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# Development of an in vitro soft tissue infection model related to percutaneous medical devices

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MADELEINE WALLERSTEDT

Department of Chemical and Biological Engineering
*Divison of Molecular Biotechnology*
CHALMERS UNIVERSITY OF TECHNOLOGY
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Development of an in vitro soft tissue infection model related to percutaneous medical devices, MADELEINE WALLERSTEDT
Department of Chemical and Biological Engineering, Chalmers University of Technology

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## Abstract

Biomaterial associated infection (BAI) is a common and serious complication in permanent implant situations or other long-term tissue-biomaterial interactions. Established infections are frequently persistent and hard to fight with current antimicrobial approaches. Formation of bacterial biofilm on the biomaterial surface is generally considered the key factor but recent studies have come to question the strong focus on surface adhered bacteria. It is suggested that bacterial colonization of tissue surrounding the device may have more importance to the persistent nature of BAI. Patients with percutaneous medical devices (e.g. external fixators and intravascular catheters) are extra sensitive to BAI. It is also well recognized that biomaterial presence itself reduces immune system efficiency. As a consequence there is a need for more research on the role of infections related to percutaneous medical devices and other BAI. This requires relevant infection models for in vitro studies to reduce the distance to animal models when testing novel antimicrobial modifications of biomaterials. However, there seems to be a lack of in vitro infection models that simulate the condition of the soft tissue surrounding percutaneous medical devices and other biomaterials.

This master thesis describes an early phase development of a novel in vitro model simulating a soft tissue infection related to percutaneous medical devices. This model makes it possible to analyze bacterial colonization of both the biomaterial and the surrounding medium mimicking soft tissue extra cellular matrix. The model comprises a collagen matrix in which a contaminated model biomaterial is incubated. Segments of silver-coated and non-coated silicon urinary catheters were used as model biomaterials. In order to mimic a contamination during a surgical procedure, the catheter segments were submerged for a short time in a diluted bacteria culture before insertion in the collagen matrix. This study included the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Bacterial quantification was performed primarily with plate count method.

Even small concentrations of the inoculation solution ($10^3$ cells/mL) resulted in bacterial growth on both the model biomaterial and in the surrounding collagen matrix. No significant antimicrobial effect was observed for the silver coating although a tendency to lower amount of bacteria was found on the coated catheter. In the surrounding media bacterial growth was similar for both materials.

More work is of course needed to develop such in vitro infection model related to percutaneous medical devices. With a model which does not discard any bacteria during the experimental steps it is possible to observe where the bacteria are located and to which amount, both on the biomaterial and in the surrounding medium. It is likely that this in vitro model better mimic the challenges for such material in the clinical situation.

Keywords: Infection, biofilm, biomaterial, in vitro model, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

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## Abbreviations

| | |
|---|---|
| BAI | Biomaterial Associated Infection |
| CFSTR | Continuous Flow Stirred Tank Reactor |
| CFU | Colony Forming Unit (a bacterial quantification measurement) |
| CLSM | Confocal Laser Scanning Microscopy |
| D/E | Dey-Engley broth (a silver neutralizer agent) |
| ECM | Extracellular Matrix |
| EPS | Extracellular Polymeric Substances |
| MBEC | Minimal Biofilm Eradication Concentration |
| MHC | Mölnlycke Health Care |
| MTP | Microtiter Plate Based (here a category of biofilm models) |
| PBS | Phosphate Buffered Saline |
| PFR | Plug Flow Reactor |
| PHMB | Polyhexamethylene Biguanide (an antimicrobial agent) |
| PW | Peptone Water |
| SEM | Scanning Electron Microscopy |
| SWF | Simulated Wound Fluid |
| TSA | Tryptic Soy Agar |

# 1 Introduction

Biomaterial Associated Infection (BAI) is a common and serious complication in permanent implant situations or other long-term tissue-biomaterial interactions [1]. Established infections are frequently persistent and hard to fight with antimicrobial strategies used today, often causing patient suffering and device failure [1].

Formation of bacterial biofilm on the biomaterial surface is generally considered the key factor of these troublesome infections [2-4]. Biofilm is a phenotype in which aggregated bacteria and bacterial DNA and proteins are embedded in a matrix of self-secreted extracellular polymeric substances (EPS) [5]. This way of living provides the bacteria with effective protection against attacks of the immune system and antimicrobial treatments [1]. Studies have shown that biofilm bacteria grow more and more resistant with time and can be 100 to 1000 times more resistant to antibiotics than free-living, so called planktonic, bacteria [1, 5]. Biofilm bacteria are also very resistant to the immune response in comparison with planktonic bacteria [1, 5]. In addition it is well recognized that biomaterial presence itself reduces the efficiency of the immune system [1, 6-8]. The relationship between host, a permanently implanted biomaterial and bacteria is often described as a race for the surface between host cells and bacteria [9]. If the host cells reach the biomaterial surface before the bacteria, tissue integration is possible which helps to protect from bacterial colonization with subsequent biofilm formation [9]. According to this generally accepted principle, the most crucial issue in fighting infection is to develop biomaterial surfaces supporting attachment of host cells (when integration is desired) but resisting bacterial attachment [1]. Currently high amounts of antibiotics are the standard treatment for BAI although it may not fully eradicate the bacteria, which in addition may cause antibiotic resistance [1]. Consequently there is a lot of research going on in new strategies to prevent and treat BAI. The focus is currently on approaches to inhibit adhesion of planktonic bacteria to the biomaterial or inhibit the formation of biofilm [1].

Recent studies have however come to question the strong focus on surface adhered biofilm-forming bacteria in BAI research [10-14]. These results suggest that bacterial colonization of tissue surrounding the device, so called peri-implant tissue, may have more importance to the persistent nature of BAI [10-14]. Since the immune system gets less effective, especially in the area surrounding the biomaterial, it is a high risk that this area is not cleared of contaminating pathogens [15, 16]. The results of Broekhuizen et al. [12, 14] show for example a higher amount of bacteria colonizing the peri-implant tissue and lower antibiotic susceptibility of these bacteria when compared to bacteria recovered from the implant. Broekhuizen et al. only describe the bacteria found in the peri-implant tissue as bacterial colonization and not biofilm, but it is becoming more and more acknowledged that several infection diseases include biofilm formation not only on solid surfaces as biomaterials or hard tissue, but also in soft tissue [17, 18]. Bacterial aggregates found in the wound bed of chronic wounds have for example been recently recognized as biofilm [17, 18]. Some studies also imply the importance of bacteria persistence inside human immune cells, which further complicates the focus on biofilm formation on the biomaterial surface and suggests that peri-implant tissue functions as an infection relapse source [15, 16, 19].

Patients with percutaneous medical devices (e.g. external fixators, permanent implants, intravascular catheters and glucose sensors) are extra sensitive to infection due to the inability to seal normally around the device and subsequent loss of an innate skin barrier [20-23]. For example are the skin entry points (pin sites) of external fixators [24, 25] and the tissue-device interface of bone-integrated prosthesis [23, 26-29] frequently infected.

As a consequence there is a need for more research on the role of infections related to percutaneous medical devices and other BAI in order to explore new treatment strategies. That in turn requires relevant infection models for in vitro studies to reduce the distance to animal models. To my knowledge there is currently no realistic in vitro infection model to simulate the condition of the soft tissue surrounding a percutaneous medical device. I strongly believe that current in vitro infection models used for these kinds of tests are inadequate because the analysis of biofilm formation is limited to the material [30-32].

# 2 Aim

The objective of this thesis project is to initiate the development of a robust and easy-to-use in vitro soft tissue infection model related to percutaneous medical devices. The intended benefit with the envisioned model is the ability to analyze bacterial colonization of both the biomaterial and the medium representing the surrounding soft tissue. The model's intended use is initial evaluation of novel antimicrobial materials or surface modifications before moving on to animal models. With the benefit of this model I believe that it possible to reduce the distance between in vitro and animal models and improve studies of product efficacy and safety.

# 3 Problem formulation

The model biomaterial is incubated in a collagen matrix after contamination with *Staphylococcus aureus* or *Pseudomonas aeruginosa*. Bacterial survival, growth and localization are subsequently analyzed primarily using plate-spread method. The problem formulation can further be broken down into the following questions:

- What biomaterials and antimicrobial modifications are possible to test?
- How should the biomaterial be inoculated to best mimic a contamination occurring before device insertion in surgical settings?
- How much bacteria will be recovered from the biomaterial and the collagen matrix respectively?
- Is the method good for testing antimicrobial efficiency?

# 4 Background

The background section describes five relevant topics for better understand this cross-disciplinary problem.

## 4.1 Percutaneous biomaterials

A biomaterial is by definition a material that interfaces with biological systems in order to "evaluate, treat, augment, or replace any tissue, organ or function of the body" [33]. Percutaneous medical devices are biomaterials penetrating the skin through a surgically created defect [23]. The concept of percutaneous medical device covers many applications. Some examples are attachment for bone-integrated prostheses, external fixation, dental implants, intravascular catheters, feeding tubes, and glucose sensors [22, 23]. Consequently the different devices are in contact with diverse physiological environments, but nonetheless do the soft tissue of the skin surround them all. All percutaneous devices are a connection between the body interiors and exteriors, which makes the surrounding tissue extra sensitive to infection [23].

