Summary

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Bacterial-infections are mostly due to bacteria in an adhering, biofilm-mode of growth and not due to planktonically-growing, suspended-bacteria. Biofilm-bacteria are much more recalcitrant to conventional antimicrobials than planktonic-bacteria due to (**1**) emergence of new properties of biofilm-bacteria that cannot be predicted on basis of their planktonic properties, (**2**) low penetration and accumulation of antimicrobials in a biofilm, (**3**) disabling of antimicrobials due to acidic and anaerobic conditions prevailing in a biofilm, and (**4**) enzymatic modification or inactivation of antimicrobials by biofilm inhabitants. In recent years, new nanotechnology-based antimicrobials have been designed to kill planktonic, antibiotic-resistant bacteria, but additional requirements than the mere killing of suspended bacteria must be met to combat biofilm-infections. The requirements to and merits of nanotechnology-based antimicrobials for the control of biofilm-infection form the focus of **Chapter 1**, yielding as a general aim of this thesis the development of novel, self-adaptive, antimicrobial polymeric nanocarriers with enhanced ability to penetrate and eradicate infectious biofilms by multi-drug resistant bacteria.

The fact that bacterial biofilms can cause persistent infections and are recalcitrant to antimicrobials, is partly due to poor penetration of antimicrobials into biofilms, which allows bacteria residing in the depth of a biofilm to survive antimicrobial treatment. In **Chapter 2**, we describe the preparation of surface-adaptive, Triclosan-loaded micellar nanocarriers showing (**1**) enhanced biofilm penetration and accumulation, (**2**) electrostatic targeting at acidic pH toward negatively charged bacterial cell surfaces in a biofilm, and (**3**) antimicrobial release due to degradation of the micelle core by bacterial lipases. First, it was established that mixed-shell-polymeric-micelles (MSPM) consisting of a hydrophilic poly(ethylene glycol) (PEG)-shell and pH-responsive poly(*β*-amino ester) (PAE) become positively charged at pH 5.0, while being negatively charged at physiological pH. This is opposite to single-shell-polymeric-micelles (SSPM) possessing only a PEG-shell and remaining negatively charged at pH 5.0. The stealth properties of the PEG-shell combined with its surfaceadaptive charge allow MSPMs to penetrate and accumulate in staphylococcal biofilms, as demonstrated for fluorescent Nile red loaded micelles using confocal-laser-scanning-microscopy. SSPMs, not adapting a positive charge at pH 5.0, could not be demonstrated to penetrate and accumulate in a biofilm. Once micellar nanocarriers are bound to a staphylococcal cell surface, bacterial enzymes degrade the MSPM core to release its antimicrobial content and kill bacteria over the depth of a biofilm. This constitutes a highly effective pathway to control blood-accessible staphylococcal biofilms using antimicrobials, bypassing biofilm recalcitrance to antimicrobial penetration.

In addition to the emergent recalcitrant property of biofilms to antimicrobials, the number of bacterial strains intrinsically-resistant to available antibiotics is alarmingly growing. In **Chapter 3**, we report that micellar nanocarriers with a PEG shell can fully penetrate staphylococcal biofilms, which can be attributed to their biological invisibility. However, when the shell is complemented with PAE, these MSPMs become positively charged in the low pH environment of a biofilm, allowing in a first instance not only deep penetration but also their later accumulation in biofilms without being washed-out, as do SSPMs lacking the pH-adaptive feature. Accordingly, bacterial killing of multi-drug resistant (MDR) staphylococcal biofilms exposed to protoporphyrinIX-loaded MSPMs and after light-activation, was superior compared with SSPMs. Subcutaneous infections in mice, induced with vancomycin-resistant, bioluminescent staphylococci could be eradicated by daily injection of photoactivatable protoporphyrinIX-loaded MSPMs in the bloodstream and light-activation at the infected site. Micelles, which were not degraded by bacterial enzymes in the biofilm, were degraded in the liver and spleen and cleared from the body through the kidneys. Thus adaptive micellar nanocarriers loaded with light-activatable antimicrobials constitute a much-needed, alternative to current antibiotic therapies.

Conventional antimicrobials are becoming increasingly ineffective for treating bacterial infection due to the emergence of MDR pathogens. In **Chapter 4**, we report on PEG-PAE micelles with conjugated antimicrobials, that can uniquely penetrate biofilms, target themselves to bacterial cell surfaces once inside the low-pH environment of a biofilm and release conjugated antimicrobials through degradation of their ester-linkage with PAE by bacterial lipases. *In vitro*, PEG-PAE micelles with conjugated Triclosan (PEG-PAE-Triclosan) yielded no inadvertent leakage of their antimicrobial cargo and better killing of MDR *Staphylococcus aureus*, *Escherichia coli* and oral streptococcal biofilms than Triclosan in solution. In mice, PEG-PAE-Triclosan micelles yielded better eradication efficacy towards a MDR *S. aureus*-infection compared with Triclosan in solution and Triclosan-loaded micelles at equal Triclosan-equivalent concentrations. *Ex vivo* exposure of multi-species oral biofilms collected from orthodontic patients to PEG-PAE-Triclosan micelles, demonstrated effective bacterial killing at 30-40 fold lower Triclosan-equivalent concentrations than achieved by Triclosan in solution. Importantly, *Streptococcus mutans*, the main causative organism of dental caries, was preferentially killed by PEG-PAE-Triclosan micelles. Thus PEG-PAE-Triclosan micelles present a promising addendum to the decreasing armamentarium available to combat infection in diverse sites of the body.

In **Chapter 5**, we aimed at investigate strategies to tackle intracellular pathogens that are extremely recalcitrant to conventional antibiotic treatments. To this end, we synthesized an amphiphilic binary antimicrobial conjugate which will undergo self-assembly into sub-stable nanoparticles. Subsequently, the sub-stable nanoparticles were encapsulated in leukocyte-like cell membrane. The resulting leukocyte-like nanocarriers possess Toll-like receptors on their surfaces and are internalized by especially by infected leukoytes. Once inside an infected leukocyte, encapsulated antimicrobial conjugated nanoparticles are released to kill intracellular staphylococci. The killing efficacy of ACN-LLNs was evaluated both *in vitro* and *in vivo*. ACN-LLNs showed a synergistic killing efficacy, superior to either single antimicrobials or the bare ACN with membrane encapsulation. This strategy contributes greatly to current antibiotic therapies to overcome the barriers towards intracellular pathogens.

Bacterial infections by antimicrobial-resistant pathogens threaten to become the number one cause of death in 2050. Therewith the optimism about infection control that arose after the discovery of antibiotics, has come to an end and new infection control strategies are direly needed. Development of new antibiotics is generally considered unlikely. In **Chapter 6**, a likelihood perspective is given, for the possibilities offered by combination and smart encapsulation of existing antibiotics, use of probiotics and phage therapy, antimicrobial peptides and nanotechnology-based antimicrobials. Combination of existing antibiotics with probiotics, antimicrobial peptides or nanotechnology-based antimicrobials may also have good perspectives for clinical infection control, also when caused by antimicrobial-resistant strains. Therewith, existing antibiotics may still be useful for several decades to come despite the occurrence of antibiotic-resistance, provided further research and development of the above strategies are focused on their downward clinical translation, carried out collaboratively within academia and industry, rather than on developing and publishing yet another, new antimicrobial compound.