Antimicrobial Polymers

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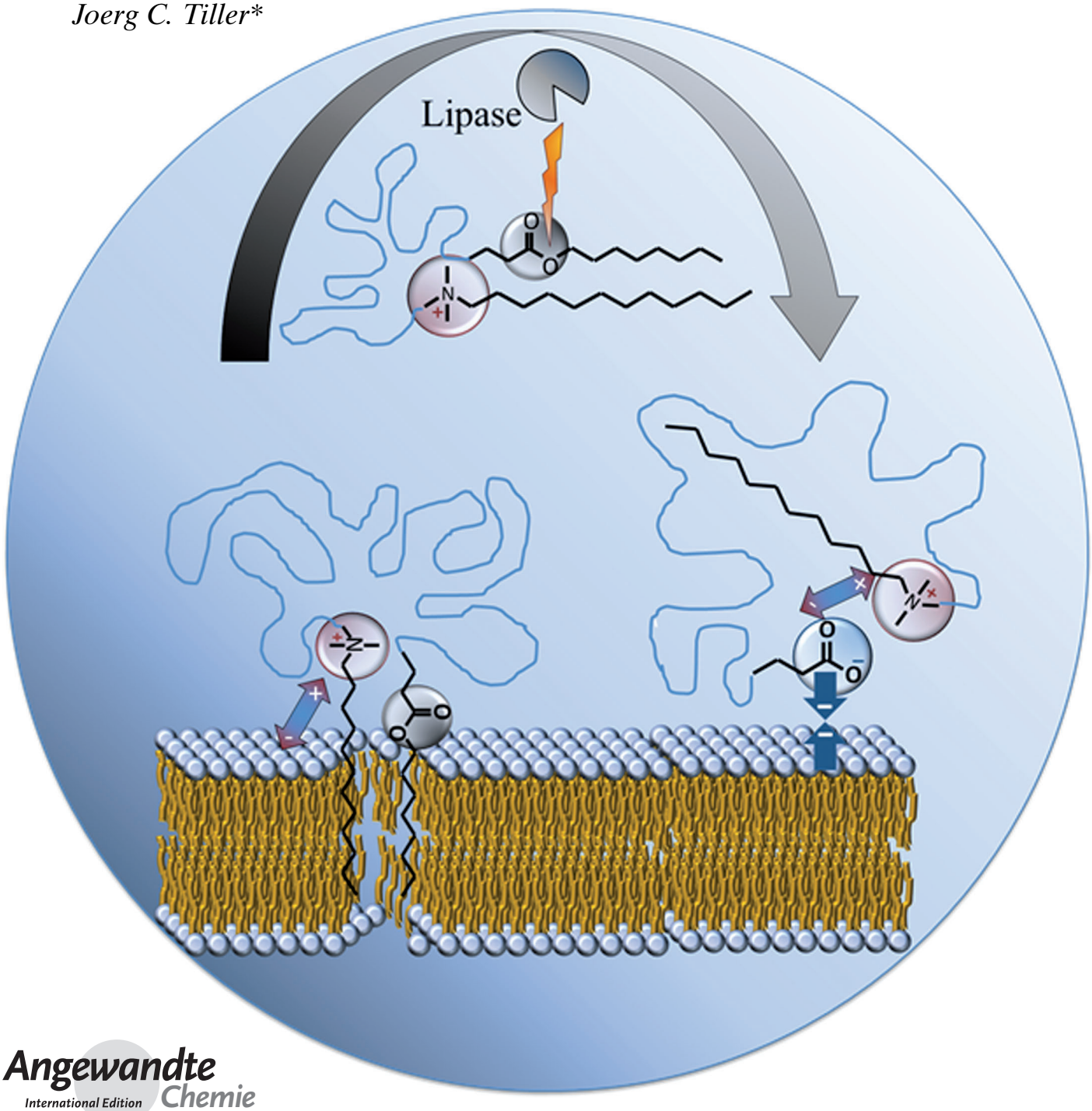
Antimicrobial Poly(2-methyloxazoline)s with Bioswitchable Activity through Satellite Group Modification**

*Christian Krumm, Simon Harmuth, Montasser Hijazi, Britta Neugebauer, Anne-Larissa Kampmann, Helma Geltenpoth, Albert Sickmann, and Joerg C. Tiller**



