Chapter 1

General introduction

Bacteria are among the most resilient organisms on the planet. They can easily adapt their metabolic and genetic activity to survive in their local environment. Different bacterial species can live in a large range of humidities, pHs, temperatures, oxygen levels, and nutrient availability. Not only can bacteria survive in a large range of environments, but also they possess the ability to evolve by mutation to obtain a strategic equilibrium with their environment (10,12,21). Despite these advantages, bacteria are rarely found isolated from community structures known as biofilms. Biofilms have a complex extracellular matrix and depending on the biofilm and its environment develops in five steps (37), which include initial bacterial adhesion, anchoring of the bacterium to the surface, formation of microcolonies including production of extracellular polymeric substances (EPS), formation of macrocolonies with three dimensional structures (i.e. mushrooms), and detachment intended for re-colonization.

It is currently estimated that over 60% of all human infections and 80% of bacterial infections, treated by physicians are due to biofilms (19,40). While some bacteria have a symbiotic relationship with their host (16,29,45), others are virulent (8,30,31) and in some cases fatal (3). The versatility of bacteria and their formation into biofilms allows bacteria to be found on most surfaces, including nutrient deficient abiotic biomaterial surfaces. Once a biofilm forms, it becomes significantly more difficult to remove or to kill the bacteria in the biofilm (48). In order to treat biomaterial associated infections different mechanisms can be used: prevention of bacterial adhesion, killing of adhering bacteria and therewith preventing biofilm formation, and killing and/or removing of a mature biofilm (7). So far previous research has been extensively focused on preventing

bacterial adhesion using surface coatings antagonistic with bacterial surfaces (33) and developing antimicrobial surface properties for killing the adhering bacteria (36,44). However, there are locations, such as the oral cavity, where an initial healthy biofilm can be subsequently change into much more pathogenic biofilms, thereby rendering these initial prevention methods irrelevant. For these cases, it is necessary to find a method to control biofilms after they have been formed. Before we can focus on biofilm removal we first need to know how to control a biofilm, and controlling a biofilm requires knowledge of the complexity within biofilms (11).

The EPS matrix of a mature biofilm is comprised of multiple compounds including, but not limited to, polysaccharides, proteins, and extracellular DNA (5,17). EPS has been characterized as one of the fundamental structural components in a biofilm, and the properties of these substances have been investigated by many studies (26,32,46). Therefore, removal of mature biofilms requires the disruption of this fundamental structural component, because killing of the biofilm alone is not enough. Killing over 95% of *Pseudomonas aeruginosa* in biofilms did not prevent the EPS matrix from mechanically recovering from yield stress (28). Along with the structural foundation provided by EPS, biofilms also have an increased resistance to antimicrobials. A multitude of mechanisms are suggested for the increased antimicrobial resistance including an increased mutation rate (14), formation of antimicrobial degrading enzymes (23), endogenous oxidative stress (4), phenotypic changes (35), and metabolic states (6). Many of these mechanisms are also associated with EPS, further emphasizing its importance in biofilm survival. Physical associations of antimicrobial penetration have been

linked to components of biofilm structure, such as EPS, but no single association was capable of explaining all of the observed phenomena (1,41) due to heterogeneous microenvironments within the biofilm. However, physical properties related to components of biofilm structure may be an alternative.

Biofilms are naturally visco-elastic containing both elastic and viscous properties (25,42), and their elastic relaxation times are largely uniform amongst different bacterial biofilms (39). In addition to its structural relevance, the EPS matrix is largely responsible for the visco-elastic response to stress (18,46). The visco-elastic response of biofilms is measured using one of two methods (creep or stress relaxation) and modeled using a combination of springs and dashpots which are either in series or parallel (Fig. 1). The movement of a biofilm under constant force, or creep, is modeled using the Burger parameters (24) or the Voigt elements (27). The measure of load at a constant distance, or stress relaxation, is modeled using the Maxwell elements (9,28). Herein, external stress using a uni-axial compression device is applied to a variety of biofilms to measure their mechanical properties and subsequent stress relaxation.

