Combatting implant-associated biofilms through localized drug synthesis

Raoul Walther, Signe Maria Nielsen, Rikke Christiansen, Rikke L. Meyer, Alexander N. Zelikin

PII: S0168-3659(18)30491-7
DOI: doi:10.1016/j.jconrel.2018.08.025
Reference: COREL 9434

To appear in: *Journal of Controlled Release*

Received date: 19 May 2018
Revised date: 10 August 2018
Accepted date: 15 August 2018

Please cite this article as: Raoul Walther, Signe Maria Nielsen, Rikke Christiansen, Rikke L. Meyer, Alexander N. Zelikin , Combatting implant-associated biofilms through localized drug synthesis. Corel (2018), doi:10.1016/j.jconrel.2018.08.025

Combatting implant-associated biofilms through localized drug synthesis

Raoul Walther[1], Signe Maria Nielsen,[2] Rikke Christiansen,[2] Rikke L. Meyer,[2]* Alexander N. Zelikin[1,2]*

[1] Department of Chemistry and [2] Interdisciplinary Nanoscience Centre (iNANO), Aarhus University, Aarhus 8000, Denmark.

Email : rikke.meyer@inano.au.dk, zelikin@chem.au.dk

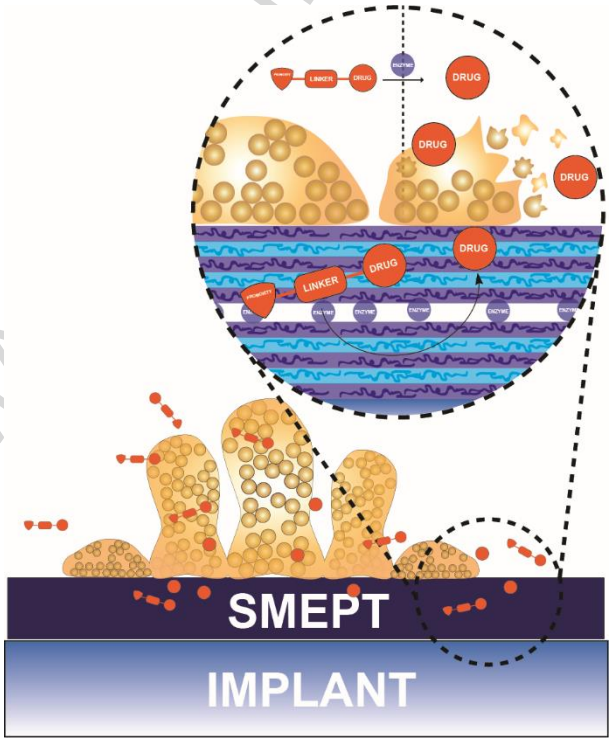
ABSTRACT

Bacterial contamination of implantable biomaterials is a significant socioeconomic and healthcare burden. Indeed, bacterial colonization of implants after surgery has a high rate of incidence whereas concurrent prophylaxis using systemic antibiotics has limited clinical success. In this work, we develop enzyme-prodrug therapy (EPT) to prevent and to treat bacteria at interfaces. Towards the overall goal, novel prodrugs for fluoroquinolone antibiotics were developed on a privileged glucuronide scaffold. Whereas carbamoyl prodrugs were not stable and not suitable for EPT, glucuronides containing self-immolative linker between glucuronic acid masking group and the antibiotic were stable in solution and readily underwent bioconversion in the presence of β-glucuronidase. Surface coatings for model biomaterials were engineered using sequential polymer deposition technique. Resulting coatings afforded fast prodrug conversion and mediated antibacterial measures against planktonic species as evidenced by pronounced zone of bacterial growth inhibition around the biomaterial surface. These biomaterials coupled with the glucuronide prodrugs also effectively combatted bacteria within established biofilms and also successfully prevented bacterial colonization of the surface. To our knowledge, this is the first report of EPT engineered to the surface of biomaterials to mediate antibacterial measures.

Keywords: Biofilm, enzyme-prodrug therapy; glucuronide; prodrug; biomaterial

INTRODUCTION

Implanted biomedical devices present a life-long risk for infections caused by bacteria that colonise the implant and form biofilms - a multi-cellular assembly of bacteria encased in a hydrated extracellular matrix. [1, 2] Within biofilms, bacteria become less susceptible to antibiotics and require markedly higher doses of therapeutic agents. Antibiotic treatment therefore often fails to clear implant-associated infections, as systemic administration cannot reach the bactericidal level without adverse effects to the patient. Revision surgery is often the only viable option. [2] The incidence of post-operative infections after joint replacements (arthroplasties) vary between 1-9% after the primary operation, and are as high as 40% after revision surgery where the implant is removed and replaced.[3] In the US alone, the cost for revision surgery of knee and hip arthroplasties is estimated to reach $ 1.6 billion by 2020. [4] Postoperative prophylaxis following hip and knee arthroplasty using systemically administered antibiotics shows little clinical success[5] highlighting the need for novel methodologies to combat biofilm infections.



It is increasingly recognized that modifications of the implants themselves may be key to the overall success of antibacterial measures. [1, 2] Development of such technologies generally takes one of two routes: i) modification of the implant surface, or ii) local delivery of high levels of antibiotics. The former approach relies on surface coatings that are non-adherent to prevent bacterial adhesion.[6] Such coatings are yet to be implemented in clinic, partly because it is difficult to prevent bacterial attachment while simultaneously ensure biocompatibility and integration of the implant with host tissue. The second route involves localized drug delivery to achieve localized, site-specific release of a drug, resulting in lower systemic exposure.[7] This is routinely achieved through engineering drug loaded coatings, hydrogel meshes or beads. However, pre-loading the implant with antibiotics has several drawbacks. The drug release must occur at a high concentration as slow release of sub-inhibitory concentrations provides a window of opportunity for development of antimicrobial resistance. [8, 9] Furthermore, the type of drug and its dosing are engineered into the implant and cannot be changed by the physician according to the individual needs of the patient and/or progression of infection. In other words, such implants are mass produced and offer no means for personalized treatment. The ideal approach for treating biofilm infections should therefore combine localized drug delivery with the flexibility of choosing when to treat, and what to treat with.

Figure 1. Schematic illustration of a bacterial biofilm on the surface of an implantable biomaterial and an approach to combat the biofilm via a localized drug synthesis using the substrate-mediated enzyme prodrug therapy (SMEPT).

Inspiration for how to accomplish this feat may be taken from anticancer research, as cancer treatment faces some of the same challenges as biofilm infections, namely achieving a drug concentration that is effective against the disease without killing the patient. Cancer treatment is therefore a prime area of biomedicine wherein localized drug delivery is highly prized. [10-13] An advanced approach to