The skin consists of three layers: epidermis, dermis, and sub cutis [34]. The epidermis contains many layers of epithelial cells and most of these are specialized to produce the protein keratin, so called keratinocytes [34]. A hardened layer of keratin covers the epidermis and provides a barrier for the body interiors to pathogens and other harmful substances. The dermis contains very few cells in comparison to the epidermis. It is instead dominated by extracellular matrix (ECM) containing various glycosylated and fibrous proteins [34]. Collagen is a fibrous protein and the dominating substance component produced by specialized cells called fibroblasts, the predominant cell type of the dermis. The sub cutis is an insulating layer consisting mainly of ECM and fat cells [34]. After insertion of a percutaneous medical device, a normal wound healing response starts. Epithelial cells migrate to the area in an attempt to seal the defect and may or may not attach to the biomaterial [23]. It is common that the epithelial cells grow towards and along the dermis border in an attempt to extrude the device. In this case, a hollow space is formed around the device that is very sensitive to infection [23]. Infection is also one of the most common reasons to why percutaneous implants fail [23]. A lot of research is going on to establish stable epidermal-biomaterial interfaces in order to avoid device failure [22, 23].

## 4.2 Antimicrobial approaches to prevent biomaterial associated infection

Pre-implantation sterilization, good hygiene, and prophylactic antibiotics are currently the standard approaches for preventing BAI, but these are often inefficient [1]. Oral administration of antibiotics is also the most common way to handle an established BAI although it may not eradicate the pathogens completely [1]. According to Temenoff and Mikos [1] it is of special importance to develop antimicrobial materials in order to reduce infections related to bacterial colonization of the biomaterial after surgery. With respect to the findings of Broekhuizen et al. [12, 14] it is, however, more important to prevent bacterial colonization of the tissue surrounding the biomaterial. There is currently much research in new antimicrobial strategies for preventing BAI, especially related to indwelling catheters and orthopedic implants [1]. Commercially available alternatives to the standard approaches are sustained release of antibiotics or other antimicrobial substances, such as silver ions, from the biomaterial surface [1]. The model biomaterials used in this study are urinary

catheters made of silicon whereof one has an antimicrobial hydrogel coating of ionic silver on both outside and lumen [35]. The silver is released during 30 days into the urinary tract according to the manufacture. The hydrogel also gives a more lubricious surface in comparison with the non-coated catheter [35].

The most common, but unsatisfactory, post-operative treatment to prevent pin site infection is plain gauze wetted with saline [25]. Impregnated dressings on pin sites, or coated pins releasing the antimicrobial agent polyhexamethylene biguanide (PHMB) have however been suggested as alternatives to reduce the risk of pin site infections [25]. Other studies suggest biomaterials releasing the novel and natural antimicrobial agent salicylic acid, which has been recognized to reduce the pathogen's ability to cause disease instead of killing it [36, 37].

Except coatings it is also possible to modify the biomaterial surface in other ways to support integration with tissue and thus prevent infections [1]. It is suggested that the ideal biomaterial should promote host cell attachment and repel bacteria. Important surface properties to consider are hydrophobicity, charge, roughness, and steric hindrance [1]. Materials with positive surface charge have for instance been shown to stimulate bacterial adhesion but prevent bacterial growth [38]. Extruding hydrophilic groups may reject bacterial adhesion and grooves may trap bacteria and support growth [1]. It has been recognized that the most promising biomaterial modifications to prevent formation of biofilms and subsequent infection are surfaces with a positive charge, with low free energy, or covered with polymer brushes [38].

### 4.3 Conventional view on biomaterial associated infections

There is a rule of thumb saying that infection is a bacterial load of $10^5$ cells per gram of host tissue [39]. However this number is strain dependent and based on counts of planktonic bacteria [39]. The number of bacterial cells to create an infection has also become questioned with increasing knowledge about bacterial biofilms [39]. The development from localized bacterial colonization into infection, and its severity, not only depends on amount of bacteria or the present species [39]. Other important factors are the efficiency of the host immune system, the number of and synergy between present pathogen species, and also their virulence (ability to cause disease) [39]. In addition it has been indicated that the maturity of the bacterial biofilm might have more importance than the amount of bacterial cells [40].

Biofilms are now known to play an important role in many persistent human infections [2, 5, 31, 41]. An approximation says that 65-80% of all chronic human infections are related to biofilm formation [31]. Some examples other than BAI are bacterial endocarditis, cystic fibrosis, pneumonia [41, 42] and, more recently recognized, chronic wound infection [17, 43]. There are three different infection categories (mostly used for implant BAI): superficial immediate infection, deep immediate infection, and late infection [1]. Superficial immediate infection develops early after insertion of the biomaterial. It is usually caused by migration of skin-dwelling microbes and an example is bacterial colonization under burn dressings. Deep immediate infection also develops early after device insertion. Skin-dwelling bacteria, which are transported to the implant site during surgery most often, cause this type of infection. Late infections can on the other hand develop months to years after the intervention. The cause is unknown but may be the spreading of a pathogen from another infection in the

body by the blood [1]. As already mentioned the conventional description of the relationship between host, a permanently implanted biomaterial and bacteria is often described as a race for the surface. If the host cells win there may be an appropriate integration of the biomaterial with the body that protects the implant from bacterial colonization [1]. The race is, however, initiated and conditioned by adsorbed proteins. The proteins in the media surrounding the material are thus important for attachment of host cells and bacteria [38].

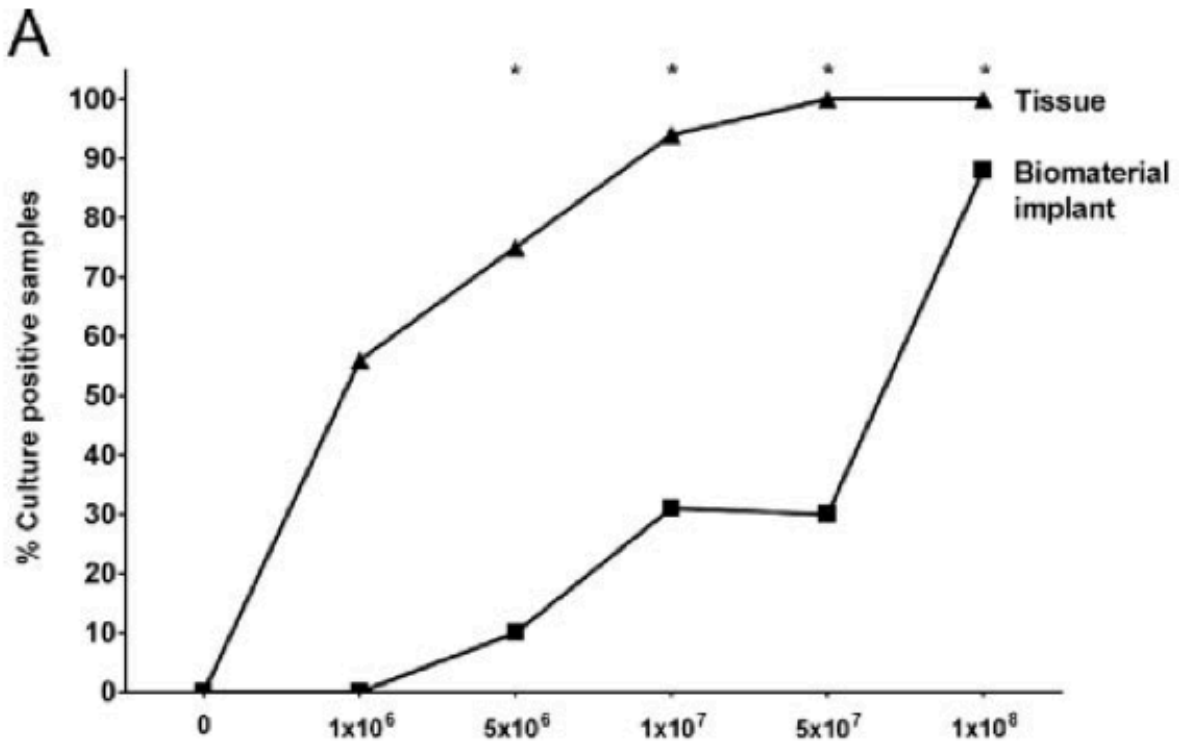
There are generally four stages described in a biomaterial-associated infection: attachment, adhesion, biofilm formation and dispersion [1]. Attachment to the biomaterial surface is reversible and caused by non-specific interactions such as hydrophobicity and surface charge. Adhesion is non-reversibly attachment to the surface and is the result of a combination of non-specific and specific interactions between the pathogen and the biomaterial surface. Non-specific interactions are based on the bacterial and biomaterial surface properties hydrophobicity and charge, whereas specific interactions are based on receptor-ligand bindings. The adhesion takes a few hours to develop and biofilm formation usually starts within a day from the initial attachment step [1]. Several different microbes can inhabit the same biofilm. In the dispersion stage may shear forces, for example from flowing blood, spread biofilm pieces locally or further away in the body [1]. It is recognized that bacteria form biofilms for several reasons; the EPS provide a stable environment to grow in; catalytic functions are facilitated when the biofilm drive the bacteria closer; and the biofilm is a protection to environmental stresses (toxins, UV-radiation, phagocytosis etc.) [44]. It is important to remember that all biofilms do not cause infections and that this phenotype also exists within the natural flora of the body. Two examples are dental plaque and the bacterial lining of the intestines [43].

