SUMMARY AND CONCLUSIONS

Biofilm formation is one of the important reasons for the decreased sensitivity of bacteria to routinely used antimicrobial agents. Biofilms are aggregates of bacterial species held together by a matrix of extracellular polymeric substances such as polysaccharides, proteins and extracellular DNA. The architecture of biofilms enables the inhabiting sessile cells to communicate with each other in a highly organized fashion. It additionally functions as a protective barrier against the host immune response and compounds such as antibiotic molecules. This is commonly referred to in literature as the biofilm diffusion barrier and is the central focus of this thesis to device strategies to overcome this.

Chapter 1 starts by shedding light on the structural organization of the bacterial biofilm and illustrates the role of 2 involved key players, namely (1) the sticky biofilm matrix which can capture and/or enzymatically disintegrate antibiotic molecules and (2) the contribution of dense cell clusters which limits the net drug influx towards deeply embedded cells. Next, we provide an overview of different strategies that are currently explored to overcome this barrier, which can be divided in 2 groups. The first type of approaches increase antibiotic penetration by packaging antibiotics into nanoparticles, thereby shielding them from interactions with biofilm matrix constituents. Besides acting as a shelter, nanoparticles can also be endowed with interesting properties for biofilm delivery, for example by including targeting modalities on their outer surface or enabling triggered release of antibiotics. On the other hand, methods that specifically interfere with the biofilm integrity can also enhance antibiotic delivery. Pharmacological approaches aiming to enzymatically degrade biofilm matrix components are one example hereof, which, however, have limited clinical significance as their applicability very much depends on the biofilm composition. In contrast, physical methods, such as those making use of ultrasonic waves or electric currents, can be used to break down biofilms regardless of the specific microbial species present. However, more clinical trials are needed to rule out detrimental side effects and define effective dose-responses. The use of laser technology to disrupt biofilm matrices comes along with certain advantages such as spatial control and its fast nature, and has been used to break down biofilms by using heat (photothermal) or mechanical damage (photomechanical). Nevertheless, clinical translation of these approaches is limited due to the possible spread of heat or the danger to disperse bacteria into the surrounding healthy tissue. A more subtle way of biofilm dispersal is of current interest to circumvent these limitations, and a possible way to achieve this is by using laser-induced vapor nanobubbles (VNB).
In Chapter 2, we explore the potential of this new technique which merges the interesting features of laser light with the light absorbing and heat generating properties of gold nanoparticles (AuNP), however without heating up the environment. Indeed, by irradiating the AuNP with very intense, short laser pulses, the AuNP temperature rises quickly to several hundreds of degrees, thereby vaporizing the liquid surrounding the AuNP. Since almost all incident laser light is converted into mechanical energy of the expanding vapor bubble, no heat is being transferred to the environment, while the collapsing bubble gives rise to localized mechanical damage. The proof-of-concept study shows that 70 nm cationic AuNP can penetrate deep into both Gram-negative (Burkholderia multivorans, Pseudomonas aeruginosa) and Gram-positive (Staphylococcus aureus) biofilms, and that laser-induced VNB can locally disturb the tightly packed sessile cells. We find out that pre-treatment of biofilms with VNB can substantially improve the efficacy of tobramycin, up to 3000-fold, depending on the organism and treatment conditions. An initial toxicity screen in an in vivo Caenorhabditis elegans model shows no significant toxicity of pristine nor laser-irradiated AuNP on the nematodes.

These promising results paved the way for further exploration of the VNB-concept in the context of biofilm associated wound infections, as a first potential application of the technique. This follow-up study is described in Chapter 3 and investigates whether VNB can enhance the efficacy of a broad range of commercially available antimicrobials used for treating wound infections, including povidone-iodine, chlorhexidine, benzalkonium chloride, cetrimonium bromide and mupirocin. The investigations are performed on biofilms of clinically relevant pathogens often found in chronic wounds, namely Pseudomonas aeruginosa and Staphylococcus aureus. We find a potentiating effect for benzalkonium chloride (~ 21x) in P. aeruginosa biofilms, and cetrimonium bromide (~ 24x) and mupirocin (~ 53x) in S. aureus biofilms, which could be increased to a complete loss of survival after repeated VNB-formation in case of mupirocin. Since VNB only give additional effects for certain combinations of antimicrobials and bacterial biofilms – which is rather surprising given our promising results obtained with tobramycin in Chapter 2 – we speculate that this can be due to the fact that there is no diffusion barrier for the other types of combinations. In order to test this hypothesis, a positive control is included in which we check the activity the antimicrobials after completely disrupting the biofilms by vortexing and ultrasound, and compare this with the effect after VNB-treatment of the biofilms. We find similar trends between the forced and VNB-mediated disruption experiments, thereby confirming our hypothesis that VNB enhances the efficiency of those antimicrobials that experience a diffusion barrier in biofilms, while this is not the case for molecules for which there is no diffusion barrier. Furthermore, in most cases, the extent of the enhanced potency after VNB-formation is similar to the enhancement obtained after
forced disruption, thereby showing the effectiveness of the subtle but powerful VNB-approach.

In the previous chapters, we showed that VNB can be used to effectively loosen up the biofilm structure by locally disrupting the bacterial cell clusters. In the last experimental study of Chapter 4, we want to address the second cause of limited biofilm diffusion, in particular the possible binding to or inactivation by constituents of the biofilm matrix, by encapsulating antibiotics into nanocarriers. More specifically, we want to explore whether the disruptive force of VNB can be used to simultaneously disrupt the biofilm structure – as shown in Chapter 2 and 3 – and trigger antibiotic release from light-responsive nanocarriers. In this way, enhanced biofilm eradication might be obtained by light-triggered release of high doses of antibiotics close to the target cells while at the same time loosening up the biofilm structure, thereby providing a complete solution to both problems contributing to the biofilm diffusion barrier. Therefore, tobramycin is encapsulated in two different types of nanocarriers, both able to generate VNB upon pulsed laser irradiation, namely AuNP functionalized liposomes and graphene quantum dots (GQD). Liposomal encapsulation of tobramycin enhances its effectiveness in P. aeruginosa biofilms, however no beneficial effects are observed after laser irradiation and VNB formation. As this is due to premature release of tobramycin upon AuNP functionalization, we next switch to GQD as an alternative light-responsive nanocarrier. Tobramycin can be efficiently loaded on GQD, and is – in contrast to the liposomal formulation – not spontaneously released from its carrier. Furthermore, similarly as observed for AuNP, GQD can locally disrupt biofilms by pulsed laser mediated VNB formation, resulting in enhanced efficacy of co-administered tobramycin. Nevertheless, laser irradiation and VNB formation in tobramycin-loaded GQD particles does not result in enhanced P. aeruginosa biofilm eradication, which was unexpectedly due to tobramycin not being released from the surface of GQD upon laser irradiation. Together our findings show the delicate balance between stability and ability to release antibiotics from light-responsive nanocarriers upon VNB-formation. Even though we were unable to design a suitable nanocarrier for this purpose yet, we believe that future research should re-evaluate the concept in other types of nanocarriers by taking into account the lessons learned from this study.

Finally, Chapter 5 discusses antimicrobial resistance in a broader international context by explaining how bacteria are becoming less susceptible to several antibiotics together with the consequences on healthcare on a global level. An overview is given of different therapeutic approaches aiming to get rid of the protection offered by biofilms, with inclusion of the VNB-approach in this field. We reflect on some pitfalls, provide advice on how to overcome these, and finally list some recommendations for future research in order to further mature the VNB-technology as anti-biofilm approach.