Abstract: *Biocides are widely used for preventing the spread of microbial infections and fouling of materials. Since their use can build up microbial resistance and cause unpredictable long-term environmental problems, new biocidal agents are required. In this study, we demonstrate a concept in which an antimicrobial polymer is deactivated by the cleavage of a single group. Following the satellite group approach, a biocidal quaternary ammonium group was linked through a poly(2-methyloxazoline) to an ester satellite group. The polymer with an octyl-3-propionoate satellite group shows very good antimicrobial activity against Gram-positive bacterial strains. The biocidal polymer was also found to have low hemotoxicity, resulting in a high HC_{50}/MIC value of 120 for S. aureus. Cleaving the ester satellite group resulted in a 30-fold decrease in antimicrobial activity, proving the concept valid. The satellite group could also be cleaved by lipase showing that the antimicrobial activity of the new biocidal polymers is indeed bioswitchable.*

Among the most threatening health issues in modern globalized society is the evolution of antibiotic-resistant microorganisms.[1] Tremendous amounts of disinfectants are used to fight pathogenic microbes but these weapons are getting dull, because they help to build up microbial resistance and are concentrated in the environment causing unpredictable long-term problems[2,3a] either directly or by their metabolites.[3] The optimal modern biocide would kill germs in the targeted area for the required period of time and then disappear.[4a] In the next best approach, a biocide would biodegrade into an inactive, nontoxic form. So far, there are only a few not fully accepted examples of biocides that are degradable.[4]

Antimicrobial polymers are a promising alternative to low-molecular-weight antibiotics and disinfectants.[5] Such macromolecules can be classified as biocide-releasing polymers, polymeric biocides, and biocidal polymers.[6] A few examples of the first two classes are designed to be biodegradable, mostly to release biocides on demand.[7] In contrast, biocidal polymers function as one molecule that is able to destabilize and eventually destroy the cell membrane of microorganisms resulting in cell lysis and cell death.[6,8a,b] Thus, even antibiotic-resistant bacteria such as Methicillin-resistent *Staphylococcus aureus* (MRSA) can be killed as effectively as nonresistant *S. aureus*.[8c] The structure of biocidal polymers, which by definition are composed of nonbiocidal repeating units, is particularly interesting, because full degradation of such polymers would inevitably result in inactive compounds.[8d,e,13] A few examples of potentially biodegradable biocidal polymers have been described very recently.[9] Only one of them has actually been explored regarding its degradation, showing deactivation only under nonnatural conditions.[10] An alternative to the above-mentioned polymers are biocides coupled as end groups to inactive polymers.[8b,11] If the polymer contains a second, non-antimicrobially active end group referred to as a satellite group (SG), the antimicrobial activity of these polymers can be controlled over several orders of magnitude.[8a] This might offer the possibility of creating an antimicrobial polymer that can be deactivated by altering a single bond in the whole molecule. The idea is based on the fact that hydrophobic SGs of appropriate length greatly activate the distal biocidal group, whereas nonbasic hydrophilic groups deactivate it. Thus, we chose to introduce an ester group as the SG, such that the active macromolecule would be rendered inactive just by the hydrolysis of this very function (Figure 1), possibly biocatalyzed by anomnipresent lipase.

The ester SG group was introduced at one polymer end by a functional initiator, which was prepared by esterification of 3-bromopropanoic acid[12] and the respective alcohol, followed by exchange of the bromide with iodide by a subsequent Finkelstein reaction with NaI. Since the activity control of the hydrophobic SG group is not easily predictable, a series of ester initiators with various alkyl residues ranging from ethyl to tetradecyl were been prepared (Table S1 in the Supporting Information).

The polymers were synthesized by cationic ring-opening polymerization of 2-methyl-2-oxazoline (MOx) and termination with the known biocidal group *N*,*N*-dimethyldodecylamine (DDA). All polymers were found to have more than 86% functionalization with SG as well as with the biocidal DDA group according to ^{1}H NMR spectroscopy.

Next, we tested whether the ester SG can be selectively hydrolyzed. Thus, the polymer starting with octyl-3-iodopropanoate (OP) OP-PMOx-DDA was treated with 0.015 M aqueous NaOH at 50 °C overnight. The control experiment was performed with an octyl bromide initiatedPMOx-DDA (O-PMOx-DDA) described in previous work.[8a] According to the ^{1}H NMR spectra only OP-PMOx-SG was chemically converted in the procedure (Figure S3). The ^{1}H NMR spectrum of the NaOH-treated OP-PMOx-DDA (Figure S3b in the Supporting Information) shows that the characteristic octyl ester signals disappear after alkaline hydrolysis. All other signals are not affected indicating that no other modification took place.