Surface properties of different bacterial species vary and influence the interaction with the biomaterial surface [1]. Gram-positive and Gram-negative are the two major classes of bacteria [1]. A phospholipid membrane and a thick peptidoglycan layer surround Gram-positive bacteria, whereas Gram-negatives have a phospholipid membrane and a thin peptidoglycan layer surrounded by yet another membrane, the so-called lipopolysaccharide layer. Both classes contain different macromolecules that can play a part in the specific interactions with biomaterial surfaces and host tissue [1]. Environmental conditions can also have an impact on these interactions. A bacterial laboratory strain may have different surface properties than the original wild type strain [1]. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are together with *Staphylococcus epidermidis, Enterobacteriaceae* and fungi the most common pathogens related to biomaterial-associated infections [1]. *P.aeruginosa* (Gram-negative) and *S. aureus* (Gram-positive) are two pathogens well known for their connection to chronic infections and biofilm formation [17]. PA01 is a common laboratory wild type strain of *P. aeruginosa* used in this study. With increased bacterial population density it produces a toxin called pyocyanin. Pyocyanin is a virulence factor with a characteristic green color which may be used as an indication that cell density is high and that the bacteria has started to develop towards the biofilm phenotype [45].

### 4.4 Questioning conventional view on biomaterial associated infections

Some investigators question the strong focus on biofilm formation on the biomaterial [12, 14]. Formation of biofilm on the device surface is of course important when considering device functionality but bacterial colonization of the tissue surrounding a biomaterial might have more importance to the infection persistence. The diagram in figure 1 is from the

results of an in vivo mouse study performed by Broekhuizen et al. [12, 14]. With low bacterial loads they could only recover culturable cells from the peri-implant tissue and not from the implant. At higher doses more colony forming units (CFU) were cultured from the peri-implant tissue than from the implant itself. They also found that bacteria persisted longer in the tissue than on the implant [12]. In a study on implants retrieved from deceased patients the investigators could also observe a lower susceptibility to antibiotics for the bacteria residing in the surrounding tissue than for the bacteria recovered from the implant [14].




**Figur 1. This image is retrieved from a mouse study performed by Broekhuizen et al. [12]. The diagram illustrates the percentages of culture-positive samples recovered from peri-implant tissue and the implant itself (collected result after 5 and 14 day respectively of incubation) after bacterial challenges (*Staphylococcus epidermidis*) between $10^6$ and $10^8$ CFU injected into implant site.**

It is estimated that 99.9% of all non-laboratory grown bacteria reside as biofilms [46]. Some investigators therefore strongly speculate that natural bacterial inocula of host tissue is "broken off pieces" of already mature biofilms, not necessarily containing a high amount of cells. For these reasons they suggest low cell-number biofilm to be used as inocula of BAI-models [47]. The culturing of biofilms with low number of cells does however require special equipment and is limited to solid surface substrates [47].

## 4.5 Model systems to study biofilm related to biomaterial associated infections

According to the result of my literary research, there is currently no in vitro infection model specialized for percutaneous biomaterials. The focus of in vitro infection experiments related to biomaterials in general is quantification of adhered bacteria to the biomaterial surface [1]. These experiments can either be performed using static or flowing conditions of the growth medium [1]. The bacteria are allowed to attach to the surface for some time and the biomaterial is subsequently neatly washed from all non-attached bacteria [1]. Model

systems for studies of biofilm formation under controlled conditions are essential in order to understand the development of this bacterial phenotype and its resistant nature. In biofilm models there is a distinction, among others, between microtiter plate (MTP)-based, flow displacement based, cell-culture based, and wound simulation, as the in vitro biofilm model systems used today [31]. In addition biofilm can also be grown in the laboratory on tissue engineered or ex vivo tissue [31].

MTP are the most commonly used model systems of biofilm formation due to the benefits of relatively low cost, high throughput, no special equipment or technical competence needed, and also the possibility to change parameters easily and multiplexing (include more than one organism or treatment in one run) [31]. The biofilm grows in the bottom or walls of the microtiter wells or on the surface of a device placed inside the wells. A drawback with MTPs can be the condition of a closed system (please refer to figure 2), which may not be satisfactory depending on what conditions of biofilm formation you want to mimic. A closed bioreactor system means that the environment is not constant, e.g. signaling molecules can accumulate and nutrition run out without regular replacement of fluid [31]. MTPs are often used to investigate effects of surface modification on biofilm formation on biomaterial surfaces [30, 48-50]. The MBEC Assay (Minimal Biofilm Eradication Concentration) is a commercialized product by Innovotech Inc. (Canada) and an alternative MTP where the biofilm is grown on pegs attached to the lid of the microtiter plate. This is mostly a way of testing biofilm eradication agents (as compared to biofilm preventing agents) but according to some investigators it is also a good tool for studying implant associated infections although only biofilm formation on the pegs are analyzed [32, 51]. Innovotech also claims that it is possible to customize the peg material for example investigating the effect of a specific antimicrobial coating for a biomaterial [52, 53].

Flow displacement model systems can be divided into continuous flow stirred tank reactors (CFSTR) and plug flow reactors (PFR) (please refer to figure 2) [54]. In the CSTR there is no change of substrate and product concentrations over time. When the flow rate is higher than the duplication rate of the bacteria, all non-surface-attached (non-sessile) bacteria are washed out [54]. In the PFR system does the feed move in a plug and also here do only sessile cells remain [54]. The benefit of these systems is that they simulate flowing conditions well and they have been useful to test antimicrobial surfaces under flow conditions [31]. The drawbacks are however higher costs and lower throughput because of special equipment and technical competence needed, high amount of substrate needed, and the inability of multiplexing. In addition it is not possible to run co-cultures with immune cells in order to test immunological factors or other in vivo aspects [31].

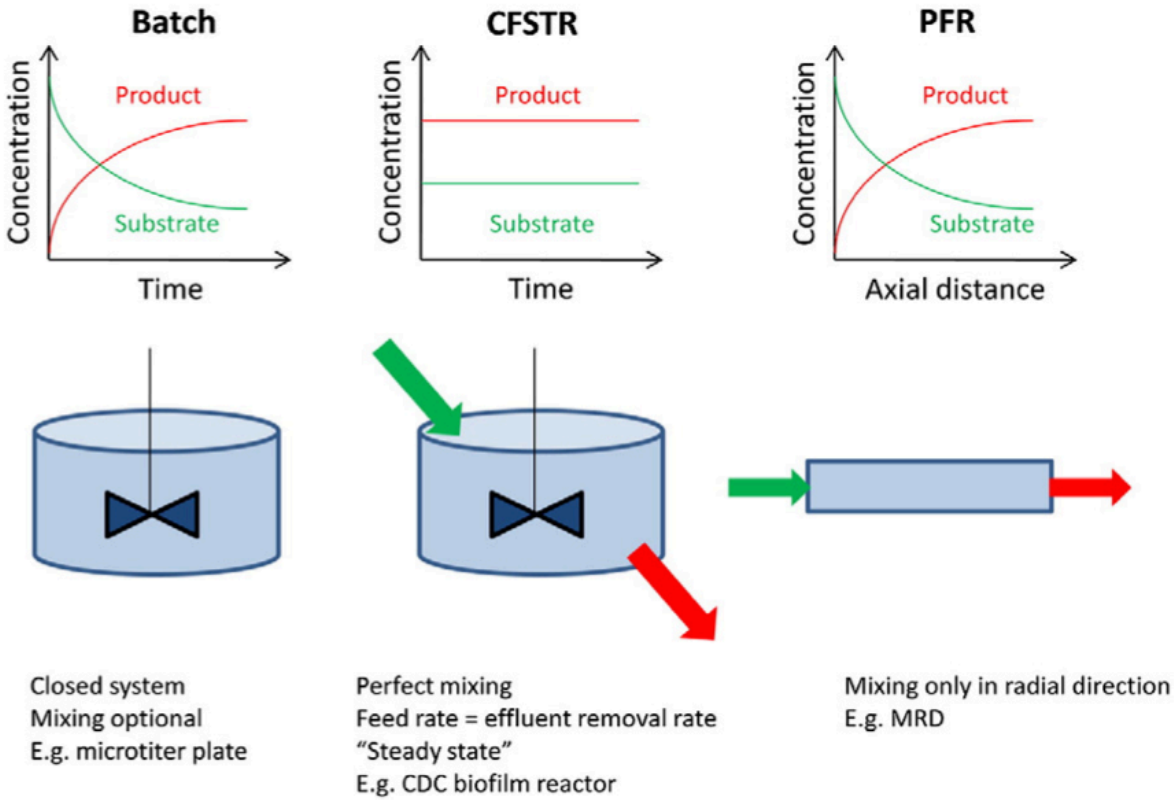



**Figure 2. Models for studies of biofilm formation can be based on different system concepts. This image illustrates the difference between closed batch, continuous flow stirred tank reactor (CFSTR) and plug flow reactor (PFR) systems. In the flow-based systems the growth medium is continuously washed away from the system and only the surface-adhered bacteria remain. [31]**

Biofilms can also form on biotic surfaces and it is possible to use host cells (human or animal derived) in vitro or even ex vivo tissue to mimic the in vivo condition. These cultures make it possible to investigate formation of biofilm and its impact on host cells. Some examples of such studies include static and flow-adhesion in vitro experiments with epithelial cells [55, 56] and ex vivo experiments with human skin [57]. There are also many in vivo biofilm model systems, especially related to BAI. In these the biomaterial can either be pre-inoculated or the animal inoculated for days, weeks or months after the insertion of the device [31]. The latter does however not mimic infections acquired during the surgical procedure [58]. As already mentioned most of the in vivo models are mainly concerned with the formation of surface adhered biofilm bacteria and not with bacterial colonization of the tissue surrounding the biomaterial. In vivo studies with only biofilm analysis of the device are performed for example in mouse and rat models [58, 59].