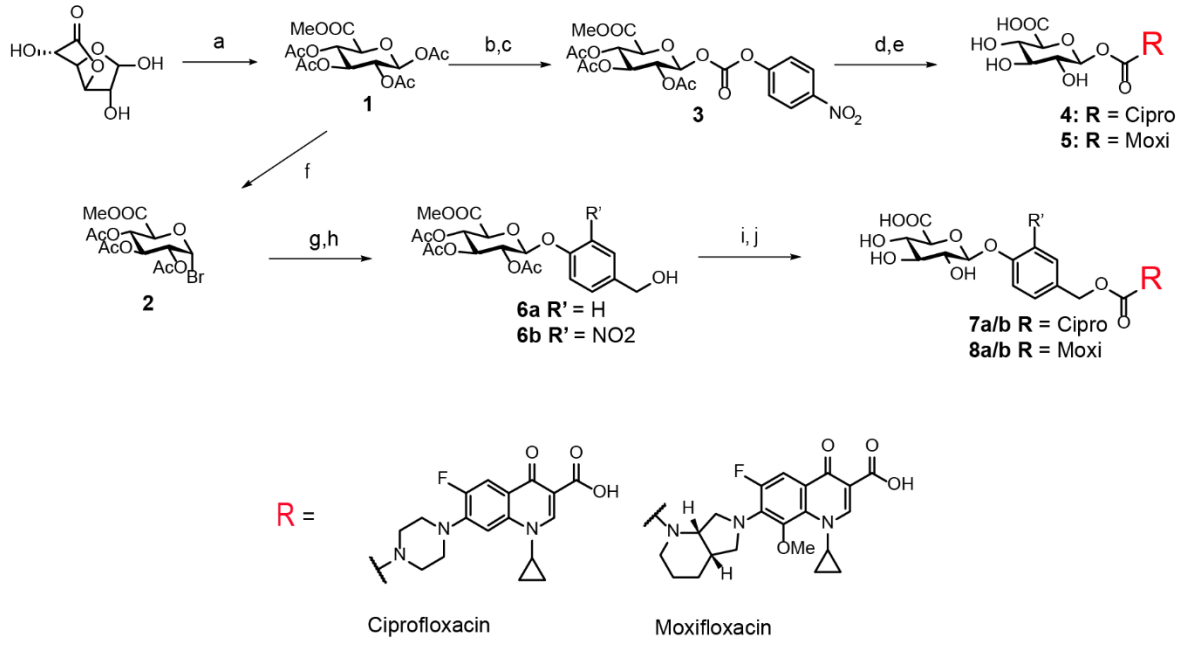
localized drug delivery is that of "enzyme prodrug therapy" (EPT), in which drug synthesis occurs locally at the treatment site [14]. A suite of such techniques has undergone intensive development for anticancer therapy over the past decades, but has gone unnoticed in the context of bacterial biofilm treatment. EPT is a two-pronged approach that relies on i) an enzyme positioned at the site for therapeutic intervention, and ii) a corresponding prodrug that is inactive in the administered form but is activated by the enzyme. Benefits of EPT include the possibility to interactively change the drug dose through optimization of prodrug feed as a step towards personalized treatment; [15] performing combination therapy treatment and on-demand drug synthesis; [16] performing localized synthesis of drugs with limited stability in physiological media [15] for which conventional drug delivery measures are futile or challenging. Success of EPT depends on both, method of enzyme localization [14] and on the choice of the prodrug [17]. Enzyme placement can be achieved surgically, [18] by localized gene expression, [19] or by engineering into the implantable biomaterial [18]. The latter approach is termed "substrate mediated EPT" (SMEPT, Figure 1) and was engineered by us and others using hydrogel biomaterials, [16] [20] electrospun fibers, [21] and surface coatings [15, 22] to achieve the synthesis of drugs with anti-proliferative and anti-inflammatory activity. [18] EPT has even been performed using bacteria to colonize tumor and therein express the enzyme for localized prodrug conversion.[23] However, to our knowledge, there are no literature examples on EPT implemented as a measure to combat bacterial biofilms.

One aspect that has limited the progress of EPT as an antibacterial measure is the lack of appropriate prodrugs. Prodrug design for antibacterial agents has focused on optimization of bioavailability and largely relied on esters as bio-precursors of the drugs. [24] Yet esterases are ubiquitous in the human body and esters are therefore not suitable to site specific prodrug activation. [17] Prodrugs that are activated by bacteria themselves [25-29] are highly important in their own right but are disadvantaged in that the level of bacterial enzymes may not be reliable for sustained conversion of prodrugs. This strategy may also plausibly lead to the development of bacterial resistance to the treatment through the loss of enzyme expression under the evolutionary pressure. In turn, the privileged prodrugs for EPT, namely glucuronides, [17] are not developed for antibacterial agents. Glucuronides are attractive in that these exhibit high aqueous solubility, are typically stable in human plasma, and exhibit a low level of mammalian cell entry.[17] The latter aspect is important in that even glucuronides of potent anticancer agents are essentially non-toxic to mammalian cells. [17] Glucuronides are also human metabolites and as such represent products of metabolism marked for fast renal elimination, that is, have short plasma half-life. [30] The latter may not be advantageous for EPT and rectified through association of prodrugs with endogenous long-circulating macromolecules, e.g. albumin.[31, 32] In this work, we focus on fluoroquinolones as broad-spectrum antibacterial agents and in one aspect of novelty, we developed glucuronide prodrugs of these agents for EPT. We then engineer surface coatings based on multilayered polyelectrolyte coatings, a facile technique of surface modification that has previously been adopted to substrates as diverse as polymers, metals, hydrogels, decellularized vasculature, etc. [33] We investigate enzyme-containing coatings in the context of prodrug conversion as well as ensuing antibacterial effects using planktonic bacteria and biofilms. For the latter, we investigate both prevention and treatment of biofilms. Taken together, successful design of prodrugs performed in this work coupled to an engineering of biomaterials surface establish the first step towards enzyme prodrug therapy as a measure to combat bacterial biofilm.

RESULTS AND DISCUSSION

Enzymatic glucuronidation in the liver affords glucuronide derivatives for a diverse range of functionalities, including aliphatic and aromatic alcohols, amines, and carboxylic groups. We chose to develop prodrugs for fluoroquinolones ciprofloxacin and moxifloxacin (Figure 2). Acyl glucuronides of these drugs are readily available from commercial sources but these are notoriously unstable and exert non-specific, possibly toxic effects via acyl migration, [34] and for these reasons were not considered in this work. Instead, we hypothesized that fluoroquinolones may afford stable N-carbamoyl glucuronides. A carbamate bond is expected to reduce electrophilicity of the carbonyl and therefore decrease the likelihood of hydrolysis, trans-acylation, and acyl migration reactions. With this hypothesis, the corresponding carbamoyl-linked fluoroquinolone glucuronides were synthesized (Figure 2). Specifically, stereoselective incorporation of the carbamate bond was achieved through kinetic control of a nitrophenyl-carbonate glucuronide **3**.[35] Carbonate exchange reaction was carried out in DMF with TEA as base of choice to deliver the cipro- or moxifloxacin derivatives **4** and **5** with excellent stereochemical integrity (as determined by ^{1}H-NMR spectroscopy, $^{3}J_{H1\text{-}H2}$ = 8.0 Hz in both cases). Resulting carbamoyl glucuronides of fluoroquinolones were suitable substrates for β-glucuronidase (β-Glu) and underwent bioconversion into the corresponding antibacterial agents (Figure 3). However, to our surprise, these prodrugs were not stable in physiological buffer (phosphate buffered saline (PBS), pH 7.4, 37°C, 24 h) and quantitatively underwent a rearrangement into non-identified side-products, as evidenced by HPLC (Figure 3). Characterization of these products of rearrangement was beyond the scope of this study and was not pursued. Instead, we focused on the development of alternative glucuronide prodrugs of fluoroquinolones with enhanced stability to be suitable for applications in EPT.