Since ^{1}H NMR data are not always sufficient to fully characterize polymer chains, we performed electrospray ionization mass spectrometry (ESI-MS) measurements of

[*] Dipl.-Ing. C. Krumm, Dipl.-Chem. S. Harmuth, M. Hijazi, B. Neugebauer, A.-L. Kampmann, Prof. Dr. J. C. Tiller
Biomaterials and Polymer Science
Department of Bio- and Chemical Engineering, TU Dortmund
Emil-Figge-Strasse 66, 44227 Dortmund (Germany)
E-mail: joerg.tiller@udo.edu
H. Geltenpoth, Prof. Dr. A. Sickmann
Leibniz-Institut für Analytische Wissenschaften –ISAS– e.V.
Otto Hahn-Strasse 6b, 44227 Dortmund (Germany)

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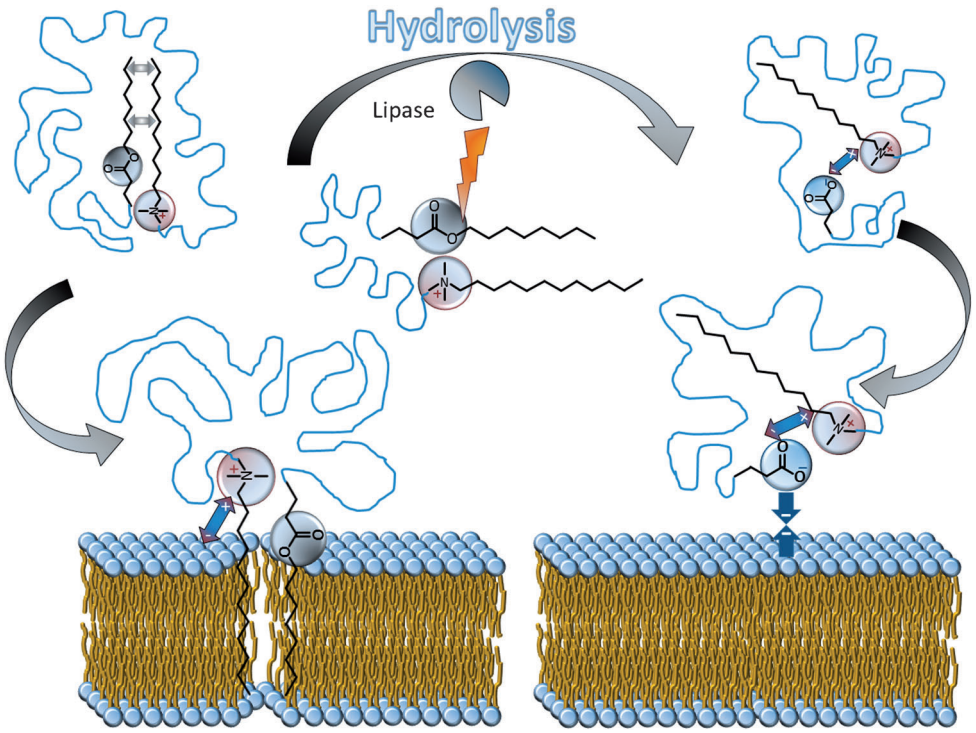

Figure 1. Schematic representation of the membrane-active biocidal PMOx controlled by the cleavable satellite group (SG) before and after hydrolysis.

OP-PMOx-DDA before and after treatment with NaOH. The measurements were performed with trifluoroacetic acid (TFA)/H_2O solutions of the polymers. As seen in Figure 2a, the mass spectrum of the polymer before hydrolysis confirms the results of the ^{1}H NMR analysis. More than 93% of the absolute counts represent mass peaks that correspond to macromolecules containing the octyl ester, a varying number of MOx repeating units, and the biocidal endgroup DDA. After the polymer was treated with NaOH all mass peaks are shifted to molecular weights corresponding to polymers that contain a COOH instead of an octyl ester group, indicating complete hydrolysis of the ester SG and no further modification of the polymer backbone. The expected negative shift in mass is 112 g mol^{-1}, because formally the octyl group (113 g mol^{-1}) is replaced by a proton (1 g mol^{-1}). All other ester SG-PMOx-DDA polymers were treated similarly.