The biofilms are usually harvested by scraping, sonication and or cycles of sonication and vortexing [31]. The most common method of bacterial quantification is the conventional plate count method. However this is quite labor-intensive and does not allow recover of viable but non-cultivable cells [31]. Other quantification methods are often microscopic visualization with Scanning Electron Microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM), often aided by colored or fluorescent dyes. Some examples of these dyes are crystal violet staining and Syto9 staining (stain both live and dead cells), and dimethylene blue (stain EPS). In addition it is also possible to use bioluminescent strains [31].

When choosing appropriate biofilm model systems it is important to select a system relevant to the questions being addressed and with resemblance to the real-life situation. Other important factors are also individual preferences of the investigators and the available resources [31]. The model platform of this project was chosen based on these factors including the recent recognized importance of bacterial colonization of peri-implant tissue in BAI [12, 14]. The platform is an in vitro biofilm model that can be categorized as a wound or soft tissue biofilm model. This model is developed and described by Werthén et al. [60] and the bacteria are incubated in a collagen matrix in the presence of simulated wound fluid (SWF) including 50% calf serum and 0.1% peptone. Microscopic studies showed that the bacterial aggregates in this model were much similar to bacterial aggregates, most probably biofilm, from clinical wound samples [60]. The model is intended to mimic the collagen rich dermis of the skin and was considered a suitable platform for an in vitro model of infections related to the soft tissue surrounding percutaneous medical devices.

# 5 Materials and method

This chapter describes how the experiments and analyses were performed. The protocol at this stage of the in vitro biomaterial-collagen infection model development is first presented, followed by the specific procedure of all experiments presented in the results section.

## 5.1 Bacterial strains and model biomaterials

Model bacteria used in this study are *Staphylococcus aureus,* ATCC:6538, and *Pseudomonas aeruginosa,* PAO1. Two different types of silicon urinary catheters, Kendall Dover Silver catheter (Covidien) and Bard all-silicone foley catheter (Bard Medical), were used as model biomaterials. The catheters were cut into segments of 2 cm using antiseptic measures.

## 5.2 Preparation of collagen matrices

For 10 mL of the collagen solution (2 mg/mL), the following ingredients were mixed in order in a 50 mL centrifuge tube; 1 mL 0.1% acetic acid; 2 mL of 10 mg/mL collagen stock solution (rat-tail collagen type I, BD Biosciences); 6.0 mL cold SWF (50% fetal calf serum and 50% physiological NaCl in 0.1% Peptone water (PW)); and 1 mL of 0.1 M NaOH. Note that the collagen solution should be mixed gently by carefully turning the test tube upside-down (no vortex) and also that the solution should be kept on ice to avoid polymerization. Subsequently 2 mL of the collagen solution was added to each well of a polystyrene 12-well microtiter plate. For complete polymerization of the collagen solution the plates were incubated at 35 °C for 1 h.

## 5.3 Culture conditions and inoculation procedure of model biomaterial

Batch cultures of bacteria were grown overnight (o/n culture) in SWF at 35 °C. The cultures were washed from proteins by centrifugation and the pellet was subsequently suspended in Phosphate buffered saline (PBS). The washed o/n culture, approx. $10^9$ colonize forming units per mL (CFU/mL), was diluted to two different inoculation concentrations, approx. $10^5$ and $10^3$ CFU/mL for the controlled contamination. In order to confirm the bacterial concentration of the inoculation solution plate count method was performed. Duplicates or triplicates of each biomaterial sample were submerged in 10 mL of inoculation solution for 30 min. The samples were subsequently removed from the inoculation solution and the

outer surface and lumen carefully rinsed with approximately 3 mL of PBS with a 20 mL syringe, excess PBS was “padded” off using a sterile compress. The biomaterial segments were handled, each with separate sterile tweezers, and added to the collagen matrices (one segment for each well) by penetrating the matrices to the bottom of the well (for experimental setup see figure 8) The plates were incubated at 35° C for 24 or 48 h with approximately 90% relative humidity.

## 5.4 Quantification of bacteria

After incubation for 24 and 48 hours the biomaterial pieces were carefully and antiseptically removed and placed in petri dishes and kept in incubator at 35 °C with 90% relative humidity during dissolution of the polymerized collagen. To dissolve the collagen 2 mL of collagenase solution (1 mg/mL in PBS) was added to each well and incubated for approximately 90 min at 35 °C until complete dissolution of the collagen. The biomaterial samples were put into so-called stomacher bags (these are then put into a stomacher machine which uses paddles to drive the microbes from the solid sample into suspension) filled with 10 mL of the silver neutralizer agent Dey-Engley broth (D/E) (room temperature) for 10 min, stomached for two min and then transferred to 15 mL centrifuge tubes. Collagen matrices samples were also transferred to 15 mL centrifuge tubes and the same amount of silver neutralizer was added. All samples were mixed with a vortex followed by centrifugation for 10 min at 4000 g, 20 °C. The resulting pellets were dissolved in 2 mL of 20% SWF in 0.1% PW.

*Plate count method:* Bacterial growth was quantified with the conventional plate count method with serial dilutions prepared in 0.1% PW and spread onto Tryptic Soy Agar (TSA) plates to assess the number of CFU per sample.

*Spectrophotometric method:* In addition a novel evaluation method of bacterial number was used in some of the experiments. This method includes spectrophotometric measurements and resazurin, an oxidation-reduction indicator for bacteria metabolism (Sigma-Aldrich, usually used as a cell viability reagent). In the spectrophotometric method one serial dilution of a control collagen matrices sample (highest estimated bacterial concentration) was made in 20% SWF in 0.1% PW for construction of the standard curve in the resazurin assay. 14 µL resazurin was added for every 1 mL of sample and also to a blank control sample. All samples were subsequently mixed with vortex and quickly added in triplicates to the wells of a polystyrene microtiter plate (96 wells) and put into a spectrophotometer (Synergy 2 Spectrophotometer, BioTek, Gen5 data analysis software). Spectrophotometric measurement of wavelength emission of 570 and 600 nm was performed for 24 h in 35 °C. Data series of 600 nm and also blank control data series were subtracted from data series of 570 nm emission. The data is obtained as curves where bacterial concentrations are proportional to time to reach linear metabolism (corresponds to logarithmic growth phase), i.e. the shorter time to stationary phase, the higher bacterial concentration in the start solution. CFU assessment is also possible with this method by comparing these time points with the standard curve constructed with number of CFU obtained from the plate count method.

## 5.5 Experiments

This section describes how the performed experiments presented in the "Results" section were performed.

### 5.5.1 Inocula attachment test using stomaching and the plate count method

Experiment with *S. aureus* and *P. aeruginosa.* No collagen matrices were prepared but inoculation procedure was performed as described in section *"*Culture conditions and inoculation procedure of model biomaterial*"*. The devices were not incubated in the collagen gel but directly put into D/E, stomached and analyzed with the plate count method.

### 5.5.2 Inocula attachment test using agar plate

Experiment with *S. aureus* and *P. aeruginosa.* No collagen matrices were prepared but inoculation procedure was performed as described in section *"*Culture conditions and inoculation procedure of model biomaterial*"*. The washed o/n culture was diluted to approx. $10^3$ CFU/mL. The inoculated model devices were then rotated on the surface of a TSA plate in a non-overlapping way. A sterile tweezer was used to gently move the device rather than compress it. The number of CFU was assessed after 24 h incubation in 35 °C.

### 5.5.3 Inocula attachment test using turbidity and resazurin

Experiment with *P. aeruginosa.* Inoculation was performed as described in section *"*Culture conditions and inoculation procedure of model biomaterial*"*. The washed o/n culture was diluted to approx. $10^1$, $10^2$, $10^3$ and $10^4$ CFU/mL. Instead of collagen matrices the biomaterial segments were incubated in a 24-well microtiter plate filled with 1.8 mL SWF in each well. 25 µL resazurin was added to every second well. Some wells were also used as blank controls. The turbidity and the color change of resazurin were observed naked-eyed after 24 and 48 h incubation at 35 °C.

### 5.5.4 Biomaterial-collagen model, experiment 1

Batch cultures of *S. aureus* were grown overnight in SWF at 35 °C and diluted to approx. $10^5$ CFU/mL. The procedure was performed as described in section 5.1-5.4. The biomaterial segments were however submerged in 10 mL of the diluted bacteria solution not washed from proteins. In addition the serial dilution was performed in in 0.1% PW. Incubation for 24 h. Bacterial quantification with the plate count method.