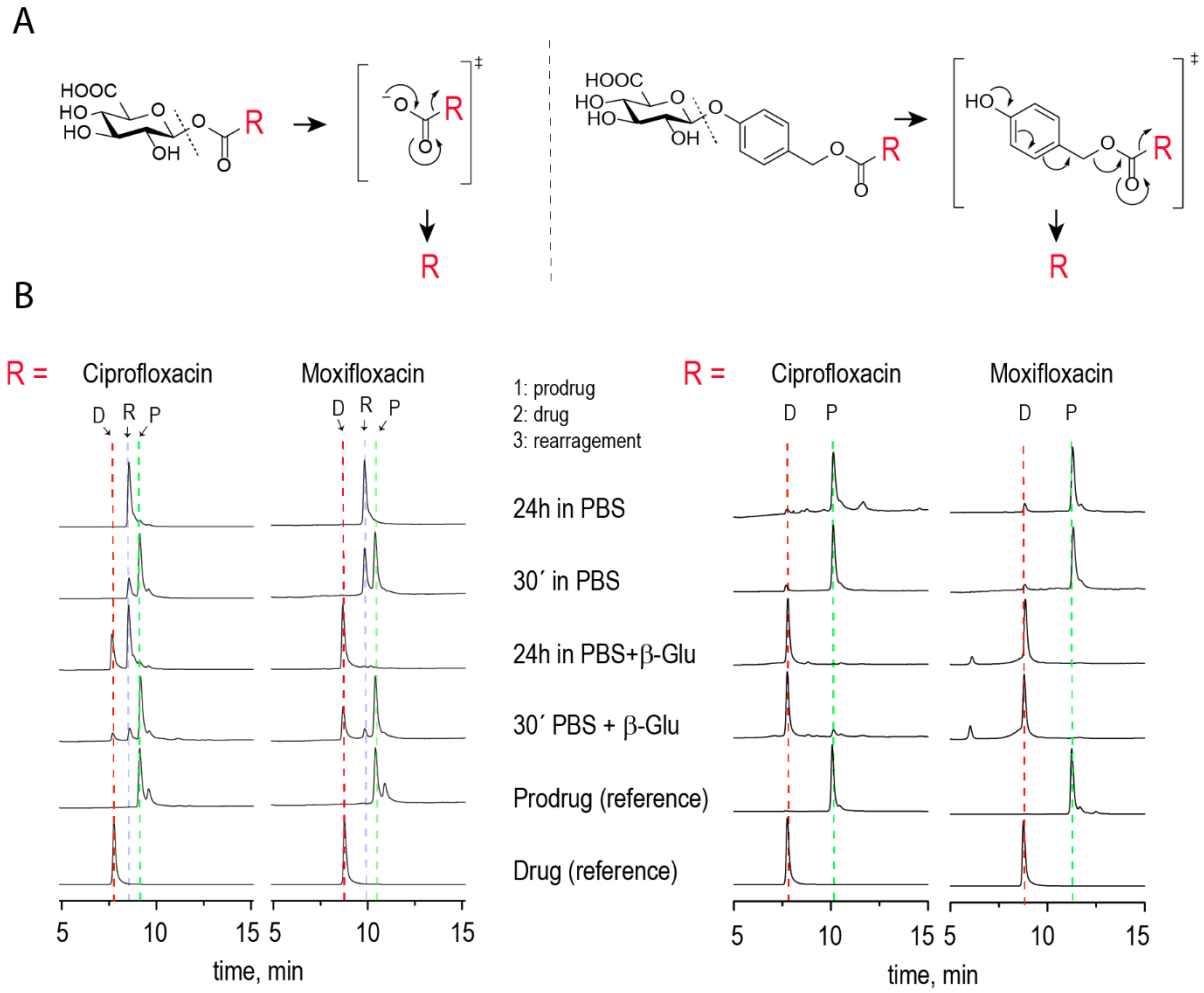
We hypothesized that glucuronide derivatives with the desired stability can be synthesized using a self-immolative linker (SIL) based on namely *p*-hydroxybenzyl alcohol (PHBA). [17, 36] SIL



methodology has undergone significant development over the past decades and is most commonly used to enhance accessibility of the scissile bond to the enzyme. Market validation of this type of SIL can be found in an academically and commercially successful Brentuximab vedotin. [37] Our vision was that this SIL would bridge glucuronic acid and fluoroquinolone using stable linkages between the SIL and enzyme-specific trigger (phenolic glucuronide) and that with the drug.

Figure 2. Schematic illustration of syntheses for glucuronide prodrugs used in this study: (A) carbamoyl prodrugs, (B) glucuronides engineered using self-immolative linker. Reagents and conditions: a) 1) NaOMe/MeOH, r.t.; 2) Ac_2O, pyr, 0ºC-r.t., 2 steps 64%; b) $H_2NNH_2{\cdot}AcOH$, DMF, r.t., 65%; c) (p-$NO_2C_6H_4)_2CO$, TEA, CH_2Cl_2, -10°C, 67%; d) Cipro- or Moxifloxacin, TEA, DMF, r.t., 72% or 46%.; e) DIEA, MeOH/H_2O, 0°C – r.t., 6% or 9%;f) HBr/AcOH, CH_2Cl_2, r.t., 72-92%; g) 4-hydroxybenzaldehyde or 4-hydroxy-2-nitrobenzaldehyde, Ag_2O, 3Å mol sieves, MeCN, r.t., dark, 45% or 98% respectively; h) $NaBH_4$, silica, $CHCl_3$/i-PrOH, 91%; i) 1)p-nitrophenylchloroformate, TEA, CH_2Cl_2, r.t. 75-85%; 2) Ciprofloxacin or Moxifloxacin, TEA, DMF, r.t., 85-90%; j) 1) NaOMe/MeOH, r.t.; 2) 2M NaOH, H_2O, 0^0 C, 2 steps 46-80%.

Synthetic methodology started with D-(+)-Glucurono-6,3-lactone which was treated with NaOMe in anhydrous methanol and subsequently acetylated to yield the protected glucuronide **1** in 64% yield (Figure 2). **1** was treated with HBr in AcOH to deliver the glycosyl bromide **2** in 72-92% yield. Glycosylations of 4-hydroxybenzaldehyde was performed in the presence of Ag_2O and powdered activated 3Å mol sieves and delivered the glycosylated product with complete β-selectivity. Subsequent reduction with $NaBH_4$ in presence of silica gel delivered the corresponding benzyl alcohols **6a** and **6b**. Silica gel turned out to be essential for the reduction in order to avoid hydrolysis of the acetyl protecting groups. The corresponding SIL linkers containing a nitro-group were analogously synthesized, and can be used for further conjugation, such as antibody conjugation.[38, 39] Next steps involved the activation of the benzylic alcohol **6** with nitrophenol chloroformate. Sequential reaction with the fluoroquinolones in presence of a mild base (TEA or Huenig's base) delivered the protected prodrugs **S6a,b** and **S7a,b** chemoselectively and without the protection of the carboxylic acid moiety of the quinolone. Deprotection via Zémplen deacetylation and then ester hydrolysis with 2M NaOH at 0ºC for 10 minutes delivered the glucuronide prodrugs **7a,b** and **8a,b** with acceptable yields. Major side product was always the dehydro-glucuronide prodrugs, which is a common side product in glucuronide synthesis.[30]



Thus obtained glucuronide derivatives of fluoroquinolones proved to combine the desired stability and being substrate to β-glucuronidase, as evidenced by the HPLC investigation, Figure 3 (and Supplementary Figure S2). In physiological buffer and in the absence of the enzyme, prodrugs revealed negligible drug release over 24 h. In contrast, in the presence of β-Glu, prodrugs underwent fast, quantitative conversion into the corresponding fluoroquinolones. Thus, synthetic efforts presented above afforded novel prodrugs for fluoroquinolone antibacterial agents with the properties desired for successful EPT. We note that this synthetic methodology is modular and at the last conjugation step, virtually any drug with a nucleophilic amine or hydroxyl can be installed onto the SIL for β-Glu-triggered drug release. This opens up greater biomedical prospects such as combination therapy whereby the same enzyme achieves concurrent bioconversion of two (or more) prodrugs. [18]

To establish implant-mediated bioconversion of the synthesized prodrugs, we considered multilayered polyelectrolyte coatings as host compartments for enzyme immobilization. Such coatings have undergone development from advent to a well-established methodology with applications in diverse areas of biomedicine. [33] In brief, sequential exposure of a surface to polyelectrolytes of alternating charge leads to immobilization of polymers at the interface in a layer-by-layer fashion. Virtually any polymer (or nano/microscopic colloid, including nanoparticles and proteins) can be deposited onto the surface; interaction forces are not limited to electrostatic association but also include hydrogen bonding, hydrophobic interactions, and covalent bonding. These coatings can be engineered as biodegradable materials for delivery of biological drugs and nucleic acids. Alternatively, surface modification can be designed to be virtually permanent, using non-degradable polymers. In our prior studies, we engineered EPT into multilayered polyelectrolyte coatings to achieve localized synthesis

of anti-proliferative drugs[22] and for delivery of nitric oxide[15], biomedical opportunities highly warranted in the context of cardiovascular stenting. We have also demonstrated that enzymes immobilized within such coatings exhibit negligible escape from the multilayered films and biocatalysis is confined to the surface coatings, being key to the desired site-specific production of therapeutics vis EPT. [15, 22, 40] Herein we propose that such nanometer-thin coatings can be deposited on the surface of an implant to prevent and/or treat bacterial biofilm (Figure 1).

