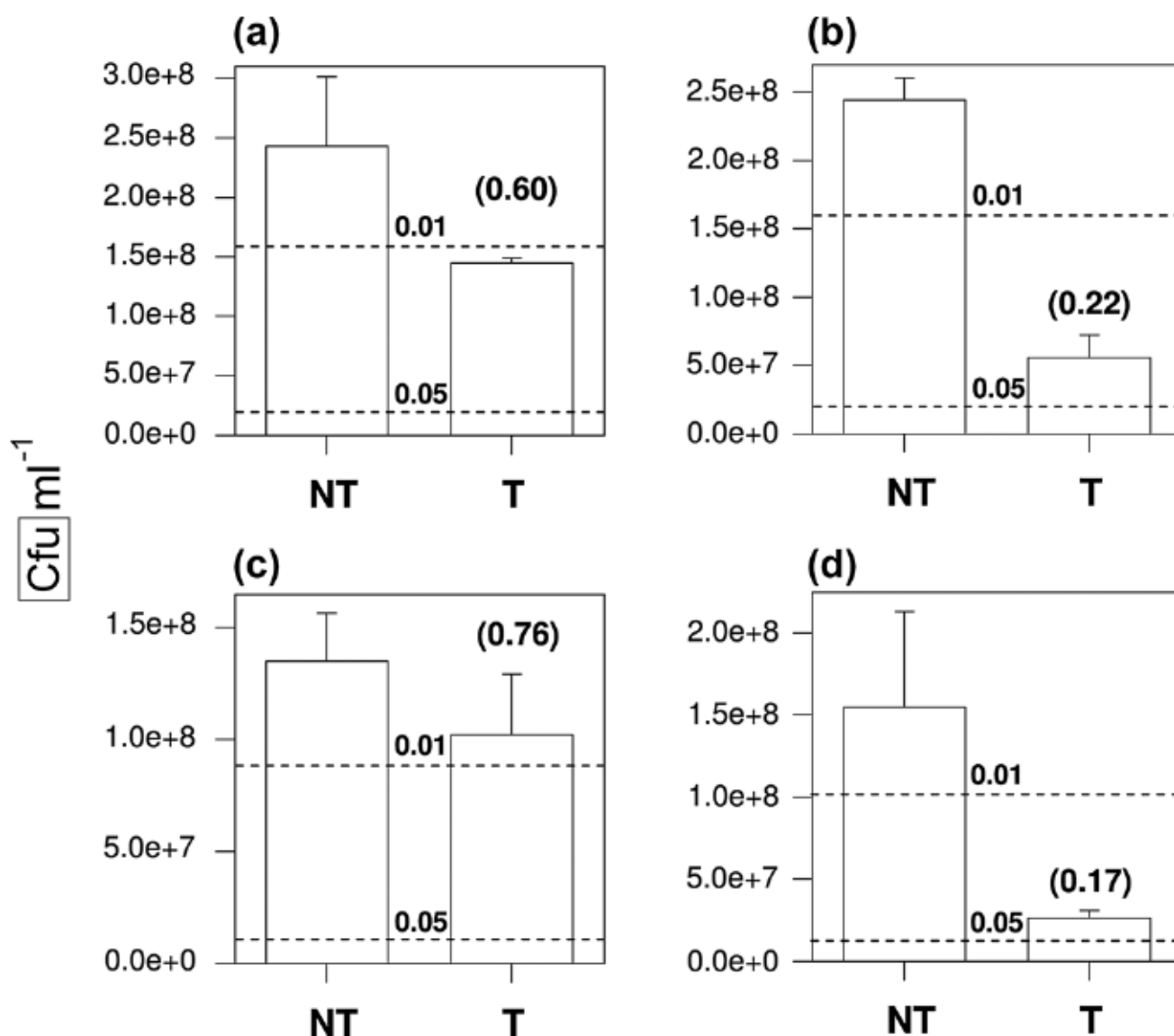


Figure 5. Killing of a biofilm of *Aa* targeted with prodrug. (a and c) Medium without CMC; (b and d) Medium with CMC. T=targeted, NT=non-targeted. For the NT conditions all the steps in the sequence of targeting reactions were performed except for the addition of the *Aa*-mAb (step i). Error bars are SD, $n=3$. For (b) and (d) $p=0.00015$ and 0.0186 , respectively. For (a) and (c) the differences between the T and NT conditions are not significant ($p>0.05$) unless the data are combined. For comparison when data from (a) and (c) are combined $p=0.0420$ for the T and NT conditions. The dotted lines in each graph are the levels of mortality produced by exposure to 0.01 or 0.05 $\mu\text{g ml}^{-1}$ ciprofloxacin in the bulk medium after a period of 18 h. The numbers in parenthesis are the fraction of cfu of T to NT.

Model predictions of the time dependence of ciprofloxacin concentration in the biofilm

The levels of killing obtained with the targeted prodrug are consistent with the dosing levels in the biofilm predicted by the model. The results of the model predictions for the targeted prodrug are presented in Figure 6. The total concentration of the prodrug targeted to the biofilm (C_T) was assumed to be the mean of the values in column 1 of Table 1. The (first order) rate constant for cleavage of the biotinylated prodrug (k_D) was measured as described in the methods (see also Supplemental material). The one-dimensional model