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# Antimicrobial and nanoparticle penetration and killing in infectious biofilms

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*Document Version*
Publisher's PDF, also known as Version of record

*Publication date:*
2019

Link to publication in University of Groningen/UMCG research database

*Citation for published version (APA):*
Rozenbaum, R. T. (2019). *Antimicrobial and nanoparticle penetration and killing in infectious biofilms*. [Thesis fully internal (DIV), University of Groningen]. Rijksuniversiteit Groningen.

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Download date: 07-10-2024

# Summary

Initial bacterial adhesion to surfaces in the human body can result in biofilm formation, which plays a critical role in bacterial infections. It is estimated that approximately 60% of all bacterial infections are caused by microbial biofilms. In a biofilm, bacteria embed themselves in a matrix of extracellular polymeric substances (EPS), acting as 'the house of the biofilm cells'. EPS consists of water, polysaccharides, proteins, extracellular DNA (eDNA) and other molecules and protects the biofilm from the human immune system, mechanical forces, penetration of antimicrobials, and desiccation. In comparison with planktonic bacteria, biofilm can be up to 1000 times more resistant to antimicrobials. Antimicrobial peptides (AMPs) have been mentioned to battle antimicrobial resistance and in order to let them penetrate into biofilms development of nanocarriers has been suggested **(Chapter 1).** Therefore, the aim of this thesis was to investigate the penetration of AMPs and nanocarriers in infectious biofilms *in vitro* and *in vivo*.

In **Chapter 2**, a protocol is presented how to grow biofilms with a well-defined thickness to match the thickness of clinically occurring biofilms in a constant depth film fermenter (CDFF). In a CDFF, biofilms are grown on the bottom of wells with set depths, while a scraper blade removes biofilm growing above the wells. Proper fixing of well-depth and use of smooth scraper blades are critical steps for growing biofilms of constant thickness over their entire surface area. Biofilm thickness can be measured with confocal laser scanning microscopy (CLSM), low load compression testing (LLCT) or optical coherence tomography (OCT). CLSM is mostly used, but relies on penetration of fluorophores and laser-light through the biofilms. This makes CLSM unsuitable for relatively thick CDFF biofilms, leaving LLCT and OCT preferred. The relatively low resolution of OCT enables to determine thickness over an entire biofilm surface area, constituting a major advantage over CLSM. The reproducible thickness of CDFF biofilms facilitates high-throughput studies and is important for studying antimicrobial penetration in biofilms.

OCT is a non-destructive tool for biofilm imaging, not requiring staining and used to measure biofilm thickness and putative comparison of biofilm structure based on whiteness distributions in OCT-images. Quantitative comparison of biofilm whiteness in OCT images, is impossible due to the auto-scaling applied in OCT-instruments to ensure optimal quality of individual images. In **Chapter 3**, we developed a method to eliminate the influence of auto-scaling in order to allow quantitative comparison of biofilms in different images. Auto- and re-scaled whiteness intensities could be qualitatively interpreted in line with biofilm characteristics expected on the basis of literature for biofilms of different strains and species, demonstrating qualitative validity of auto- and re-scaling analyses. However, specific features of pseudomonas and oral dual-species biofilms were more prominently expressed after re-scaling. Quantitative validation was obtained by relating average auto- and re-scaled whiteness intensities across biofilms with volumetric bacterial densities in biofilms, independently obtained using enumeration of bacterial numbers per unit biofilm volume. Opposite to auto-scaled average whiteness intensities, re-scaled intensities of

different biofilms increased linearly with independently determined volumetric bacterial densities in the biofilms. Herewith, the proposed re-scaling of whiteness distributions in OCT-images significantly enhances the possibilities of biofilm imaging using OCT.