### 5.5.5 Biomaterial-collagen model, experiment 2

Experiment with *S. aureus* and performed as described in section 5.1-5.4. The biomaterials were thus submerged in 10 mL of the diluted bacteria solution washed from proteins Incubation for 24 h. Bacterial quantification with the plate count method.

### 5.5.6 Biomaterial-collagen model, experiment 3

Experiment with *P. aeruginosa* and performed as described in section 5.1-5.4. Incubation for 24 h. Bacterial quantification with the plate count method.

### 5.5.7 Biomaterial-collagen model, experiment 4

Experiment with *P. aeruginosa* and performed as described in section 5.1-5.4. Incubation for 24 h. Bacterial quantification performed with spectrophotometric method as described in section *"Quantification of bacteria"*.

### 5.5.8 Biomaterial-collagen model, experiment 5

Experiment with *P. aeruginosa* and performed as described in section 5.1-5.4. Incubation for 48 h. Bacterial quantification performed with spectrophotometric method as described in section *"Quantification of bacteria"*.

# 6 Results

To study the amount of bacterial colonization/biofilm formation on the biomaterial and in the surrounding medium respectively, model devices were contaminated with either *S. aureus* or *P. aeruginosa* and incubated in a collagen matrix. Segments of silver-coated and non-coated silicon urinary catheters were used as model devices. The devices were submerged for a short time in a diluted batch culture of bacteria (named inoculation solution) before insertion in the collagen matrix. Inocula attachment tests were performed without incubation in a collagen matrix to get an estimation of amount of attached bacteria to the model devices after submersion in inoculation solution with various bacterial concentrations. In both the attachment and the model tests the inoculation solution was most often (one experiment deviate) washed from proteins to better mimic a real situation. Proteins in the inoculation solution can initiate and condition the bacterial attachment [1]. Bacterial quantification was performed primarily with the plate count method or with a novel spectrophotometric method.

## 6.1 Inocula attachment tests

Three different inocula attachment tests were performed without incubation in a collagen matrix in order to get an estimation of the amount of attached bacteria to the silver-coated and non-coated biomaterials after submersion in inoculation solution with various bacterial concentrations. (See figure 3-5) The experiments were also performed in order to get an indication of suitable bacterial concentrations, high enough to get bacterial attachment to the biomaterials, but low enough to mimic a real contamination during the surgical procedure. In addition, different methods of quantifying bacterial attachment were investigated. To some extent the effect from time of submersion was also investigated.

The first inocula attachment test used stomaching and plate count. It was performed with both *S. aureus* and *P. aeruginosa*. The purpose was to investigate the amount of bacterial attachment on model devices submerged in an inoculation solution of approximately $10^5$ CFU/mL for 5 and 10 minutes. Figure 3 indicates that 5 or 10 minutes of inoculation in approximately $10^5$ CFU/mL gives bacterial attachment between $10^3$ and $10^5$ CFU on both the silver-coated and the non-coated device. No significant differences can be observed between the different parameters but there are nonetheless small tendencies. There is a tendency to lower attachment with time for *S. aureus* and the opposite for *P. aeruginosa.* There also seems to be a tendency to higher amount of bacterial attachment on the silver-coated device for both bacteria.

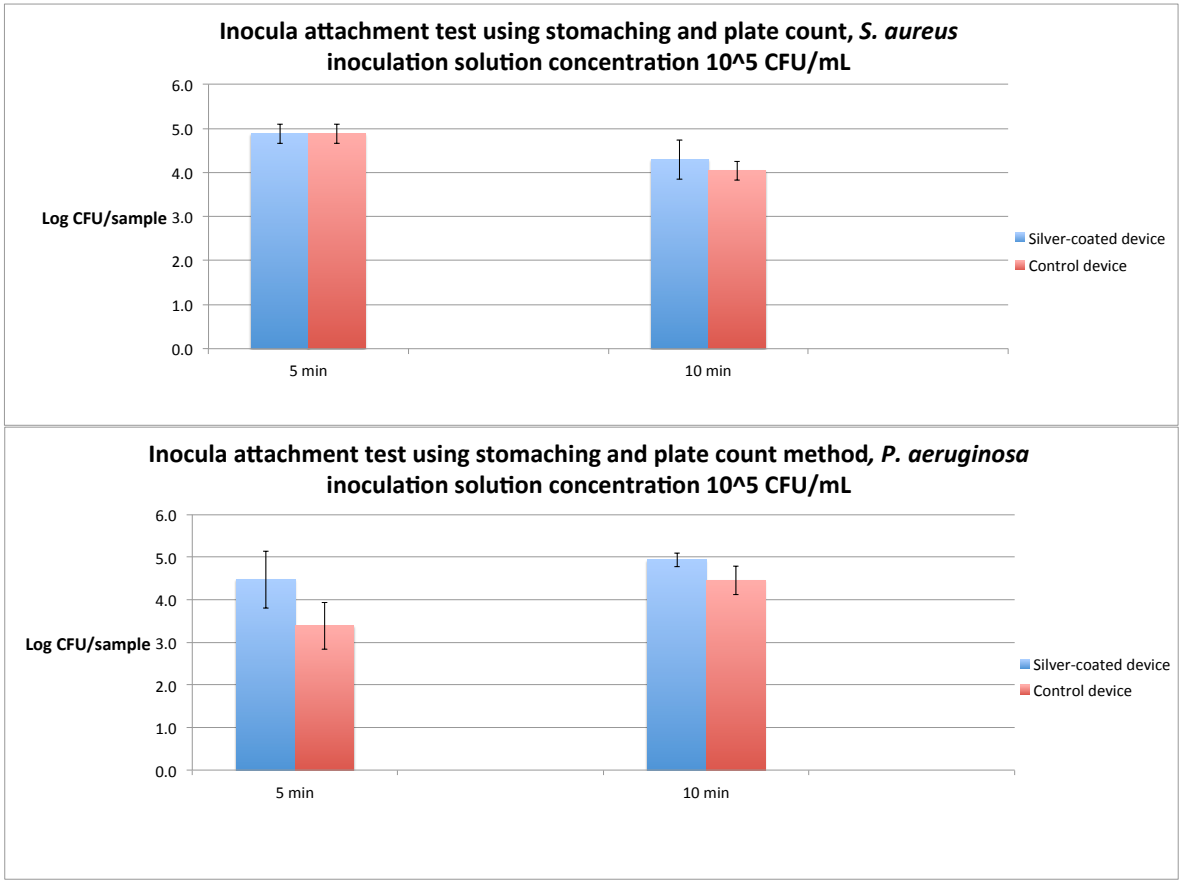



**Figure 3. This figure illustrates the amount of bacterial attachment on model devices submerged in an inocula solution of $10^5$ CFU/mL (washed from proteins) for 5 and 10 minutes. Note that this test was performed without incubation in the collagen matrix. Attached bacteria were recovered using stomaching procedure and quantified with the plate count method. The upper diagram is the result from *S. aureus* inoculation. The bottom diagram is the result from *P. aeruginosa* inoculation. Data is shown as mean value ± SD (n=2).**

The second inocula attachment test was named “inocula attachment test using agar plate”. It was performed with both *S. aureus* and *P. aeruginosa*. The model devices were submerged in an inoculation solution of approximately $10^3$ CFU/mL for 30 minutes. After this step the devices were gently rotated on the surface of an agar plate in a non-overlapping way. The data (see figure 4) indicates that the *S. aureus* inoculated devices results in more CFU on the agar plate after 24 h incubation than *P. aeruginosa*. In addition the result also indicates that silver-coated devices results in more CFU for *S. aureus* when compared to the control device. The control devices do however seem to result in more CFU for *P. aeruginosa* when compared to the silver-coated device.

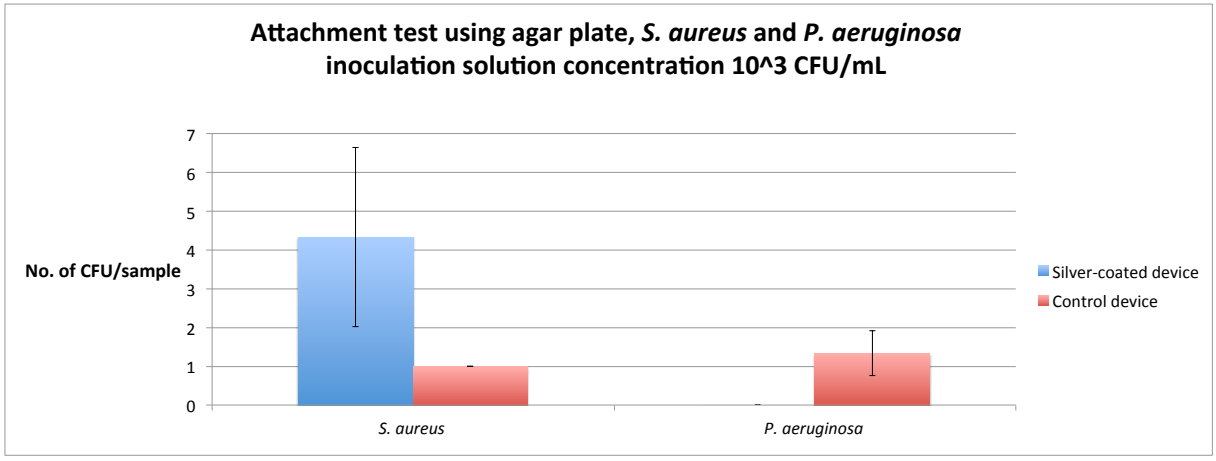



**Figure 4. This attachment test was performed with both *S. aureus* and *P. aeruginosa*. The model devices were submerged in an inoculation solution (washed from proteins) of approximately $10^3$ CFU/mL for 30 minutes. After this step the devices were rotated on the surface of a TSA plate in a non-overlapping way. The number of CFU were counted after incubation for 24 h in 35 °C. Data is shown as mean value ± SD (n=3).**