To assemble multilayered coatings, we used poly(styrene sulfonate) and poly(allylamine). This polyelectrolyte pair is among the most well-studied and is known to form "permanent" coatings. [41] [42] [43] To characterize polymer build-up and enzyme immobilization, we used quartz crystal microbalance, Figure 4A. Polymer deposition leads to a change in the resonance frequency of oscillation of the crystal. Indeed, sequential exposure of the crystal to polyelectrolytes of opposite charge led to an expected build-up of polymers at the interface. Exposure of the film to a solution of β-Glu (feed concentration of 20 mg/L) afforded a pronounced change in the ΔF, indicative of protein immobilization. Sauerbrey equation was used to calculate the mass of the immobilized enzyme which was found to be 0.5 mg/m^2. This enzyme coverage is well under the level reported previously for the protein immobilization on PSS/PAH multilayers[44] and can be increased via the choice of enzyme feed concentration during the assembly[15] to enhance the biocatalytic output of the coating.

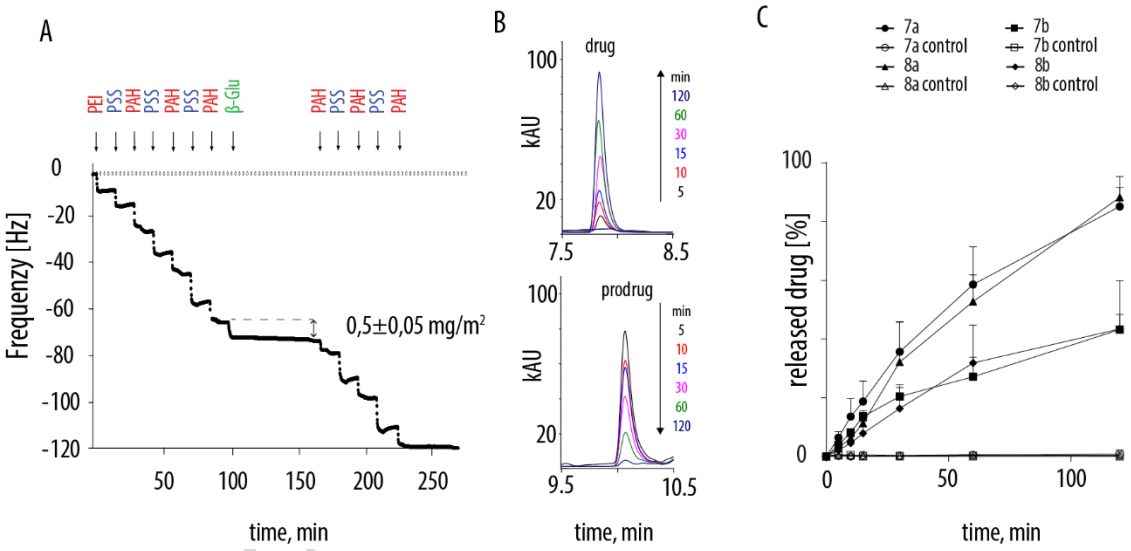


Figure 4. (A) Quartz crystal microbalance data illustrating assembly of the multilayered surface coatings using PSS and PAH with an immobilized β-glucuronidase enzyme; (B) HPLC data illustrating conversion of the glucuronide prodrug of ciprofloxacin 7a by the enzyme immobilized within the multilayered coatings; (C) Quantitative data on the release of antibiotics from the corresponding prodrugs using PSS/PAH multilayered coatings with and without an immobilized enzyme, filled and unfilled symbols, respectively. 7a : ciprofloxacin, PHBA; 7b: ciprofloxacin, nitro-PHBA SIL; 8a : moxifloxacin, PHBA; 8b: moxifloxacin, nitro-PHBA SIL. Data represented as mean ± SD, N = 3. For experimental details, see Experimental section.

To validate enzymatic activity of the assembled coatings, polyelectrolytes and the enzyme were immobilized in the wells of conventional tissue culture polystyrene 96-well plates. First, enzymatic catalysis was quantified for β-Glu in solution and within multilayered coatings using a fluorogenic substrate, resorufin-β-D-glucuronide. These experiments revealed that Michaelis-Menten's constant

K_m and catalysis rate constant k_{cat} were near identical for the immobilized enzyme and that in free solution, as would be expected for a non-covalent immobilization of the enzyme within a biomaterial (Table 1). Bioconversion of the prodrugs for ciprofloxacin and moxifloxacin was quantified in PBS using HPLC, Figure 4B (for the corresponding graph of **8a** see Supporting Figure S3). Over 2 h of observation, the peak corresponding to the prodrug was gradually decreasing whereas that for the product of enzymatic reaction progressively increased. This observation illustrates continuous bioconversion of the prodrug by the immobilized enzyme under SMEPT conditions. Quantitatively, prodrug conversion was near-complete within 2 h of observation, Figure 4C. Glucuronide prodrugs engineered using nitro-containing SIL revealed a significantly lower kinetics of drug release (p<0.01), which agrees well with expectations based on prior reports on the subject.[17, 45] Finally, control experiments using multilayered coating lacking the enzyme revealed negligibly low bioconversion of the prodrugs.

Table 1: Comparison of enzymatic kinetic parameters of enzyme in solution vs SMEPT for resorufin-β-D-glucuronide as model substrate. Data represented as mean ± SD for 3 independent experiments.

	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [$s^{-1}\mu M^{-1}$]
Solution	2.65 ± 0.11	24.7 ± 1.5	0.107 ± 0.002
SMEPT	2.65 ± 0.25	24.8 ± 6.4	0.107 ± 0.018

SMEPT to combat planktonic bacteria.

The antibacterial effect mediated by prodrugs and biocatalytic coatings was first tested against planktonic *Escherichia coli*. Bacterial growth was measured in broth supplemented with prodrugs in microwell plates with biocatalytic coatings on the bottom and sides of the wells. Prodrugs exerted no antibacterial effect in the absence of the biocatalytic coating, while prodrugs incubated with the biocatalytic coating inhibited *E. coli* growth at similar concentrations as the parent antibiotics (Figure 5), resulting in similar minimal inhibitory concentrations (MIC) for prodrugs and pristine antibiotics (Table 2). This was also true for the application of SMEPT against a clinically relevant pathogen responsible for implant-associated infections, namely *Staphylococcus aureus* (Table 2) highlighting clinical relevance of our findings. This result is highly encouraging as it validates the chemical design of the prodrugs. It also illustrates that a biocatalytic coating can convert different drugs from the panel of glucuronides (in this case, ciprofloxacin or moxifloxacin), and that the drug dose can be controlled through the prodrug concentration. The opportunity to control the synthesis rate and drug choice without changing the composition of the coating is unique for biomaterials functionalized with EPT.

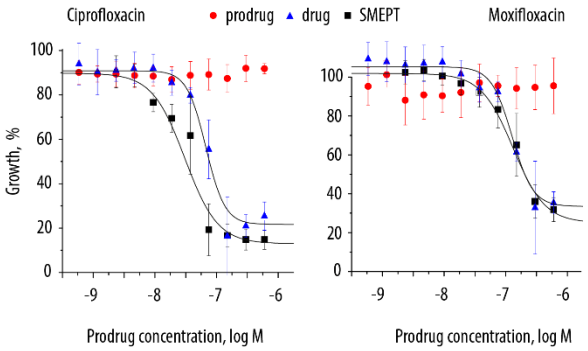


Figure 5: Dose response curves of ciprofloxacin and moxifloxacin (▲)[a] and respective prodrugs in presence (■)[a] and absence (●)[b] of biocatalytic surface coating against *E. coli*. Growth was determined by OD_{600} measurement after 24h incubation and normalized against non-inhibited growth control. Lines are guides to the eye only. [a] data presented as mean ± SD, n = 3; [b] data presented as mean ± SD of quadruplets, n = 2.

Table 2. Minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) for ciprofloxacin, moxifloxacin, and corresponding glucuronide prodrugs (**7a** and **8a**, respectively) in the presence or absence of the biocatalytic coatings. [a] N= 3; [b] not determined; [c] N = 2; [d] N = 6.

		E. coli		*S. aureus*	
Drug		**MIC (nM)**[a]	**MBC (nM)**[a]	**MIC (µM)**[d]	**MBC (µM)**[a]
Ciprofloxacin		150	n.d.[b]	3.8	7.5
7a		>600[c]	>600[c]	>240	>240
7a + SMEPT		75	75-150	7.5	30-60
Moxifloxacin		300	n.d.[b]	<0.5	0.5
8a		>600[c]	>600[c]	>60	>60
8a + SMEPT		300	300-600	0.9	1.9

Inhibition of bacterial growth in the vicinity of a coated surface.

