

Bacteria-Triggered Release of Antimicrobial Agents**

Vitaly V. Komnatnyy, Wen-Chi Chiang, Tim Tolker-Nielsen, Michael Givskov, and Thomas E. Nielsen*

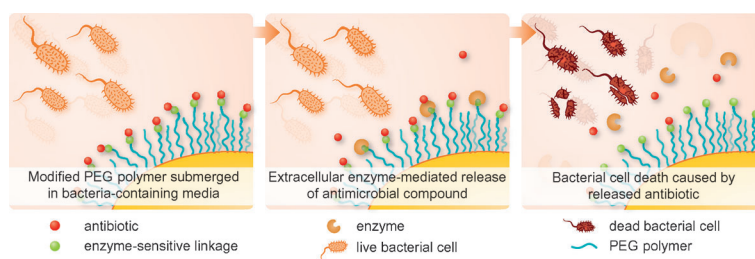
Dedicated to Professor Morten Meldal on the occasion of his 60th birthday

Abstract: Medical devices employed in healthcare practice are often susceptible to microbial contamination. Pathogenic bacteria may attach themselves to device surfaces of catheters or implants by formation of chemically complex biofilms, which may be the direct cause of device failure. Extracellular bacterial lipases are particularly abundant at sites of infection. Herein it is shown how active or proactive compounds attached to polymeric surfaces using lipase-sensitive linkages, such as fatty acid esters or anhydrides, may be released in response to infection. Proof-of-concept of the responsive material is demonstrated by the bacteria-triggered release of antibiotics to control bacterial populations and signaling molecules to modulate quorum sensing. The self-regulating system provides the basis for the development of device-relevant polymeric materials, which only release antibiotics in dependency of the titer of bacteria surrounding the medical device.

Medical devices employed in healthcare practice are often susceptible to microbial contamination.^[1] Pathogenic bacteria may attach themselves to device surfaces of catheters or implants by formation of chemically complex biofilms, which may be the direct cause of device failure. Treatment of device-related infections is often difficult as pathogens exhibit a high degree of antibiotic resistance in the biofilm mode-of-life.^[2] For the most severe cases, surgery and replacement of the indwelling device may be the only option for patient survival.^[3] In this context, the development of new methods

for functionalization of material surfaces that prevent biofilm formation would clearly have significant commercial potential and socioeconomic benefits. Along these lines, various methods, such as impregnating the device material, for example, polyurethane walls of central-venous catheter, with bactericidal components, along with different coating strategies, have been developed.^[4]

Here, we present a new approach for the bacteria-triggered release of antimicrobial compounds (Scheme 1) from poly(ethylene glycol) (PEG) materials, thereby paving the way for a new generation of bacteria-repellent materials. Extracellular lipases are particularly abundant at sites of bacterial infection, as indicated by high amounts of anti-lipase



Scheme 1. The concept of bacteria-triggered enzymatic release of antibiotics from chemically modified polymers.

in anti-sera obtained from cystic fibrosis patients suffering from *Pseudomonas aeruginosa* infection.^[5] We therefore envisioned that active or proactive compounds attached to polymeric surfaces using lipase-sensitive linkages, such as fatty acid esters or anhydrides, could be released in response to infection. We communicate our recent investigations on such bacteria-responsive materials and report a novel lipase-triggered release system for the control of bacterial populations and modulation of quorum sensing.

It is commonly believed that once biofilms are formed, bacterial cell-to-cell signaling, frequently referred to as quorum sensing (QS), plays an essential role for bacteria in their evasion of the host immune system and resistance to antibiotic treatment.^[6] Although host phagocytic leukocytes (PMNs) and administered antibiotics can normally clear acute infections that originate from planktonic bacteria, those measures fail to eradicate bacteria present in biofilms because of protection offered by the extracellular biofilm matrix. Given the importance of quorum sensing and cell-cell signaling for infectious processes, release systems that can be triggered under carefully controlled conditions could