The last inocula attachment test used turbidity and the color change of resazurin, an indicator of cell metabolism (blue represents no growth whereas stronger red represents more metabolism/growth) to assess bacterial attachment to biomaterials. The test was only performed with *P. aeruginosa*. The purpose was to find an appropriate bacterial concentration of the inoculation solution. The model devices were submerged in one inoculation solution of either $10^1$, $10^2$, $10^3$ or $10^4$ CFU/mL for 30 min. Instead of collagen matrices the devices were incubated in microtiter plate wells filled with SWF. Observations of turbidity and color change took place after 24 and 48 h and are illustrated in figure 5. In addition the *P. aeruginosa* toxin pyocyanin was also used as an indicator of bacterial growth. After approximately 24 hours of incubation it is possible to note some turbidity for the control samples (compared to the blank samples) in the wells with biomaterial inoculated in $10^4$ and $10^3$ CFU/mL solutions. Resazurin does not give any color indication of bacterial growth. After 48 h the well with the silver coated biomaterial inoculated in a $10^2$ CFU/mL solution, show turbidity and pyocyanin formation but no color change of resazurin, whereas the control sample shows the opposite.

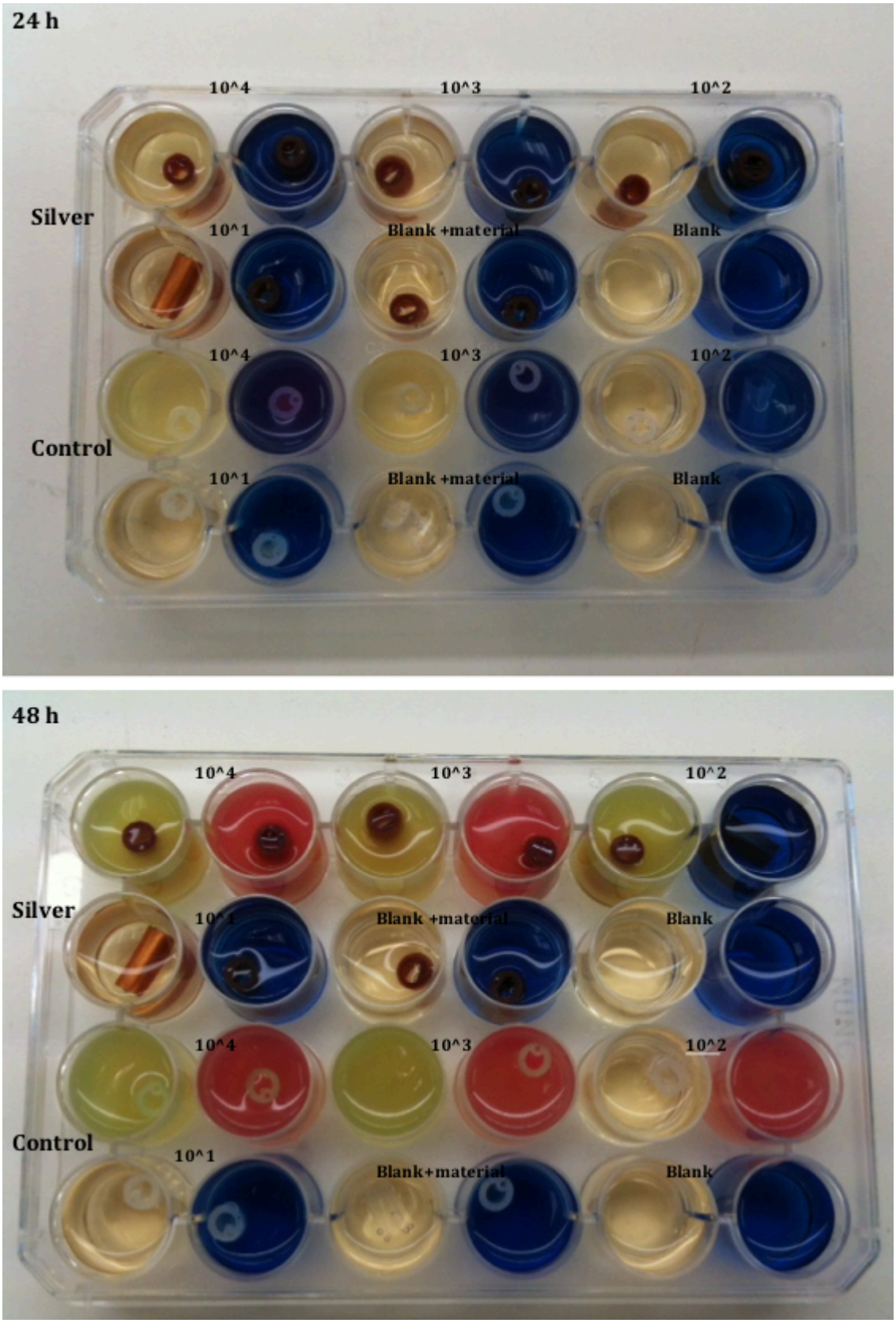



**Figure 5. This attachment test was performed with only *P. aeruginosa*. The purpose was to find an appropriate concentration of the inoculation solution. The model devices were submerged in an inoculation solution (washed from proteins) of either $10^1$, $10^2$, $10^3$ or $10^4$ CFU/mL for 30 min. The devices were incubated in a microtiter plate filled with SWF instead of collagen matrices. To every second well, a blue color indicator for cell metabolism, resazurin was added. The turbidity and the formation of the green bacterial toxin pyocyanin, or the color change of resazurin (blue represents no growth whereas stronger red represents more growth) were observed after 24 and 48 h.**

## 6.2 Biomaterial-collagen model, experiment 1-5

The result from five experiments with different parameters of the biomaterial-collagen model is presented here. The purpose of all experiments was to investigate bacterial growth after a controlled contamination of the device. Bacterial numbers were analyzed both on the model biomaterial and in the collagen matrix, representing the surrounding host tissue. The purpose was also to investigate if any antimicrobial effect of the silver-coated model device could be detected in this test model.

Model experiment 1 and 2 (see figure 6) were performed to investigate the robustness of the model design and to test if proteins in the inoculation solution could affect the outcome. These experiments were only repeated for *S. aureus.* Experiment 1 was performed before the washing step of the inoculation solution had been considered. The model devices were submerged in an inoculation solution, not washed from proteins, of approximately $10^5$ CFU/mL for 10 minutes and incubated in a collagen matrix for 24 h. Stomaching and the plate count method were used for bacterial quantification. Experiment 1 was performed twice to investigate the reproducibility of the model. In experiment 2 the inoculation solution was washed from proteins. All samples resulted in bacterial growth both on the model biomaterial and in the surrounding collagen matrix. No significant antimicrobial effect was observed for the silver coating although a small tendency to lower amount of *S. aureus* is found on the coated catheter, inoculated in a solution without proteins. In the surrounding media bacterial growth was similar for both materials.

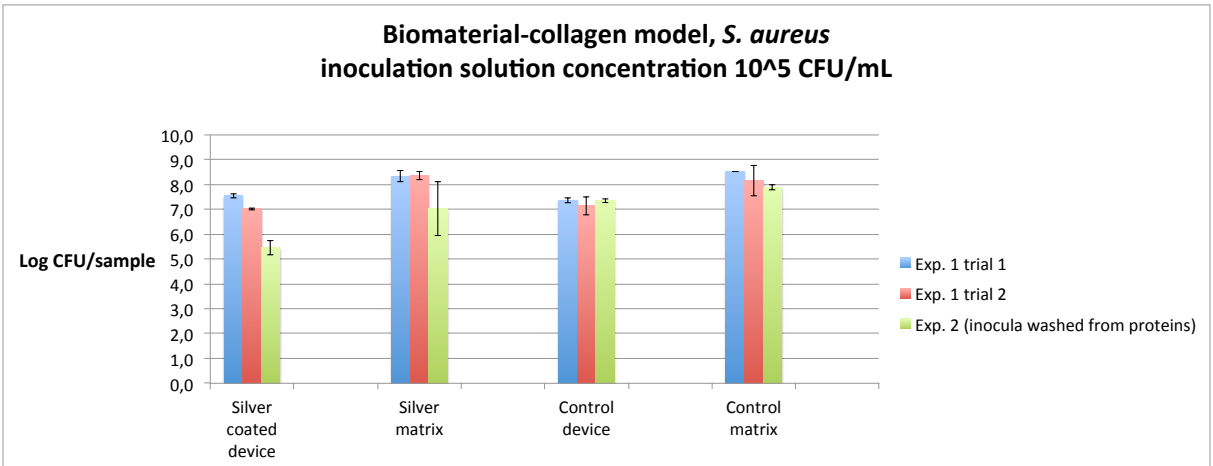



**Figure 6. Amount of recovered CFU from device and matrix respectively after 24 h incubation of *S. aureus* inoculated device in collagen matrix. Bacterial quantification was performed with stomaching and the plate count method. Devices in experiment 1 were inoculated in $10^5$ CFU/mL solution not washed from proteins. Devices in experiment 2 were inoculated in approximately $10^5$ CFU/mL solution washed from proteins. Data shown as mean value ± SD (n=2).**