Encouraged by the antibacterial effects on planktonic species, we next performed a "zone of inhibition" assay to show that the diffusion of active drugs from biocatalytic coating was fast enough to eliminate bacterial growth in the vicinity of the surface. Metallic disks (mimicking implantable biomaterials) were coated with the multilayered polyelectrolyte coatings containing β-Glu and placed on top of the agar gel in the wells of 12-well plates. Agar plugs were amended with moxifloxacin glucuronide **8a** resulting in a concentration 10×MIC (9 µM, 3.9 mg/L). *S. aureus* were spread on the agar surface before placing the disc with the coating facing the agar. After 24 h, bacterial growth was imaged using a digital camera, Figure 6. These experiments fully validated antimicrobial effects exerted by model metallic implants equipped with an enzyme for localized synthesis of antibiotics. Specifically, neither the prodrug nor the biocatalytic coating had any antimicrobial activity on their own. But when combined, active antibiotics were synthesized in the coating, resulting in inhibition of bacterial growth around the implant, and in some cases in the entire volume of the well. These data illustrate that the coatings successfully covert the prodrug for ensuing antibacterial activity of the synthesized drug.

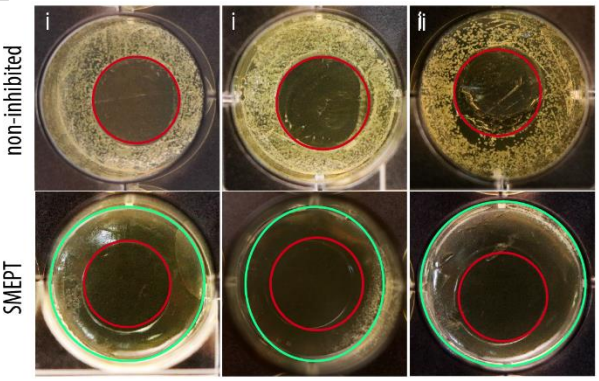


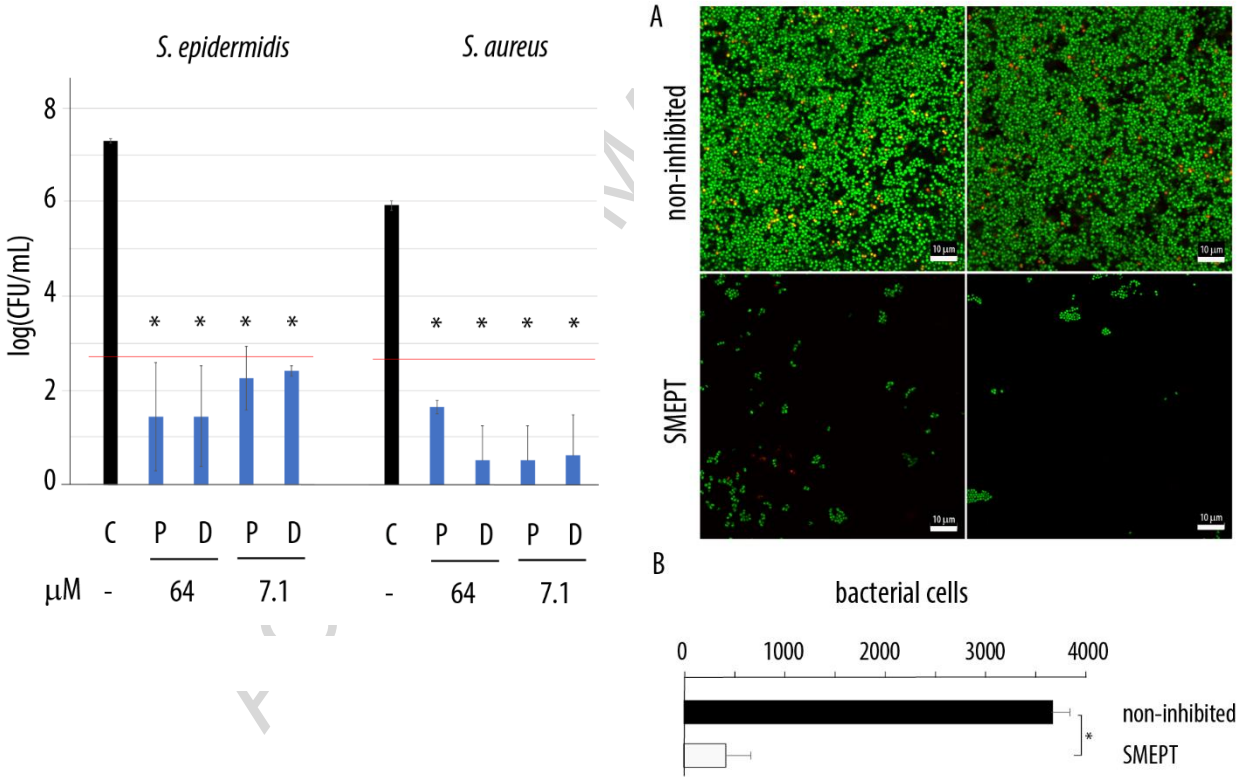
Figure 6. "Zone of inhibition" experiment whereby biocatalytic surface coatings were deposited on metallic disks and used to convert externally added prodrug into moxifloxacin. Bacterial growth was imaged after 24 h of culture and removal of the disk. Metallic disk geometry marked with red circle whereas the zone of inhibition of bacterial growth with a green circle. Non-inhibited proliferation was measured for samples (i) lacking prodrug and (ii) in the presence of the prodrug but for metal disks without biocatalytic coating.

Inhibition of biofilm growth

Next, we aimed to test SMEPT as a platform to combat bacterial biofilms. In these experiments, we used glucuronide prodrugs for moxifloxacin and biofilm forming bacteria *S. epidermidis* and *S. aureus*. Biocatalytic surface coatings were deposited on the peg lids that were immersed into bacterial culture in 96-well plates to allow for the biofilm formation. Subsequently, established biofilms were exposed to a solution of moxifloxacin drug or glucuronide for 24 h before quantification of viable bacteria, Figure 7. This experiment revealed that SMEPT resulted in an antibacterial effect closely matching that of the parent drug provided in solution, and this was true for both *S. epidermidis* and *S. aureus* species.

Independently, we tested SMEPT for prevention of biofilm formation in which case biocatalytic surfaces were inoculated with a bacterial culture in the presence of the prodrug, and prodrug was subsequently present during the 24 h incubation before biofilms were visualized. These experiments were conducted under flow conditions in microfluidic chambers, and biofilms were imaged by confocal laser scanning microscopy, Figure 8. We observed that biocatalytic conversion of prodrugs performed by the surface coating was an effective way to prevent bacterial colonization.

Figure 7. SMEPT to combat established bacterial biofilm. Quantification of live bacteria (in colony forming units) for *S. epidermidis* and *S. aureus* biofilms established on the surface of peg lids with a deposited biocatalytic coating for localized prodrug conversion and subsequently exposed to moxifloxacin drug (D) or prodrug (P).



Taken together, our work presents the design and development of enzyme-prodrug therapy associated with implantable biomaterials towards prevention and treatment of bacterial colonization. Key to the overall success was chemical design of prodrugs that are both stable at physiological conditions and undergo enzymatic conversion into corresponding antimicrobial agents (ciprofloxacin, moxifloxacin). Model implants equipped with tools of EPT successfully exerted antimicrobial effects on planktonic bacteria and were active to both, prevent and treat bacterial biofilms formed by *S. aureus* and *S. epidermidis*. For translational studies, the developed glucuronide prodrugs are attractive in that prodrugs of this type are nature-inspired, typically have an excellent safety profile, and have been used

in diverse EPT settings. Compared to the conventional coatings with preloaded drugs, EPT enables full flexibility to choose appropriate drugs, alternate between drugs, and administer the treatment continuously or intermittently. Such flexibility is particularly important to achieve the highest efficacy in treatment of biofilm. Future work on SMEPT as a platform to prevent and/or treat implant associated bacterial infections will focus on *in vivo* validation of this technology, which is the subject of ongoing research.