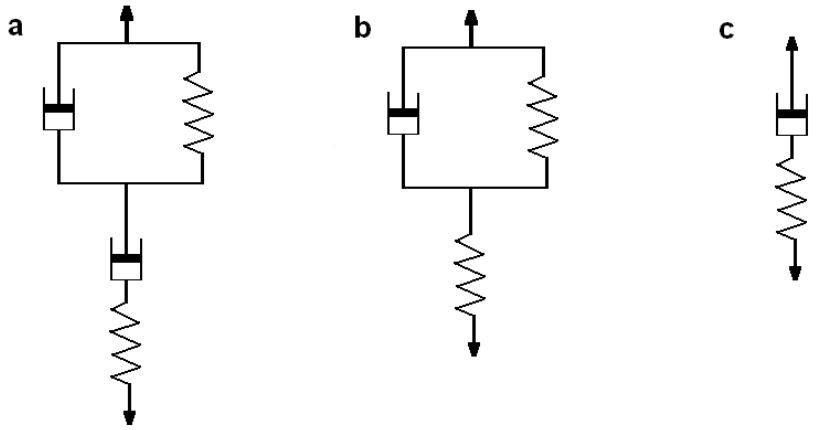

Figure 1. Orientation of springs and dashpots used to model visco-elastic properties in biofilms. a) Burger parameter b) Voigt element c) Maxwell element.

The best way to study biofilms is to obtain naturally occurring biofilms from their flourishing environments. One of the most commonly occurring biofilms in the human body is dental plaque, yet obtaining large samples of intact natural dental plaque in a reproducible manner is both difficult and expensive in both time and money. Biofilm models, including those for dental plaque, focus on replicating the natural environment. However, it is very difficult or almost impossible to have a purely natural model (47). Biofilm growth models involve cycles of feast and famine (20), use of a chemostat (22) or use of a flow system (15). Although an *in vitro* model with a completely natural environment may be out of reach, we hypothesize that comparing the mechanical properties of *in vitro* biofilms to those of naturally occurring biofilms maybe a valid criterion to validate the correlation to naturally occurring biofilms. Mechanical properties, such as stress relaxation, have an advantage over metabolic and compositional properties, as they are more consistent over a broad

spectrum of bacterial biofilms (39). However, mechanical properties remain abstract and have not yet been associated with biofilm structures or physical properties such as antimicrobial penetration.

A distinct difference between naturally occurring biofilms and those created *in vitro* are the laboratory techniques used in harvesting and growing bacteria and biofilms. Centrifugation is a commonly used laboratory technique to harvest and wash planktonic bacterial cells after culturing from frozen bacterial stocks. Centrifugation, in essence, involves compacting bacteria into a pellet, causing collisions and shear forces on bacterial cell surfaces, possibly altering bacterial surface properties. A wide variety of forces (roughly ranging from 1000 to 12000 × *g*) (2,38,43) have been used without mentioning a reason for a particular choice. High centrifugation speeds are currently preferred to collect as many bacteria as possible, verifying for bacterial cell viability but assuming it does not cause any other bacterial cell damage (13,38). Yet, studies have shown that both surface charge (34) and virulence (2) of microorganisms could be affected from high speed centrifugation. Therefore, accurately modeling mechanical properties of naturally occurring bacterial biofilms requires further investigation into the causes of centrifugal damage.

The aim of this thesis is to better understand the visco-elastic properties of mature biofilms. Therefore we have investigated the following questions:

1- Are laboratory techniques important for the natural growth of bacteria and formation of biofilms.
2- Is there a particular biofilm constituent which is responsible for the visco-elastic properties of a biofilm.
3- Can visco-elastic properties of a biofilm be used for the prediction of antimicrobial penetration.

This has been achieved by measuring the visco-elastic properties of *in vitro* biofilms of *Streptococcus oralis*, *Actinomyces naeslundii*, *Pseudomonas aeruginoa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus mutans* under different growth conditions. While growing *in vitro* biofilms the effect of surface damage due to centrifugation on bacterial properties and on the visco-elastic properties was also studied.

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