approximates the biofilm as a homogeneous slab. Thus, the effect of diffusion within the biofilm colonies or between colonies in the lateral plane of the biofilm was not included. A justification for this simplification is presented in the Supplementary material.

The diffusion coefficient of ciprofloxacin (D) has the greatest effect on the dosing levels, consistent with its effect on the level of killing (Figure 5). Predictions based on the diffusion of ciprofloxacin in medium without and with CMC are presented in Figure 6a and b, respectively. The predicted dosing levels shown in Figure 6 were obtained without the inclusion of non-specific adsorption of ciprofloxacin to the biofilm. The incorporation of non-specific adsorption into the model decreases the predicted level of dosing. Quantitatively, the effect is

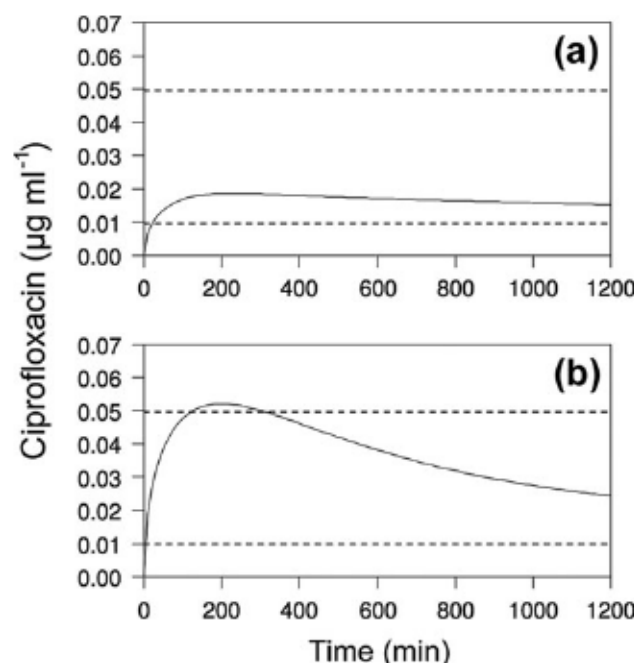


Figure 6. Model predictions of the time course of change of free ciprofloxacin in the biofilm after targeting the prodrug. (a) Ciprofloxacin diffusion based on the viscosity of water; (b) ciprofloxacin diffusion based on the increase in viscosity obtained by adding CMC. The dotted straight lines in both the graphs indicate the 0.01 and 0.05 $\mu\text{g ml}^{-1}$ levels.

relatively small. However, the reduced dosing levels predicted enhance the correspondence between the levels of killing obtained with the targeted prodrug and those expected based on dosing with ciprofloxacin in the bulk medium (see Supplementary material).