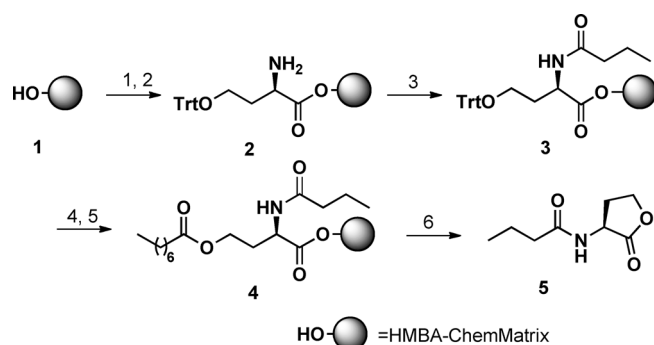
[*] Dr. V. V. Komnatnyy, Prof. Dr. T. E. Nielsen
Department of Chemistry, Technical University of Denmark
Kgs. Lyngby, 2800 (Denmark)
E-mail: ten@kemi.dtu.dk

Dr. W.-C. Chiang, Prof. Dr. T. Tolker-Nielsen, Prof. Dr. M. Givskov
Department of International Health
Immunology and Microbiology, University of Copenhagen
Copenhagen, 2200 (Denmark)

Prof. Dr. M. Givskov, Prof. Dr. T. E. Nielsen
Singapore Centre on Environmental Life Sciences Engineering
Nanyang Technological University
637551 Singapore (Singapore)

[**] DSF Center for Antimicrobial Research (Danish Council for Strategic Research) is gratefully acknowledged for financial support. We thank Dr. Susanne Wilhelm, Heinrich-Heine-University Düsseldorf, for providing the *P. aeruginosa* *lipA* *lipC* *estA* mutant.

Supporting information for this article is available on the WWW under <http://dx.doi.org/10.1002/anie.201307975>.



Scheme 2. Solid-phase synthesis of homoserine-containing ester **4** and lipase-triggered release of C₄-HSL **5**: 1) Fmoc-O-trityl-homoserine, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT), *N*-methyl-imidazole, DMF, 1 h, RT; 2) 20% piperidine (DMF, RT); 3) butanoyl chloride, NEt₃, CH₂Cl₂, 1 h, RT; 4) 5% TFA in CH₂Cl₂, 1 h, RT; 5) octanoyl chloride, triethylamine, DMAP, CH₂Cl₂, 1 h, RT; 6) lipase from *Pseudomonas fluorescens* (Fmoc = 9-fluorenylmethoxycarbonyl, DMF = *N,N*-dimethylformamide, TFA = trifluoroacetic acid, DMAP = 4-dimethylaminopyridine, and HMBA = 4-hydroxymethylbenzoic acid).

become biomedically relevant to treat and diagnose populations of Gram-negative bacteria, such as *P. aeruginosa*, through QS modulation.^[7] Our attention turned to the acyl-homoserine lactone (AHL) molecules produced by *P. aeruginosa*, *N*-3-oxo-dodecanoyl-L-homoserine lactone (3O-C₁₂-HSL) and *N*-butyryl-L-homoserine lactone (C₄-HSL), which directly or indirectly control the expression of multiple virulence factors, secondary metabolites, swarming motility, and biofilm development.

The homoserine-containing construct **4** (Scheme 2) was assembled on HMBA-linked ChemMatrix polymer,^[8] which conveniently serves as a representative model system for PEG-based materials (HMBA = 4-hydroxymethylbenzoic acid). Like related materials, this polymer allows diffusion of biological macromolecules, such as enzymes and substrates.^[9]

We envisioned how lipase-treatment of **4** upon ester hydrolysis would liberate a free hydroxy group and undergo a spontaneous cyclization, possibly favored by the acidic interior of the lipase, and thereby release C₄-HSL. We chose the C₄-HSL signal molecule because this allows easy assessment of the effect of the release system on bacteria as described below. In addition, the system can easily be modified to release quorum quenching compounds.

