EPS and water in biofilms

Hou, Jiapeng

SUMMARY

Planktonic life is dangerous for most bacterial strains and species and adhesion to surfaces is often considered a survival mechanism. Once adhering, a cascade of events is triggered that involves amongst others, the production of an extracellular matrix leading to the formation of what is generally called a “biofilm”. Biofilms grow on almost every surface to cause various clinical, industrial and environmental problems. Complete killing or removal of biofilms is often difficult because bacteria protect themselves in a biofilm mode of growth within a self-produced matrix that is composed of extracellular polymeric substances (EPS) and water. However, effective treatments against this protective mode of growth remain to be developed. Therefore, this thesis aims to study the fundamental mechanisms of biofilm resistance to physical stress and the role of EPS and water. To this end, both microscopic and spectroscopic methods were applied on biofilms of different bacterial strains in order to study the distribution, structure and function of EPS and water in biofilms under various environmental stress conditions.

In **CHAPTER ONE,** we hypothesize about the way bacteria become aware of their adhering state. We consider that bacteria deform under the influence of the adhesion forces exerted upon them when adhering to a substratum surface. Surface enhanced fluorescence has convincingly demonstrated the existence of minor cell wall deformation upon adhesion that can act as a trigger for an adhering bacterium to start EPS-matrix production, as a hallmark of the transition between planktonic and sessile phenotypes. For staphylococci, EPS-matrix production appears related with the magnitude of the adhesion forces felt. The importance of the EPS-matrix is ubiquitously present throughout the entire process of biofilm development, from facilitating initial bacterial adhesion to maintaining biofilm structural integrity during growth and offering “back up” resources in case of nutrient depletion during biofilm aging. During the entire biofilm life cycle, EPS protects the biofilm against chemical attacks such as from antimicrobial treatment and through its viscoelastic properties, against mechanical stresses. The viscoelastic response of biofilms to external mechanical stresses can be modeled using three Maxwell elements representing the flow of water, more viscous EPS and bacteria repositioning in a deformed biofilm. Bacterial repositioning is more prominent in open structured biofilms than in more condensed ones with an impact on antimicrobial penetration and detachment. The main advantages of stress relaxation to determine biofilm structure and composition over microscopic techniques are that it yields quantitative data covering an area of several square millimeters. Biofilms are also slippery due to their EPS-matrix, but whether the slipperiness of biofilms serves any physiological role or not is unknown. Summarizing, this chapter reviews the events occurring during the transition from bacterial adhesion to EPS-matrix production and biofilm formation from a physico-chemical perspective, offering new concepts like bacterial adhesion force-sensing and cell wall deformation as a trigger for the development of sessile bacterial phenotypes.

In order to further understand the role of water in biofilms, we first studied water as occurring on a surface under physical stress. To this end, we applied a tribochemist, consisting of a tribometer and FTIR spectrometer to study the relation between mechanical sliding stress and IR absorption characteristics of water on a surface (**CHAPTER TWO)**. Water absorption spectra were studied on germanium (Ge) and silicon (Si) crystals under mechanical sliding stress. Surface-thermodynamic analyses suggested that water molecules bind to both surfaces with their hydrogen groups. XPS showed that Ge-crystal surfaces

providing optimal lubrication, were comprised of a mixture of –O and =O functionalities, while Si-crystal and quartz surfaces solely possessed –O functionalities. Comparison of infrared absorption-bands of the crystals in water indicated less bound water layers on hydrophilic Ge- than on hydrophobic Si-crystal surfaces, while absorption-bands for free water on the Ge-crystal surface indicated a much more pronounced presence of structured, free water clusters near the Ge-crystal than near Si-crystal surfaces. Therefore, we concluded that the presence of structured, free water clusters is essential for water-based lubrication. Prevalence of structured water clusters can be regulated by adjusting the ratio between surface electron-donating and electron-accepting groups and between –O and =O functionalities.