Discussion

The results demonstrate the feasibility of killing a pathogenic biofilm using a targeted cleavable prodrug. The targeted prodrug approach precisely localizes drug release to the site of the pathogen infection. Thus, with respect to controlled release strategies, this approach offers the best option for limiting the absolute amount of drug released, while optimizing the selective killing of the pathogen. Selective killing of an invasive pathogenic species would provide benefits for treatment of mucosal infections such as periodontal disease, in which the indigenous microbiota can promote health (Roberts & Darveau 2002).

The dosing level in the biofilm using the targeted prodrug approach results from the balance between the increase in the free drug as it is cleaved and the loss by diffusion into the bulk medium. In the case of ciprofloxacin there is also a significant loss of the free drug as it is adsorbed non-specifically to the biofilm. The model indicates that the rate of cleavage for the glutathione conjugated prodrug at pH 8 is close to optimal to obtain an extended uniform dosing period. The model predicts that at higher cleavage rates there is a dosing spike which is then compensated for by lower doses at later times. At lower rates of cleavage non-specific adsorption of ciprofloxacin to the biofilm delays the onset of dosing, which then never reaches the level attained at higher cleavage rates. Cleavage at pH 8 is suitable for studies in rat models of periodontitis (Graves et al. 2008) since the pH of rat saliva is close to 8 (Smith et al. 2010). The pH of human saliva ranges from about 6.5 to 7.5 (Aframian et al. 2006). The present authors have preliminary evidence that the pH triggered prodrug cleavage rate may be 'tunable' by appropriate design of the chemistry of the functionalities adjacent to the cleavable bond. This is based on the cleavage rate obtained if the prodrug is conjugated to a Cys-Gly dipeptide (37% is cleaved at pH 7 within 120 min compared to 41% of the glutathione coupled prodrug cleaved at pH 8). It is not currently known why the specific group conjugated to the maleimide of the prodrug can influence the rate of base catalyzed cleavage.

The targeting scheme facilitated *in vitro* validation but is not practical for *in vivo* studies. One obvious improvement would be to conjugate the prodrug directly to a targeting Ab. However, the transport of biomolecules the size of Ab into oral biofilms is hindered substantially by their size, as the results presented in

Figure 3 show. Hindered transport into an oral biofilm consortium has been described previously (Thurnheer et al. 2003). Targeting peptides coupled to a killing agent have been used for selective killing of bacterial pathogens (Qiu et al. 2005; Eckert et al. 2006; Kaplan et al. 2011; Liu et al. 2011; Mai et al. 2011). Molecules the size of these peptides penetrate rapidly into biofilms (Thurnheer et al. 2003). The ciprofloxacin prodrug could be readily coupled to peptides synthesized with a terminal Cys residue, thus rendering it into an antimicrobial suitable for *in vivo* studies.

There are two primary mechanisms which could be used to increase the lethality of the prodrug. The simplest approach would be by multiple dosing. The success of this would depend on the dosing schedule and the turn over rate of the cell wall target. The second primary means would be to increase the loading of the targeted prodrug. The data in Figure 4 indicate that with two prodrugs conjugated directly to the Aa-mAb the loading would be increased by a factor of 3.7. The model predicts that this would produce a dosing level of $0.1 \mu\text{g ml}^{-1}$ over a 12 h period. This dose is sufficient to reduce viable Aa biofilm cells by 99% according to measurement with ciprofloxacin in solution (data not shown), while still limiting the drug released to within the ng range. The results presented in Figure 3, and in the insert of Figure 4, suggest that the targeting sites on cells in the interior of the Aa microcolonies are not saturated, and that this is due to hindered transport of the relatively large Ab. If this is the case then loading could be increased substantially by coupling the prodrug to a low molecular weight targeting agent, such as a peptide.

The effects of flow were not tested or modelled. Flow in areas of periodontal infection is expected to be dominated by a variable rate of formation and the potentially complex local transport characteristics of gingival crevice fluid (Griffiths 2003). The model can incorporate the effects of flow if the basic parameters of the flow regime are known. An advantage of the targeted prodrug approach is that, since drug is released in close proximity to the cell wall of targeted cells, transport can be modelled by diffusive transport within a fluid boundary layer (Harrison et al. 2003), while for controlled release devices that are inserted into the oral cavity, modelling of transport to the intended site of action has to include a more detailed description of the flow profile.

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