EPS provides biofilms with viscoelastic properties, causing time-dependent relaxation after stress-induced deformation, according to multiple characteristic time-constants. These time-constants reflect different biofilm (matrix) components. Since viscoelasticity of biofilms has been related with antimicrobial penetration, but not yet with bacterial killing, this study aimed to relate killing of *P. aeruginosa* in its biofilm-mode of growth by three antimicrobials with biofilm viscoelasticity **(Chapter 4)**. *P. aeruginosa* biofilms were grown for 18 h in a CDFF, either with mucin-containing artificial sputum medium (ASM), artificial sputum medium without mucin ($ASM^-$), or Luria-Bertani broth (LB). This yielded 100 µm thick biofilms, that differed in their amounts of matrix eDNA and polysaccharides. LLCT followed by three-element Maxwell analyses, showed that the fastest relaxation component, associated with unbound water, was most important in LB grown biofilms. Slower components due to water with dissolved polysaccharides, insoluble polysaccharides and eDNA, were most important in relaxation of ASM grown biofilms. $ASM^-$ grown biofilms showed intermediate stress relaxation. *P. aeruginosa* in LB grown biofilms were killed most by exposure to tobramycin, colistin or an antimicrobial peptide, while ASM provided the most protective matrix with less water and most insoluble polysaccharides and eDNA. Concluding, stress relaxation of *P. aeruginosa* biofilms grown in different media revealed differences in matrix composition that, within the constraints of the antimicrobials and growth media applied, correlated with the matrix protection offered against different antimicrobials.

Development of antimicrobial dendritic polymers is considered promising as an alternative infection control strategy. Therefore, this work aimed to determine the role of dendron peripheral composition in their penetration into *P. aeruginosa* biofilms **(Chapter 5)**. For antimicrobial dendritic polymers to effectively kill bacteria residing in infectious biofilms, they have to penetrate and accumulate deep into biofilms. *P. aeruginosa* biofilms were exposed to red-fluorescent dendrons with different peripheral compositions. Dendrons with $NH_3^+$ peripheral groups accumulated faster into *P. aeruginosa* biofilms than dendrons with OH or $COO^-$ at their periphery, and accumulated near the top of the biofilm, due to electrostatic double-layer attraction with negatively-charged biofilm components. Distribution of dendrons with OH and $COO^-$ peripheral groups was more even across the depth of the biofilms. Unlike dendrons with $NH_3^+$ groups at their periphery, dendrons with OH or $COO^-$ peripheral groups, lacking strong electrostatic double-layer attraction with biofilm components, were largely washed-out during exposure to phosphate buffered saline (PBS) without dendrons. Thus penetration and accumulation of dendrons into biofilms is controlled by their peripheral compositions through electrostatic double-layer

interactions, which is important for the development of new antimicrobial or antimicrobial-carrying dendritic polymers.

In **Chapter 6** we investigated the release mechanism of AMPs from monolaurin lipid nanocapsules (ML-LNCs) and possible antimicrobial synergy of ML-LNCs with the AMPs DPK-060 and LL-37 against *Staphylococcus aureus* biofilm *in vitro* and in a therapeutic, murine, infected wound healing model. Zeta potentials demonstrated that AMP release from ML-LNCs was controlled by the AMP concentration in suspension. Both AMPs demonstrated no antimicrobial efficacy against four staphylococcal strains in a planktonic mode, while a checkerboard assay showed synergistic antimicrobial efficacy when ML-LNCs and DPK-060 were combined, but not for combinations of ML-LNCs and LL-37. Similar effects were seen for growth reduction of staphylococcal biofilms, with antimicrobial synergy persisting only for ML-LNCs at the highest level of DPK-060 or LL-37 adsorption. Healing of wounds infected with bioluminescent *S. aureus* Xen36, treated with ML-LNCs alone, was faster when treated with PBS, while AMPs alone did not yield faster wound healing than PBS. Faster, synergistic wound healing due to ML-LNCs with adsorbed DPK-060, was absent *in vivo*. Summarizing, antimicrobial synergy of ML-LNCs with adsorbed AMPs as seen *in vitro*, is absent in *in vivo* healing of infected wounds, likely because host AMPs adapted the synergistic role of the AMPs added. Thus, conclusions regarding synergistic antimicrobial efficacy, should not be drawn from planktonic data, while even *in vitro* biofilm data bear little relevance for the *in vivo* situation.

In **Chapter 7,** the results of this thesis are discussed regarding the penetration of nanocarriers into biofilms and the use of AMPs against biofilms. Future perspectives for antimicrobial treatments are proposed, such as investigating synergistic interactions of antimicrobials with antibiotics or other antimicrobial compounds. Ongoing research about new antimicrobials therapies and strategies to deliver antimicrobials into the biofilms are still direly needed to keep treating biofilms infections in the future.