Next, the antimicrobial activity of the polymers was explored with ubiquitous and clinically relevant bacterial strains, the Gram-positive bacterium *Staphylococcus aureus* and the Gram-negative bacterium *Escherichia coli*. The minimal inhibitory concentration (MIC) was determined from a factor two dilution series starting with a concentration of 2500 μg mL^{-1}.

As seen in Figure 2c (front columns) the antimicrobial activity of the polymers strongly depends on the length of the alkyl chain of the ester SG. In accordance to previous work,[8a] short alkyl chain SGs, such as the ethyl ester, and very long alkyl chain SGs, such as the tetradecyl ester, decrease the biocidal potency of the macromolecules against *S. aureus*. The highest antimicrobial activity was found for the octyl ester SG (Figure 2c). Determination of the MIC values for *E. coli* revealed that only the OP-PMOx-DDA was active against this bacterium (Table S2 in the Supporting Information). After ester cleavage the antimicrobial activity of the polymers against both microorganisms (see Figure 2c, back columns) is completely eliminated. These results indicate that it is indeed possible to deactivate a biocidal polymer by cleaving a single bond and applying the concept of the SG effect. In the control experiment with O-PMOx-DDA, which has no cleavable SG, the MIC value of this polymer before and after treatment with NaOH remained the same.

Since the length of the PMOx chain has little impact on the molar activity as previously shown,[13] we prepared an OP-PMOx-DDA with a shorter chain length of 30 repeating units, expecting a higher antimicrobial activity with respect to mass concentration. It was found that this polymer is indeed more active against *S. aureus* (MIC = 40 μg mL^{-1}) but not against *E. coli* (MIC = 1250 μg mL^{-1}). To broaden the knowledge on the antimicrobial spectrum, this polymer was also tested against *B. subtilis* (MIC = 40 μg mL^{-1}), *S. epidermidis* (MIC = 40 μg mL^{-1}), *S. mutans* (MIC = 156 μg mL^{-1}), and *P. aeruginosa* (no activity). Apparently, the antibacterial activity of OP-PMOx$_{30}$-DDA is specific for Gram-positive bacteria. Hydrolysis of the SG group of the macromolecule (weight loss of less than 3 wt%) resulted in an MIC against *S. aureus* of 1250 μg mL^{-1}, indicating no full deactivation upon ester cleavage but still an activity decrease by a factor of 30. These results confirm previous findings, where a decyl SG afforded high antimicrobial activity of DDA-PMOx polymers, while these polymers with a shorter methyl SG showed 30 times lower activity.[8a] Investigations of the interactions of these polymers with liposomes revealed that the effect of the SG can be attributed to controlling the adhesion of the polymers onto and their insertion into cell membranes.[11] The additional carboxylic group might even increase the deactivating effect of short alkyl chain SGs due to electrostatic repulsion by the negatively net-charged outer surface of the bacterial cytoplasmic membrane (Figure 1).

Having established that cleaving the ester bond of the satellite group of ester-PMOx-DDA deactivates the whole polymer, we now explored the possibility of hydrolyzing this group in a natural environment. Typically, nature hydrolyzes hydrophobic esters with omnipresent lipases. In order to