Figure 8. SMEPT to prevent bacterial colonization. Representative microscopy images (A) and corresponding bacterial cell count (B) for the growth of the bacterial biofilm for *S. aureus* in microfluidic chambers in the presence or absence of moxifloxacin prodrug at a concentration of 10 times MIC (3.9 g/L). A Wilcoxon Signed Rank test confirmed a statistically significant decrease in bacterial cells following prodrug treatment (P=0.0015). Scale bars in panel A are 10 μm.

Acknowledgements

ANZ acknowledges funding from the European Research Council Consolidator Grant (ERC-2013-CoG 617336 BTVI). The authors wish to acknowledge Camilla B. Andersen (Aarhus University) for assistance in NMR experiments.

Experimental section

Detailed experimental procedures for all syntheses and compound characterization: see Supporting information.

Analytical HPLC was performed on a Shimadzu LC-2010A HT equipped with a Ascentis® Express Peptide ES-C18 column with 2.7 µm particles, a length of 150 mm and an internal diameter of 3.0 mm from Supelco Analytical. HPLC mobile phase A was ultrapure H_2O supplemented with 0.1 % TFA (v/v) and mobile phase B acetonitrile (MeCN) supplemented with 0.1 % TFA (v/v). Ultrapure water (MQ) was obtained from Milli Q direct 8 system (Millipore). Polymers used for SMEPT coatings were poly(ethyleneimine) (PEI 25 kDa, branched), poly(sodium-4-styrene sulfonate) (PSS, 70 kDa), poly(allylamine hydrochloride) (PAH, 17 kDa).

Enzymatic prodrug hydrolysis

Hydrolysis of the prodrugs was carried out by incubating the prodrugs at a final concentration of (20 µg/mL) with e.coli *β*-glucuronidase (Sigma, G 7646) (1.0 µg/mL) in 10 mM PBS buffer solution pH = 7.4. Aliquots were taken out at given time points (30 min, 120 min, 1 d), enzyme was precipitated in cold MeOH (2 mL), and the samples were centrifuged (1400 rcf, 4°C, 5 min). The supernatant was transferred, the solvent was removed *in vacuo*, and the sample dissolved in 50 µl H_2O/MeCN (95/05), and analyzed via HPLC. **Method A:** Elution was performed starting with solvent B 5% to B 100% over 15 min at T = 40°C at a flow rate of 0.4 mL/min. Detection was performed by UV detector (254 nm and 280 nm). Stability tests were carried out according to the hydrolysis protocol, without the addition of enzyme.

Quartz crystal microbalance

For the Quartz crystal microbalance (QCM) experiments, a QSense E4 (Biolin Scientific) was used. AT-cut SiO_2 crystals with fundamental resonance frequency of 5 MHz were stored in 2 % sodium dodecyl sulfate (SDS) solution overnight. Prior to use, the crystals were rinsed with MQ-water, blow dried with N_2 and were UV-sterilized for 30 minutes. The crystals were mounted in the chambers of the QCM and the 3rd overtone of the resonant frequency was used for frequency-monitoring. The crystals were coated with polyelectrolytes at a concentration of 0.1 g L^{-1} in HEPES buffer at pH 7.4 and containing 150 mM NaCl. The final layer composition was PEI-$(PSS/PAH)_3$-β-glucuronidase-$(PAH/PSS)_{2.5}$ The concentration of the enzyme in the feed solution was 20 mg L^{-1}. Each layer was allowed to adsorb for 5 minutes, except from the enzyme, which was allowed to adsorb for 1 hour. After each deposition step, the crystal was rinsed three times with HEPES buffer. The next deposition step was initiated, when the frequency had been stable for 5 minutes after the last washing step.

SMEPT prodrug hydrolysis

Sequential polymer deposition was performed in standard tissue culture polystyrene 96-well plates. All polymers were dissolved in HEPES with 150 mM NaCl buffer pH = 7.4 at a final concentration of 0.1 g/L. The LbL multilayers PEI-$(PSS\text{-}PAH)_3$-β-Glu-$(PSS\text{-}PAH)_{2.5}$ were assembled by adding a starting layer of PEI (100 µL per well) and then alternating layers of PSS and PAH (100 µL per well). As a control of prodrug stability in presence of LbL multilayers, multilayers lacking the enzyme were assembled with the general structure PEI-$(PSS\text{-}PAH)_5$. Each layer was allowed to adsorb for 5 min followed by a two time washing step with HEPES buffer (100 µl per well). The enzyme *β*-Glu was adsorbed from a 20 mg/L solution in HEPES (100 µL per well) for 1 h at r.t. For evaluation of enzymatic activity of the biocatalytic coatings, after completion of the buildup, the multilayers were incubated in PBS (180 uL per well) for 30 min, washed twice with PBS, and subsequently incubated with the prodrugs at a final concentration of 200 µM in 100µL PBS at 37°C for 2 h. 10µL aliquots were taken out at the given time points, diluted in 90 µL MQ/MeCN (95/05 v/v%) and analyzed with HPLC **Method A**.

Determination of enzymatic kinetic parameters

Resorufin β-D-glucuronide was incubated at varying substrate concentrations at a final enzyme concentration of 1μg/mL or in presence of SMEPT coatings (see previous section for assembly conditions) in 100 μL PBS (10 mM, pH = 7.4) in Nunc FluoroNunc 96-well plates-black. Fluorescence was measured over 30 min at 2 min intervals (at $\lambda_{ex}/\lambda_{em}$ 570 nm/ 585nm). The initial rate was plotted against substrate concentration and analyzed with GraphPad Prism 7. Fluorescence was converted to concentration based on a standard curve (Supporting Figure S4). Enzyme concentration for the immobilized enzyme was estimated based on the surface area (148.9 mm^2) of a well in a 96-well plate and the amount of enzyme per area (0.5 mg m^{-2}) calculated from the QCM measurements.

Bacteria Cultivation

Escherichia coli K12 was obtained from DSMZ culture collection and grown in TSB medium (16g/L). *Staphylococcus aureus* DSM20231, *E. coli* K12 DSM498, and *S. epidermidis* 1457 were cultured on tryptic soy broth (TSB) broth (Sigma-Aldrich, St. Louis, MO, USA) agar plates for 24 h at 37°C. Liquid cultures for flow experiments were prepared in Erlenmeyer flasks containing 10 mL TSB broth by inoculating the media with a single bacterial colony and incubating overnight at 37°C with shaking at 180 rpm.

MIC/MBC determination

A culture of a single colony was set up in 10 mL TSB medium and incubated overnight at 37^0 C. A dilution series of the desired antibiotic in triplicates in standard tissue 96-well plates was performed. The bacteria was seeded to the standard tissue 96-well plates at a final concentration of 5×10^5 cells/mL (OD_{600} ~ 0.04-0.05 for *E.coli*) and incubated at 37^0 C for 18±2 h. Then the OD_{600} was measured and 10ul of wells with no apparent growth were spotted onto agar plates containing TSB and incubated for 24 h. Wells with no apparent growth determined by OD was the MIC. Wells with no visible growth on the agar plates determined the MBC.

CFU count

To test if the prodrug is effective in treating bacterial biofilms, a colony forming units (CFU) assay is employed. Two concentrations of moxifloxacin and two corresponding concentrations of the glucuronide prodrug of moxifloxacin **8a** were tested. Biofilms of *S. aureus* DSM20231 and *S. epidermidis* 1457 were grown on peg lids coated with PSS/PAH/β-glucuronidase following the same procedure as for coating of the 96-well plates described above. 160 μL adjusted bacterial culture with OD600 = 0.5 was added to the wells of a 96-well plate. The peg lid was inserted in the plate and the plate was incubated at 37 °C for 30 minutes. The peg lid was transferred to a new plate with 160 μL fresh TSB medium in each well and the plate was incubated at 37 °C for 24 h. A treatment plate with moxifloxacin and prodrug was prepared with drug concentrations of 7.1 μM and 64 μM. For moxifloxacin, this corresponds to 2.85 mgL^{-1} and 25.7 mgL^{-1}, respectively, and to 5.18 mgL^{-1} and 46.8 mgL^{-1} for the prodrug, respectively. The peg lid was inserted in the treatment plate and the plate was incubated at 37 °C for 24 h. Two washing plates with 180 μL PBS in each well were prepared, and excess antibiotics were rinsed off the peg lid by placing the lid in each washing plate for one minute at a time. The peg lid was transferred to a new plate containing 180 μL PBS in all wells. The plate was sonicated in a sonication bath for 10 minutes to rattle of the cells in the biofilm. A 1:10 series dilution of the wells from the sonicated plate was made, resulting in the final well in the series being diluted by a factor of 10^8. 10 μL from each well in the sonication plate and from the dilution plate were placed on TSB agar plates and incubated at 37 °C for 24 h. Three technical replicates were included by taking 10 μL from the same well three times. Next day, the number of bacterial colonies in the plates were counted. The experiment was repeated twice for each strain, so that the number of biological replicates is three.