To test the approach we employed *E. coli* MH205 as a QS monitor strain. This monitor contains an *ahyR/ahyI-gfp* reporter system, which responds readily to the presence of extracellular C₄-HSL, with the *E. coli* strain serving as a lipase-negative background. We prepared a set of experiments where beads **4** (resin loading of about 0.3 mmol g⁻¹) were suspended in bacterial growth medium in concentrations corresponding roughly to 100 μM C₄-HSL at full liberation. As controls, a 2.5 μM solution of C₄-HSL-supplemented growth medium as well as an unsupplemented growth medium were included. Rewardingly, beads **4** and the presence of *Pseudomonas fluorescens* lipase were indeed found to induce substantial activation of the genetically engineered QS monitor MH205, compared with medium devoid of added lipase and unsupplemented media (Figure 1).

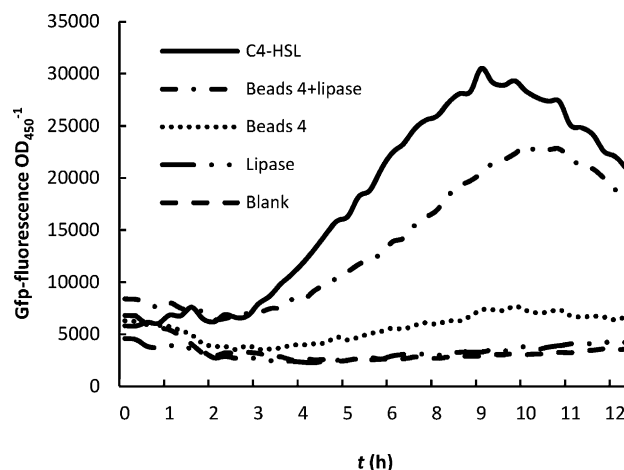
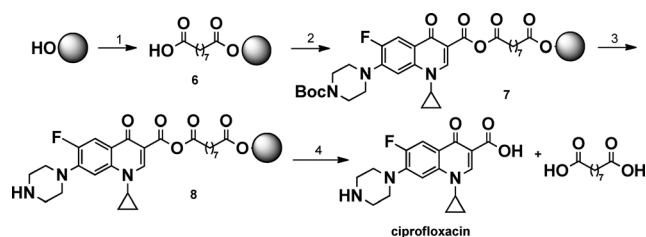


Figure 1. C₄-HSL-dependant activation of the *ahyR/ahyI-gfp* reporter system in *E. coli* (OD₄₅₀ = optical density at 450 nm).

Having developed a surface that releases functional C₄-HSL in response to defined lipase-activity, we shifted our focus to a system capable of also releasing antimicrobial drugs. Ciprofloxacin was chosen because of its function as a broad-spectrum antibiotic. This compound contains a carboxylic acid moiety which can be used as an anchoring element for chemical synthesis of lipase-labile bonds. Along these lines, mixed anhydride construct **8** was assembled on ChemMatrix resin according to the synthetic sequence shown at the Scheme 3. This construct is completely stable under



Scheme 3. Solid-phase synthesis of mixed anhydride **8** and bacteria-triggered release of ciprofloxacin: 1) azelaic anhydride, *N*-methyl imidazole, CH₂Cl₂, 2 h; 2) Boc-ciprofloxacin, bis(trichloromethyl)carbonate, triethylamine, CH₂Cl₂, 2 h; 3) TMSOTf, CH₂Cl₂, 2 h; 4) *Pseudomonas fluorescens* lipase (Boc = *tert*-butoxycarbonyl and TMSOTf = trimethylsilyltrifluoromethanesulfonate).

physiological pH conditions, but upon shifting to basic media both ciprofloxacin and the azelaic acid spacer (itself an antimicrobial compound) were released from the support (Scheme 3). We envisioned that the presence of extracellular bacterial lipases would catalyze the hydrolysis of the mixed anhydride bond and thus liberate the antibiotic.