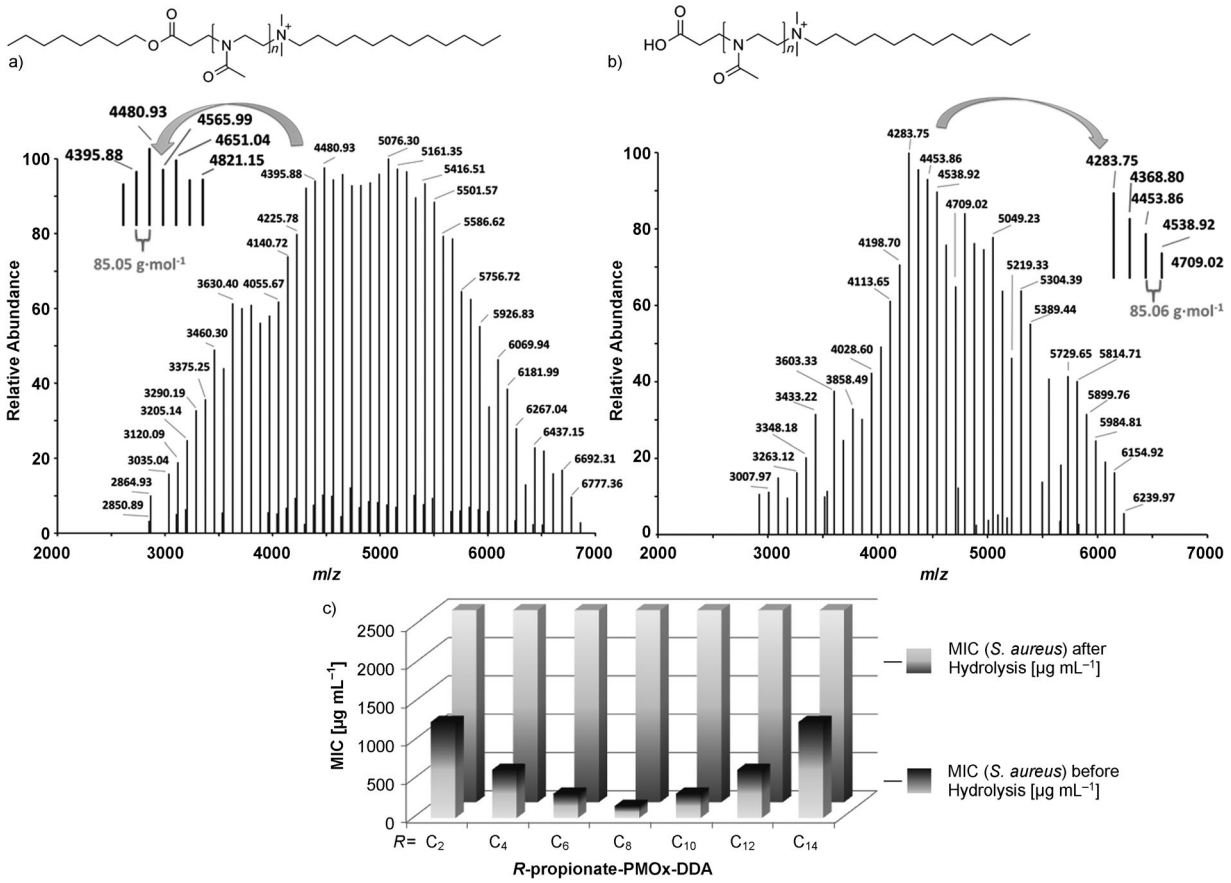

Figure 2. a) Electrospray ionization mass spectra of OP-PMOx-DDA a) before and b) after alkaline treatment. The peaks shown correspond to the isotopes with the lowest molecular weight. c) Minimal inhibitory concentrations of the antimicrobial telechelic PMOx before and after hydrolysis.

monitor the lipase-induced cleavage of the ester SG, the hydrolysis experiment with lipase was performed by treating the most reactive polymer OP-PMOx$_{30}$-DDA with lipase in phosphate-buffered D_2O at pH 7 at 37 °C and the whole mixture was periodically analyzed by ^{1}H NMR spectroscopy.

The degree of hydrolysis was calculated by following the decrease of the integral of the signal at 1.56 ppm. The degradation curve seen in Figure 3 shows that the ester is slowly hydrolyzed by the lipase reaching 50 % conversion after 10 days.

Next, the lipase-catalyzed reaction was carried out in aqueous phosphate buffer to directly measure the impact of SG ester hydrolysis on the biocidal efficacy. The mixture of buffer, lipase, and polymer was tested for its antimicrobial activity after reaction times of 3 and 7 days. The control mixture without polymer shows no antibacterial effect against *S. aureus*. The MIC (*S. aureus*) values of the polymer after 3 and 7 days of contact with lipase were found to be 156 and 312 µg mL^{-1}, respectively, while the polymer treated with the buffer without lipase retains full antibacterial efficacy. This clearly shows that lipase indeed deactivates the antimicrobial activity of the polymer.