The test runs in experiment 3, 4 and 5 were performed with *P. aeruginosa.* The purpose was to investigate a lower inoculation solution concentration to better mimic a realistic contamination in a surgical setting. Previous tests had indicated that this could be possible. In addition, beside the plate count method, experiment 4 and 5 include the novel spectrophotometric method for bacterial quantification. Inoculation of the model devices was performed in $10^3$ CFU/mL for 30 min followed by incubation in collagen matrix for 24 or 48 h. The plate count method of experiment 4 and 5 failed, due to too few dilution steps, but the spectrophotometric data could be used for CFU assessments. Figure 7 shows bacterial growth in all samples. This means that even low inoculation solution

concentrations ($10^3$ cells/mL) resulted in bacterial growth on both the model biomaterial and in the surrounding collagen matrix. No significant antimicrobial effect was observed for the silver coating although a tendency to lower amount of *P. aeruginosa* was found on the coated catheter, especially after 48 h of incubation. In the surrounding medium bacterial growth was similar for both materials. Both experiment 4 and 5 had one replicate of the control samples that contained more bacteria than the others, indicated by the green color from the virulence factor pyocyanin (see figure 8).

The same experiments were also performed with *S. aureus,* but bacterial quantification partly failed with both quantification methods (data not shown). The plate count method did however indicate bacterial amount of less than $10^3$ CFU on both devices and less than $10^4$ CFU in all matrices. It was also possible to tell from spectrophotometric data that bacteria were present in all samples when compared to a blank control.

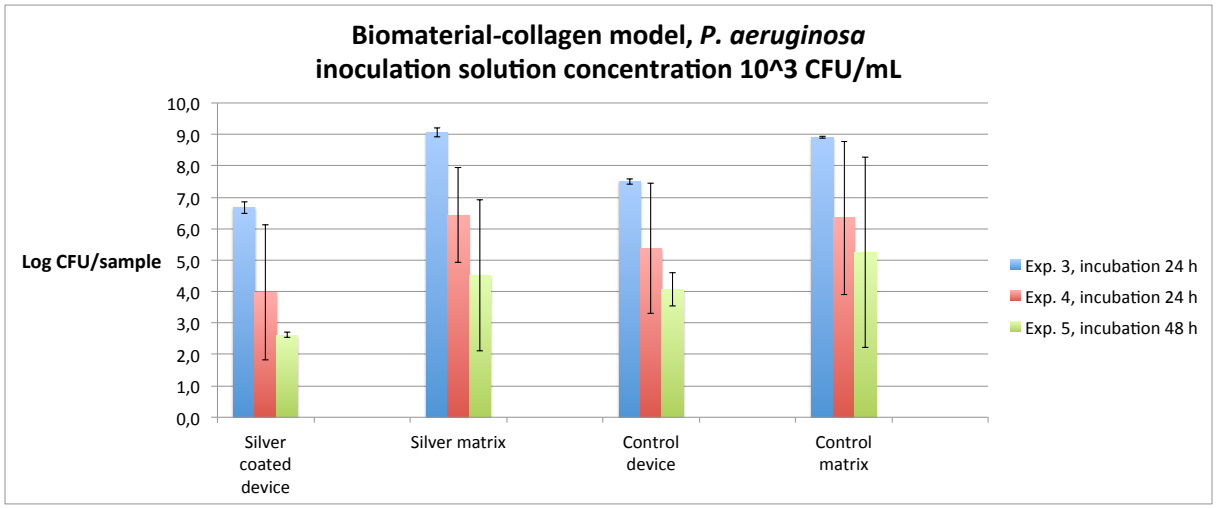



**Figure 7. Number of CFU on the device and in the matrix respectively after 24 h incubation of *P. aeruginosa* inoculated device in collagen matrix. Bacterial quantification was performed with the plate count method in experiment 3 and spectrophotometric method in experiments 4 and 5. The model devices were submerged in an inoculation solution (washed from proteins) of approximately $10^3$ CFU/mL for 30 minutes. Data is shown as mean value ± SD (n=2) for experiment 3 and mean value ± SD (n=3) for experiments 4 and 5.**

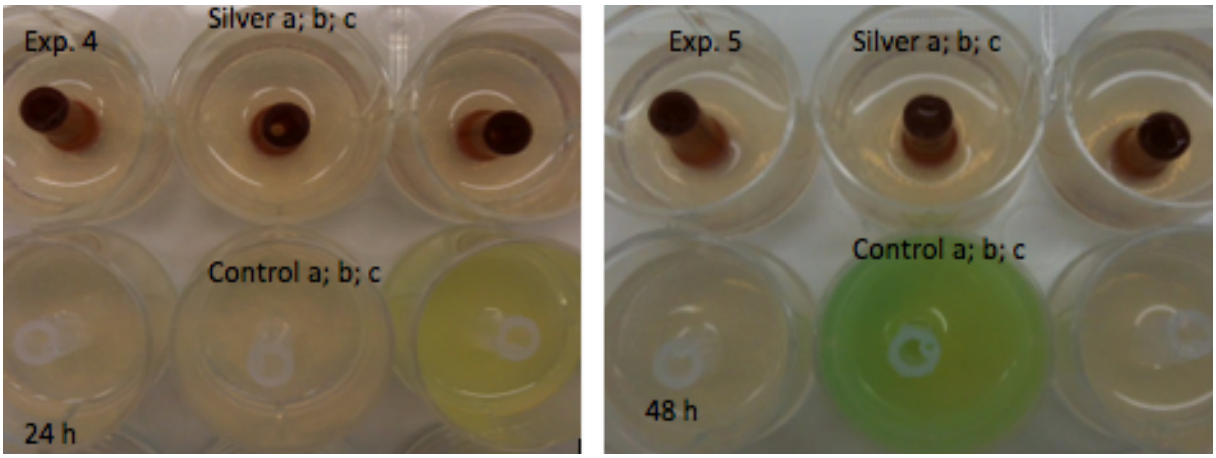



**Figure 8. This figure illustrates the set-up of the biomaterial-collagen model with three replicates. Note that the left image is from exp. 4 with 24 h incubation in the gel and the right image from experiment 5 with 48 h incubation. The model microbe is *P. aeruginosa.* The characteristic green virulence factor pyocyanin of *P. aeruginosa*, is an indication of increased bacterial population density.**

# 7 Discussion

The discussion is divided into four parts, each related to one of the problem formulation questions addressed. Section 7.2. referring to the problem formulation about inoculation of the model devices, also bring up other important procedures in the model such as incubation in the collagen matrix and the bacterial quantification methods.

## 7.1 What biomaterials and antimicrobial modifications are possible to test?

The model biomaterials, the silver-coated and the uncoated silicon catheters were chosen for more than one reason; the products were easy accessible to a relatively low cost which facilitated pilot experiments, one product could in addition be cut into many samples; silver is one of the most common antimicrobial substances incorporated in biomaterials; and it was possible to find a suitable control product. At project initiation it was intended to include more model devices with different antimicrobial approaches but unfortunately due to lack of time and accessibility this could not be done. Future model development work could however comprise testing of other antimicrobial concepts. It would especially be interesting to study concepts considered effective in studies were only bacterial colonization of the device has been investigated. It would also be interesting to elaborate with physicochemical surface properties, for example by impregnating or coating the devices with different substances before inoculation. The effect of biomaterial release of antibiotics and other antimicrobial agents such as PHMB and salicylic acid could also be tested in the model. The effect of pin site dressings impregnated with various antimicrobial agents could be screened in order to find an effective strategy to treat pin site infections.

## 7.2 Inoculation of the biomaterial and other important procedures in the model

*Test microorganisms:* The bacteria used in this study, *S. aureus* and *P. aeruginosa,* were chosen because of their relevance in biomaterial infections. Further the specific strains were chosen due to their approval and accessibility in the laboratory where the experiments were performed. Future development of the biomaterial-collagen infection model could comprise other microbes also relevant in BAI such as *Staphylococcus epidermidis* and *Candida* spp. In these types of tests it is important to include relevant species. For example the surface properties of the microbe can have a great impact on both the interaction with the biomaterial surface (in both the inoculation and incubation step) and the collagen matrix through non-specific and specific interactions [1].