As to this end, we investigated if the antibiotic-coated beads **8** could actually kill strains of *P. aeruginosa*. This biofilm-forming opportunistic pathogen produces and secretes two lipases LipA and LipC,^[10] in addition to an outer membrane-located esterase EstA.^[11] We assessed the viability (colony forming units mL⁻¹) of *P. aeruginosa* bacteria

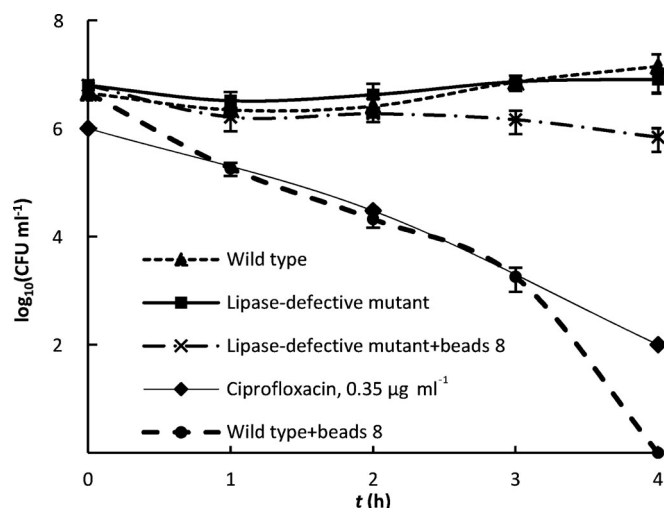


Figure 2. Viability of *P. aeruginosa* wild-type strain and *lipA lipC estA* triple mutant in presence of ciprofloxacin beads **8** (CFU = colony-forming unit).

in cultures supplemented with beads **8**. As a negative control we used a *P. aeruginosa lipA lipC estA* triple mutant, which is unable to produce the extracellular LipA, LipC, and EstA lipolytic enzymes. The killing kinetics of the *P. aeruginosa* wild-type strain in the presence of pure ciprofloxacin ($0.35 \mu\text{g mL}^{-1}$) suggested that the concentration of ciprofloxacin released from the beads approaches this value. In comparison, the mean concentration of ciprofloxacin in serum 12 h after dosing with 500 mg is typically around $0.2 \mu\text{g mL}^{-1}$. As shown in Figure 2, the wild-type strain was completely killed in the presence of beads **8** within 4 h, whereas the population of the lipase-defective mutant was only insignificantly decreased in the presence of beads **8**.

The polymer material modified with amide-bonded ciprofloxacin, so that the drug could not be released by the lipase or growth medium, did not display any antibiotic effect, neither against the wild-type strain, nor for the lipase-defective mutant (see the Supporting Information).

In the present study we have shown the feasibility of bacteria-triggered release of bioactive compounds, including QS signals and antimicrobial drugs, from chemically modified surfaces. The active compound precursor (or pro-drug) can be synthesized directly on the surface via standard solid-phase synthesis techniques. A key structural feature of the precursor is the presence of a cleavable anhydride bond which can be hydrolyzed in bacterial media by extracellular lipases. The lipase is eventually provided by the amassing bacteria as they approach the surface at the stage of early biofilm formation. Many compounds with a diverse range of biological activities may in principle be released, including compounds that will either stimulate or block QS, and subsequently sensitize bacteria to the action of the immune system and antibiotics. The approach may address some of the key challenges for the treatment of bacterial infectious disease, including both the

mode of delivery, dosage of antibiotics at the site of infection, and development of antibiotic resistance. The self-regulating system provides the basis for the development of device-relevant polymeric materials, which only release antibiotics in dependency of the titer of bacteria surrounding the medical device. In the future, we hope that this concept can be developed to construct anti-biofilm or repellent coatings for indwelling devices which can more effectively clear infections caused by pathogenic bacteria.