Another important feature of biocidal polymers is their toxicity against eukaryotic cells (cytotoxicity). This is often tested with red blood cells by determining the HC_{50} value.[14] The hemocompatibility test was performed with erythrocytes from porcine blood and a HC_{50} value of (4730 ± 220) µg mL^{-1} was measured for OP-PMOx$_{30}$-DDA. Thus, the octyl ester not only acts as bioswitchable activating/deactivating satellite group, but also improves the polymer's hemocompatibitily by an order of magnitude over that of previously described biodical PMOx-DDA derivatives.[8b] The selectivity HC_{50}/MIC for *S. aureus* is 120 ± 5, which is comparable to the best recently described hemocompatible and antimicrobial polymers.[15]

The HC_{50} of the coupling product of the octyl initiator and DDA (OP-DDA), that is, the smallest molecule containing both satellite and biocidal endgroup, was found to be 81 µg mL^{-1}, which is a 60 times higher hemotoxicity than that of OP-PMOx$_{30}$-DDA. Thus, OP-DDA is not only six times less active against *S. aureus*, but also more cytotoxic. Additionally, the HC_{50} of the hydrolyzed OP-PMOx$_{30}$-DDA increased to a value of 21 000 µg mL^{-1}, proving that the switch of the satellite group lowers the impact on living prokaryotic as well as eukaryotic cells.

In closing, we could control both antibacterial activity and the cytotoxicity of a polymer by attaching a biocleavable satellite group. Hydrolysis of the single ester group of OP-PMOx$_{30}$-DDA achievable under lipase catalysis converts the biocidal activity against *S. aureus* from very good to weak, according to the definition given by Fortuniak et al.[16] We believe that this concept might be applicable to other

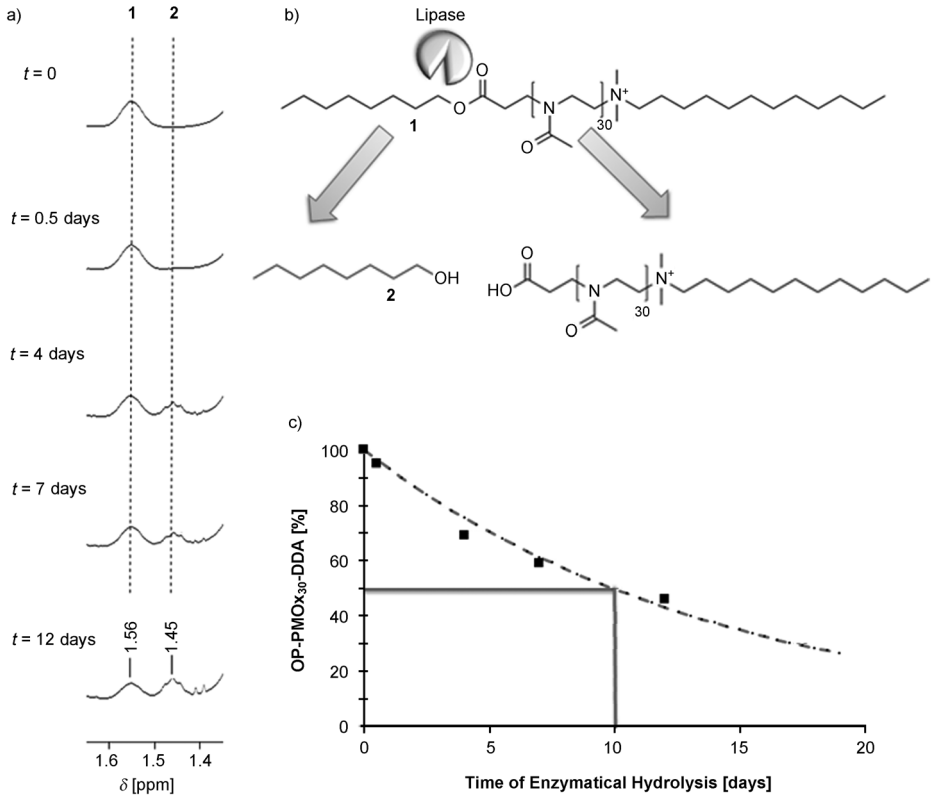

Figure 3. a) A graphical summary of the performed ^{1}H NMR investigation of the hydrolysis of *n*-octanol in D_2O catalyzed by lipase. b) Overview of the enzymatically catalyzed hydrolysis. c) Plot of the postulated endgroup hydrolysis catalyzed by lipase.

biocides, such as antibiotics, and even to other bioactive agents.

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