Zone of inhibition experiment

Flow cells and metal disks were coated according to general procedure in 2.3. In short, alternate layers of polyelectrolytes were adsorbed onto the metal disk or in the wells of the flow cell for five minutes and subsequently rinsed with MilliQ water. After buildup of three PSS/PAH bilayers, β-glucuronidase was allowed to adsorb for 1 h at 37°C. The polyelectrolyte layer was subsequently completed with 2.5 bilayers of PAH/PSS, giving the multilayer PEI-$(PSS\text{-}PAH)_3$-β-glucuronidase-$(PAH/PSS)_{2.5}$. Agar plugs were prepared by casting 1 mL TSB agar into each well in 12-well cell culture plates (TC Plate 6 Well, Sarstedt, Nümbrecht, Germany). Prodrug of moxifloxacin **8a** was dissolved in TSB broth, diluted and added to the agar plugs at a final concentration of 9 μM (3.9 mg/L, corresponding to 10 X MIC), and allowed to diffuse for 1 h into the agar. A single colony of *S. aureus* DSM20231 was added to 4 mL saline (0.85 %), vortexed thoroughly and adjusted to OD_{600} = 0.1. Bacteria were spread on the agar surface by dipping a sterile cotton swab into the bacterial solution and streaking evenly onto the agar. After 10 min incubaction at 37°C, a metal disk coated with multilayered polyelectrolyte coatings containing β-Glu was placed in the center of each well and incubated for 24 h at 37°C. Subsequently, bacterial growth was imaged and evaluated using a digital camera.

Biofilm prevention under flow

A six-channel flow-cell was cast in Poly(DiMethylSiloxane) (PDMS) by a mix of SYLGARD 184 PDMS Base and SYLGARD 184 Curing Agent 10:1 and poured onto a pre-made silicon waver negative template (196 to 224 micron). After heating to 110°C for 15 min, the flow cells were cut from the template. Holes for tube connectors were made with a biopsy puncher (0.5 mm). The flow cell was attached to a high precision microscope cover glass using a corona treater and heated to 110°C for 30 min to cure the bonding. The microfluidics device was assembled and the channels were coated with the layer-by-layer technique as described previously. An overnight-culture of *S. aureus* was diluted to OD_{600} = 0.1 in one mL TSB, and moxifloxacin prodrug **8a** was added to a final concentration of 3.9 mg/mL. The bacterial-prodrug mixture was injected into the flow chamber and incubated at 37°C for two hours. The microfluidics device was connected to a syringe pump and a continuous flow of sterile TSB broth containing moxifloxacin prodrug **8a** (3.9 mg/mL) was applied at a flow rate of 1.5 μL/min. and incubated for 24 h at 37°C. The biofilm was rinsed in PBS for 30 min. and stained with LIVE/DEAD® stain (BacLight L7007 bacterial viability kit for microscopy, Invitrogen, Thermo Fisher, Waltham, MA USA) according to the manufacturer's protocol and imaged by confocal laser scanning microscopy (CLSM) (Zeiss LSM 700, Carl Zeiss AG, Oberkochen, Germany). The experiment was carried out in triplicates (three biological replicates with 3-5 technical replicates from each) and the number of bacterial cells in each image was assessed with ImageJ.

Data Analysis

Numerical data is represented as mean ± SD and calculated based on the number of independent experiments stated. All data was analyzed with a combination of Microsoft Excel 2010, OriginPro 8, or GraphPad Prism 7. Statistical analysis of the biofilm prevention experiment was performed with STATA (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP.) using a Wilcoxon Signed Rank test.

References

[1] H.-C. Flemming, J. Wingender, U. Szewzyk, P. Steinberg, S.A. Rice, S. Kjelleberg, Biofilms: an emergent form of bacterial life, Nat. Rev. Microbiol., 14 (2016) 563.

[2] H. Koo, R.N. Allan, R.P. Howlin, P. Stoodley, L. Hall-Stoodley, Targeting microbial biofilms: current and prospective therapeutic strategies, Nat. Rev. Microbiol., 15 (2017) 740.

[3] S. Corvec, M.E. Portillo, B.M. Pasticci, O. Borens, A. Trampuz, Epidemiology and new developments in the diagnosis of prosthetic joint infection, Int. J. Artif. Organs, 35 (2012) 923-934.

[4] S.M. Kurtz, E. Lau, J. Schmier, K.L. Ong, K. Zhao, J. Parvizi, Infection burden for hip and knee arthroplasty in the United States, J Arthroplasty, 23 (2008) 984-991.

[5] P. Thornley, N. Evaniew, M. Riediger, M. Winemaker, M. Bhandari, M. Ghert, Postoperative antibiotic prophylaxis in total hip and knee arthroplasty: a systematic review and meta-analysis of randomized controlled trials, CMAJ open, 3 (2015) E338-343.

[6] B. Indrani, P.R. C., K.R. S., Antifouling Coatings: Recent Developments in the Design of Surfaces That Prevent Fouling by Proteins, Bacteria, and Marine Organisms, Adv. Mater., 23 (2011) 690-718.

[7] N.J. Hickok, I.M. Shapiro, Immobilized antibiotics to prevent orthopaedic implant infections, Adv. Drug Deliv. Rev., 64 (2012) 1165-1176.

[8] D.I. Andersson, D. Hughes, Microbiological effects of sublethal levels of antibiotics, Nat. Rev. Microbiol., 12 (2014) 465-478.

[9] E. Wistrand-Yuen, M. Knopp, K. Hjort, S. Koskiniemi, O.G. Berg, D.I. Andersson, Evolution of high-level resistance during low-level antibiotic exposure, Nat. Commun., 9 (2018) 1599.

[10] J.B. Wolinsky, Y.L. Colson, M.W. Grinstaff, Local drug delivery strategies for cancer treatment: gels, nanoparticles, polymeric films, rods, and wafers, J. Control. Release, 159 (2012) 14-26.

[11] M.W. Tibbitt, J.E. Dahlman, R. Langer, Emerging Frontiers in Drug Delivery, J. Am. Chem. Soc., 138 (2016) 704-717.

[12] T. Htay, M.W. Liu, Drug-eluting stent: a review and update, Vasc. Health Risk Manag., 1 (2005) 263-276.

[13] R. Wessely, New drug-eluting stent concepts, Nat. Rev. Cardiol., 7 (2010) 194.

[14] B. Städler, A.N. Zelikin, Enzyme prodrug therapies and therapeutic enzymes, Adv. Drug Deliv. Rev., 118 (2017) 1.

[15] A.K. Winther, B. Fejerskov, M. ter Meer, N.B.S. Jensen, R. Dillion, J.E. Schaffer, R. Chandrawati, M.M. Stevens, L.J. Schultze Kool, U. Simonsen, A.N. Zelikin, Enzyme Prodrug Therapy Achieves Site-Specific, Personalized Physiological Responses to the Locally Produced Nitric Oxide, ACS Appl. Mater. Interfaces, 10 (2018) 10741-10751.

[16] A.C. Mendes, A.N. Zelikin, Enzyme Prodrug Therapy Engineered into Biomaterials, Adv. Funct. Mater., 24 (2014) 5202-5210.

[17] R. Walther, J. Rautio, A.N. Zelikin, Prodrugs in medicinal chemistry and enzyme prodrug therapies, Adv. Drug Deliv. Rev., 118 (2017) 65-77.