Received: September 10, 2013

Revised: October 18, 2013

Published online: November 29, 2013

Keywords: lipases · polymers · quorum sensing · solid-phase synthesis · surface chemistry

- [1] a) K. K. Lai, S. A. Fontecchio, *Am. J. Infect. Control* **2002**, *30*, 221–225; b) M. Lee, J. F. Donovan, *J. Endourol.* **2002**, *16*, 241–244; c) R. M. Donlan, J. W. Costerton, *Clin. Microbiol. Rev.* **2002**, *15*, 167–193.
- [2] a) N. Barraud, B. G. Kardak, N. R. Yepuri, R. P. Howlin, J. S. Webb, S. N. Faust, S. Kjelleberg, S. A. Rice, M. J. Kelso, *Angew. Chem.* **2012**, *124*, 9191–9194; *Angew. Chem. Int. Ed.* **2012**, *51*, 9057–9060; b) P. S. Stewart, J. W. Costerton, *Lancet* **2001**, *358*, 135–138.
- [3] a) K. Lewis, *Antimicrob. Agents Chemother.* **2001**, *45*, 999–1007; b) M. R. Parsek, P. K. Singh, *Annu. Rev. Microbiol.* **2003**, *57*, 677–701.
- [4] a) J.-Y. Wach, S. Bonazzi, K. Gademann, *Angew. Chem.* **2008**, *120*, 7232–7235; *Angew. Chem. Int. Ed.* **2008**, *47*, 7123–7126; b) F. Baneyx, D. T. Schwartz, *Curr. Opin. Biotechnol.* **2007**, *18*, 312–317; c) M. Yoshinari, T. Kato, K. Matsuzaka, T. Hayakawa, K. Shiba, *Biofouling* **2010**, *26*, 103–110; d) P.-H. Chua, K.-G. Neoh, E.-T. Kang, W. Wang, *Biomaterials* **2008**, *29*, 1412–1421; e) Z. Shi, K. G. Neoh, E. T. Kang, C. Poh, W. Wang, *J. Biomed. Mater. Res.* **2008**, *86*, 865–872; f) L. C. Gerber, F. M. Koehler, R. N. Grass, W. J. Stark, *Angew. Chem.* **2012**, *124*, 11455–11458; *Angew. Chem. Int. Ed.* **2012**, *51*, 11293–11296.
- [5] M. L. Vasil, L. M. Graham, R. M. Ostroff, V. D. Shortridge, A. I. Vasil, *Antibiot. Chemother.* **1991**, *44*, 34–47.
- [6] a) J. S. Dickschat, *Nat. Prod. Rep.* **2009**, *27*, 343–369; b) H.-S. Joo, M. Otto, *Chem. Biol.* **2012**, *19*, 1503–1513; c) C. Fuqua, E. P. Greenberg, *Nat. Rev. Mol. Cell Biol.* **2002**, *3*, 685–695.
- [7] a) T. Bjarnsholt, M. Givskov, *Philos. Trans. R. Soc. London Ser. B* **2007**, *362*, 1213–1222; b) T. Praneenarat, A. G. Palmer, H. E. Blackwell, *Org. Biomol. Chem.* **2012**, *10*, 8189–8199; c) W. R. J. D. Galloway, J. T. Hodgkinson, S. Bowden, M. Welch, D. R. Spring, *Trends Microbiol.* **2012**, *20*, 449–458; d) C. A. Lowery, N. T. Salzameda, D. Sawada, G. F. Kaufmann, K. D. Janda, *J. Med. Chem.* **2010**, *53*, 7467–7489.
- [8] For complete synthetic protocols see Supporting Information.
- [9] Enzymatic hydrolysis of substrates bound to the PEG-derived materials has been widely utilized in one bead-two compound assays. For an overview, see: M. Meldal, *Curr. Opin. Chem. Biol.* **2004**, *8*, 238–244.
- [10] F. Rosenau, K. E. Jaeger, *Biochimie* **2000**, *82*, 1023–1032; A. Martinez, P. Ostrovsky, D. N. Nunn, *Mol. Microbiol.* **1999**, *34*, 317–326.
- [11] S. Wilhelm, J. Tommassen, K. E. Jaeger, *J. Bacteriol.* **1999**, *181*, 6977–6986.