Next, in **CHAPTER THREE**, we compared the responses of staphylococcal biofilms of an EPS producing (ATCC 12600) and non-EPS producing (5298) *Staphylococcus aureus* strain to fluid shear and mechanical sliding stress. Confocal Laser Scanning Microscopy (CLSM) confirmed absence of calcofluorwhite-stainable EPS in biofilms of *S. aureus* 5298. ATR-FTIR spectroscopy combined with tribometry indicated that the polysaccharide production per bacterium in the initial adhering layer was higher during growth at high shear than at low shear and this increased EPS production extended to entire biofilms, as indicated by tribometrically measured coefficients of friction (CoF). CoFs of biofilms grown under high fluid shear were higher than when grown under low shear, likely due to wash-off of polysaccharides. Measurement of a biofilm's CoF implies application of mechanical pressure that yielded an immediate increase in polysaccharide band area of *S. aureus* ATCC 12600 biofilms due to their compression that decreased after relieving pressure to the level observed prior to mechanical pressure. For biofilms grown under high shear, this coincided with a higher %whiteness in Optical Coherence Tomography (OCT) images indicative of water outflow, returning back into the biofilm during stress relaxation. Biofilms grown under low shear however, were stimulated during tribometry to produce EPS, also after stress relieve. Knowledge of factors that govern EPS production and water flow in biofilms will allow better control of biofilms under mechanical challenge and understanding of the barrier properties of biofilms toward antimicrobial penetration.

The whiteness analysis of OCT images applied in **CHAPTER THREE** cannot be quantitatively related with the measured biofilm components due to the auto-scaling applied in OCT-instruments to ensure optimal quality of individual images. In **CHAPTER FOUR**, we developed a method to eliminate the influence of auto-scaling in order to allow quantitative biofilm comparison 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 the different biofilms, 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, therewith quantitatively validating the re-scaling developed. Herewith the proposed re-scaling of the whiteness distributions in OCT-images significantly enhances the possibilities of OCT biofilm imaging.

Water is essential to biofilms but its role has been underestimated. Therefore, the role of water is summarized in **CHAPTER FIVE**, based on literature and the contributions of this thesis to the existing literature. Between 77-97 wt% of biofilms mass is due to water, occurring in channels, pores and bacterial clusters. Nevertheless, water in biofilms is a largely understudied topic and therefore this review focuses on how water in biofilms can be detected, the structural features of biofilm in which water is retained and the functions water fulfills in a biofilm. Dry-weight comparison with the weight of hydrated biofilms, FTIR and Raman micro-spectroscopy are the only techniques to affirmatively identify and quantitate water in biofilm structures, while NMR, microscopic and other imaging techniques do not yield directly quantitative results or rely on the assumption that channels and pores are indeed water-filled. Definition of “channels” and “pores” in the literature is rather loose, while moreover whether or not a channel can perform its transport function depends on the size of the molecule or particle to be transported. This review proposes a minimal channel width of three times the Debye-Huckel length to prevent electrostatic interactions of particles or molecules to be transported with the channel shores and a channel width to length ratio smaller than 0.3 to justify its transport function. Different than channels, this review attributes a storage and buffer function to pores.

FUTURE PERSPECTIVE

Whereas this thesis has focuses on the overall presence of water and EPS in biofilms, biofilms are known to be heterogeneous and so is the distribution of water and EPS. This heterogeneous distribution and its mapping seem like a worthwhile continuation of this study and might be done with Raman micro-spectroscopy, possibly combined with a tribometer. Moreover, it will be a challenge to develop a whiteness analysis method based on 3D, OCT images in order to study the distribution of water channels and pores in a biofilm matrix, as well as their structural changes under physical stress. Since water channels also facilitate the penetration of antimicrobials, it will also be interesting to study the relation between local antimicrobial concentration within biofilm matrices and the biofilm structural response to that chemical stress.