[18] B. Fejerskov, M.T. Jarlstad Olesen, A.N. Zelikin, Substrate mediated enzyme prodrug therapy, Adv. Drug Del. Rev., 118 (2017) 24-34.

[19] J. Zhang, V. Kale, M. Chen, Gene-directed enzyme prodrug therapy, AAPS J., 17 (2015) 102-110.

[20] M.T.J. Olesen, A.K. Winther, B. Fejerskov, F. Dagnaes-Hansen, U. Simonsen, A.N. Zelikin, Bi-Enzymatic Embolization Beads for Two-Armed Enzyme-Prodrug Therapy, Adv. Therap., 2 (2018) 1800023.

[21] R. Chandrawati, M.T.J. Olesen, T.C.C. Marini, G. Bisra, A.G. Guex, M.G. de Oliveira, A.N. Zelikin, M.M. Stevens, Enzyme Prodrug Therapy Engineered into Electrospun Fibers with Embedded Liposomes for Controlled, Localized Synthesis of Therapeutics, Adv. Healthc. Mater., 6 (2017) 1700385.

[22] B. Fejerskov, N.B.S. Jensen, B.M. Teo, B. Städler, A.N. Zelikin, Biocatalytic Polymer Coatings: On-Demand Drug Synthesis and Localized Therapeutic Effect under Dynamic Cell Culture Conditions, Small, 10 (2014) 1314-1324.

[23] P. Lehouritis, G. Hogan, M. Tangney, Designer bacteria as intratumoural enzyme biofactories, Adv. Drug Deliv. Rev., 118 (2017) 8-23.

[24] J. Rautio, H. Kumpulainen, T. Heimbach, R. Oliyai, D. Oh, T. Jarvinen, J. Savolainen, Prodrugs: design and clinical applications, Nat. Rev. Drug Discov., 7 (2008) 255-270.

[25] R. Liu, P.A. Miller, S.B. Vakulenko, N.K. Stewart, W.C. Boggess, M.J. Miller, A Synthetic Dual Drug Sideromycin Induces Gram-Negative Bacteria To Commit Suicide with a Gram-Positive Antibiotic, J. Med. Chem., 61 (2018) 3845-3854..

[26] G. Cado, R. Aslam, L. Séon, T. Garnier, R. Fabre, A. Parat, A. Chassepot, J. Voegel, C, B. Senger, F. Schneider, Y. Frère, L. Jierry, P. Schaaf, H. Kerdjoudj, Metz, M. Boutigue, H, F. Boulmedais, Defensive Biomaterial Coating Against Bacteria and Yeasts: Polysaccharide Multilayer Film with Embedded Antimicrobial Peptide, Adv. Funct. Mater., 23 (2013) 4801-4809.

[27] M. Cloutier, D. Mantovani, F. Rosei, Antibacterial Coatings: Challenges, Perspectives, and Opportunities, Trends Biotechnol., 33 (2015) 637-652.

[28] V.V. Komnatnyy, W.-C. Chiang, T. Tolker-Nielsen, M. Givskov, T.E. Nielsen, Bacteria-Triggered Release of Antimicrobial Agents, Angew. Chem. Int. Ed., 53 (2014) 439-441.

[29] M. Tanihara, Y. Suzuki, Y. Nishimura, K. Suzuki, Y. Kakimaru, Y. Fukunishi, A novel microbial infection- responsive drug release system, J. Pharm. Sci., 88 (1999) 510-514.

[30] A.V. Stachulski, X. Meng, Glucuronides from metabolites to medicines: a survey of the in vivo generation, chemical synthesis and properties of glucuronides, Nat. Prod. Rep., 30 (2013) 806-848.

[31] B. Renoux, F. Raes, T. Legigan, E. Peraudeau, B. Eddhif, P. Poinot, I. Tranoy-Opalinski, J. Alsarraf, O. Koniev, S. Kolodych, S. Lerondel, A. Le Pape, J. Clarhaut, S. Papot, Targeting the tumour microenvironment with an enzyme-responsive drug delivery system for the efficient therapy of breast and pancreatic cancers, Chem. Sci., 8 (2017) 3427-3433.

[32] T. Legigan, J. Clarhaut, B. Renoux, I. Tranoy-Opalinski, A. Monvoisin, J.-M. Berjeaud, F. Guilhot, S. Papot, Synthesis and Antitumor Efficacy of a β-Glucuronidase-Responsive Albumin-Binding Prodrug of Doxorubicin, J. Med. Chem., 55 (2012) 4516-4520.

[33] A.N. Zelikin, Drug Releasing Polymer Thin Films: New Era of Surface-Mediated Drug Delivery, ACS Nano, 4 (2010) 2494-2509.

[34] S.L. Regan, J.L. Maggs, T.G. Hammond, C. Lambert, D.P. Williams, B.K. Park, Acyl glucuronides: the good, the bad and the ugly, Biopharm. Drug Dispos., 31 (2010) 367-395.

[35] W.H. Bunnelle, Reagents for Stereoselective Preparation of N-Carbamyl β-d-Glucuronides, J. Org., 76 (2011) 5429-5432.

[36] P.L. Carl, P.K. Chakravarty, J.A. Katzenellenbogen, A novel connector linkage applicable in prodrug design, J. Med. Chem., 24 (1981) 479-480.

[37] P.D. Senter, E.L. Sievers, The discovery and development of brentuximab vedotin for use in relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma, Nat. Biotech., 30 (2012) 631-637.

[38] S.C. Jeffrey, J.B. Andreyka, S.X. Bernhardt, K.M. Kissler, T. Kline, J.S. Lenox, R.F. Moser, M.T. Nguyen, N.M. Okeley, I.J. Stone, X.Q. Zhang, P.D. Senter, Development and properties of beta-glucuronide linkers for monoclonal antibody-drug conjugates, Bioconjug. Chem., 17 (2006) 831-840.

[39] S.M. Lehar, T. Pillow, M. Xu, L. Staben, K.K. Kajihara, R.V. Andlen, L. DePalatis, H. Raab, W.L. Hazenbos, J.H. Morisaki, J. Kim, S. Park, M. Darwish, B.C. Lee, H. Hernandez, K.M. Loyet, P. Lupardus, R.N. Fong, D.H. Yan, C.C. Halouni, E. Luis, Y. Khalfin, E. Plise, J.C. Heong, J.P. Lyssikatos, M. Strandh, K. Koefoed, P.S. Andersen, J.A. Flygare, M.W. Tan, E.J. Brown, S.M. Ariathasan, Novel antibody-antibiotic conjugate eliminates intracellular S. aureus, Nature, 527 (2015) 323-328.

[40] S.O. Andreasen, B. Fejerskov, A.N. Zelikin, Biocatalytic polymer thin films: optimization of the multilayered architecture towards in situ synthesis of anti-proliferative drugs, Nanoscale, 6 (2014) 4131-4140.

[41] Z. Feldötö, I. Varga, E. Blomberg, Influence of Salt and Rinsing Protocol on the Structure of PAH/PSS Polyelectrolyte Multilayers, Langmuir, 26 (2010) 17048-17057.

[42] A.P. Johnston, A.N. Zelikin, L. Lee, F. Caruso, Approaches to quantifying and visualizing polyelectrolyte multilayer film formation on particles, Anal. Chem., 78 (2006) 5913-5919.

[43] H. Kerdjoudj, N. Berthelemy, S. Rinckenbach, A. Kearney-Schwartz, K. Montagne, P. Schaaf, P. Lacolley, J.-F. Stoltz, J.-C. Voegel, P. Menu, Small Vessel Replacement by Human Umbilical Arteries With Polyelectrolyte Film-Treated Arteries: In Vivo Behavior, J. Am. Coll. Cardiol., 52 (2008) 1589-1597.

[44] Z. Feldötö, M. Lundin, S. Braesch-Andersen, E. Blomberg, Adsorption of IgG on/in a PAH/PSS multilayer film: Layer structure and cell response, J. Colloid Interface Sci., 354 (2011) 31-37.

[45] A. Alouane, R. Labruere, T. Le Saux, F. Schmidt, L. Jullien, Self-Immolative Spacers: Kinetic Aspects, Structure-Property Relationships, and Applications, Angew. Chem. Int. Ed., 54 (2015) 7492-7509.

TOC image