*MTP model system:* A 12 well microtiter plate was chosen to work with because it was easy to prepare the collagen matrix in it and also to apply the model devices. 2 mL collagen solution gives a gel height of approximately 0.6 cm and the 2 cm model devices were pushed down to the bottom of the gel. This approach made it easier to have somewhat better control of the amount of surface area in contact with the gel. There is however a risk that the model devices are put down skewed, which could affect the result. There is also a risk that the pieces fall down into the gel during the application procedure. To better control this, a possible approach could be to create some sort of device support, e.g. a lid with holes or a pin site dressing. Another alternative could be to use customized biomaterial pegs, with or without surface modification, in the MBEC device from Innovotech.

*Inoculation bath:* The available surface area is important in the inoculation procedure of the model devices and can affect the amount of attached bacteria. Therefore it is important to make all the devices in one experiment of the same length. Other surface properties of the material such as hydrophobicity and surface charge of the biomaterial and bacteria also have importance to the degree of attachment. It is recognized that it takes a few hours for the bacteria to develop irreversible adhesion to a biomaterial surface from the initial reversible attachment phase [1]. Attachment is based on non-specific interactions whereas adhesion develops due to a combination of non-specific and specific interactions [1]. From this I interpret that specific interactions become more important with time. For this reason and also because the inoculation solution is free of proteins, I speculate that non-specific interactions might be more important during the 30 min of inoculation.

*Amount of attached bacteria after inoculation bath:* The exact amount of bacteria added to the collagen matrix is not possible to know. Future experiments could, however, involve a step where a lot of replicates are tested to get a significant average value of the bacteria attached during the inoculation. The average value will most likely vary depending on type of surface and bacterial specie. This step could be performed as the first or second attachment tests in this study. It could be beneficial to have an idea about how much bacteria that is incubated in the collagen gel and the difference between the test sample and control. If a novel antimicrobial approach such as positively charged surface, promotes the attachment of certain bacteria but discourage growth [38], the initial bacterial load of this sample could be higher than the control. Even if the approach was effective to some extent, there is a possibility that this would not be shown due to the higher and maybe unrealistic initial bacterial load resulted from the inoculation bath of the sample that promotes bacterial attachment but prevents growth.

*Bacterial concentration of the inoculation solution:* The exact quantification of bacteria on the material was not the purpose of the performed inocula attachment experiments. These were primarily planned to get an understanding of how low inoculation solution concentrations it was possible to use. It was reasoned that this bacterial concentration should be high enough to certainly get bacterial attachment but low enough to better simulate a contamination in a surgical setting. With sterile equipment and hygiene demands it is more likely that a contamination occurring during a surgical procedure involves a small number of bacteria. The inocula attachment test with turbidity and resazurin indicator (see figure 5) was performed in order to find an appropriate concentration of the inoculation solution. It gave an indication that it was possible to use a bacterial concentration of $10^3$ CFU/mL, at least for *P. aeruginosa* in the biomaterial-collagen model. However, the experiment needs to be repeated to draw any firm conclusions. I recommend that future work in the development of the model should comprise more replicates and initially a higher inoculation concentration (about $10^3$ CFU/mL). From there, in small steps, it would probably be possible to find the smallest but most robust (i.e. more reproducible amount of bacterial attachment at least for the same biomaterial or surface modification) inoculation concentration of the devices.

It is suggested that most bacteria (in real life) that inoculate a host probably already exist in the biofilm phenotype [47]. A low cell-number biofilm might be a better approach for inoculation in future infection models [47]. This does however require a more simple way to

grow biofilms containing low cell numbers. In addition with recent findings, suggesting that the maturity of a biofilm is more important than the amount of cells in the outbreak of an infection [40], it could be that the exact bacterial concentration may be of less importance in future inoculation solutions.

*Mimic an airborne contamination:* To mimic a contamination from the air in the surgical setting, inoculation solutions washed from proteins were prepared. Attached proteins to a biomaterial can have a strong influence on the surface properties. Also in order to better mimic the real situation, excess inoculation solution on the devices was removed. Liquid drops of the inoculation solution on the material probably have a much higher bacterial concentration than the dry biomaterial and should not have been there in a "dry contamination".

*Alternative techniques of inoculation:* A way to circumvent the problem of adding different amount of bacteria to the collagen gel could be to investigate alternative methods to device submersion in an inoculation solution. Alternatives to explore in order to develop a more robust inoculation procedure could for example be a recently described technique [61] of spraying an aqueous suspension of bacterial cells onto the biomaterial. Another approach could be direct appliance of bacteria to the matrix. Even if no bacteria would be recovered from the biomaterial, such a model could possibly be used to test the effect of released antimicrobial substances.

*Time of the inoculation bath:* The time in the inoculation bath was chosen randomly but theoretically it was reasoned that a longer "bath" would result in more attached bacteria to the biomaterial. This was investigated to some extent in the attachment tests although a clear influence on amount of bacterial could not be found. The first attachment test (see figure 3) verified that it was possible to use an inoculation solution with bacterial concentration of $10^5$ CFU/mL. It also indicated, to some extent, that time had not much impact on the amount of bacterial attachment at this high cell concentration. No further study on the time aspect was performed in order to justify this because time was at first not considered as important as the inoculation solution concentration. By considering the difference between bacterial attachment (reversible) and adhesion (non-reversible) it could in future experiments be interesting to elaborate with even longer times of inoculation of the biomaterial. This could perhaps also facilitate lower bacterial concentrations of the inoculation solution and also affect the amount of bacteria "leaving" the biomaterial surface during incubation in the collagen matrix.

*Time of incubation in collagen matrix:* The incubation time of the inoculated device in the collagen matrix was set to 24 h since this is the time period often used in the original collagen model for growing biofilm. It is questionable if 48 hours of incubation is relevant to the model since the antimicrobial coating is a more preventative measure for infection. The natural immune system would most likely have more or less effect in preventing the bacterial growth. However, it is interesting to see the development of the bacterial growth after 48 hours and how the conditions in the collagen model after this time period might simulate the conditions of a heavy colonized area in a real life situation. In future experiments it would be interesting to investigate longer incubation times to test

antimicrobial approaches on more mature biofilms and also to test if released antimicrobial substances from the biomaterial may need some time to show any effect.

*Harvesting method:* Stomaching was used to harvest bacteria from the model devices mostly due to practical reasons of accessibility but also that this was possible because the materials were soft. More solid materials are not suitable for stomaching. Other approaches could be only vortexing, or cycles of sonication and vortexing. In future experiments it would be interesting to investigate which harvest method that is most beneficial.

*Bacterial quantification methods:* The plate count method measures bacterial amount in CFU whereas the novel spectrophotometric method gives a measure of bacterial amount based on the bacterial metabolism. The latter method can be interpreted into number of CFU with a standard curve. The spectrophotometric method is still a novel and un-optimized bacterial quantification tool but very promising. It is especially useful in experiments with many samples and replicates, or when it is hard to know which dilutions to spread for plate count. It is estimated (by experience during the development work) that the spectrophotometric model can cover bacterial concentrations from $10^2$ up to $10^7$ CFU/mL (depending on organism tested) in one run. In the biomaterial-collagen model is the content of every well divided in two samples to be analyzed and it can be hard to know appropriate dilutions for plate count. This makes the spectrophotometric method much labor-saving and I would recommend this method in future development, at least for pilot experiments, of the biomaterial-collagen model. In addition the plate count method and other, more illustrative methods of bacteria/biofilm quantification, could be used to verify the results of pilot experiments. Stain techniques (e.g. crystal violet, Syto9, and dimethylene) could for example be used followed by more or less advanced microscopic techniques (e.g. SEM and CLSM).

*Virulence factors:* Some novel antimicrobial strategies, such as virulence inhibitors, do not kill the pathogen but instead reduce its virulence [36]. There might be some analyze method that could be used to investigate the amount of released virulence factors of the bacteria in the biomaterial-collagen gel model. The toxins can however remain, such as the green pyocyanin, even if the bacteria die so an even better study could in the future perhaps involve a combined cell-culture based model with host cells in order to measure host cell response to biofilm formation. An example could be a co-culture with epithelial cells.

## 7.3 How much bacteria will be recovered from the biomaterial and the collagen matrix?

Even small inoculation solution concentrations ($10^3$ cells/mL) resulted in bacterial growth on both the model biomaterial and in the surrounding collagen matrix (See figure 6 and 7). In addition all experiments within this system recovered more bacteria from the matrix than the model device. Due to large deviations both within samples and experimental trials, it is not yet possible to certainly tell if this always is the case. The result does, however, support the importance of investigating bacterial growth/biofilm formation in the surrounding media when testing new antimicrobial approaches. I speculate that the deviations may depend on that the model device contained a lumen where a piece of collagen matrix can get stuck when the device is removed after incubation. A way to get around this problem is to wedge the material into two pieces. This would also make it easier to notice attached matrix substance. This is of course not a problem with other device types without an open lumen.

Deviations could however also result from varying amounts of attached bacteria in the inoculation procedure of the devices.

If the resulted amount of CFU on the agar plate in the second attachment test (see figure 4) is a reflection of the number of attached bacteria, the result indicates that *S. aureus* more easily attach to both the silver-coated and the non-coated device than *P. aeruginosa*. In addition the result in that case also indicates that *S. aureus* attach more easily to the silver-coated device than the non-coated device. The result can for example depend on biomaterial or bacterial surface properties or possibly less tolerance to silver ions for the Gram-negative *P. aeruginosa*. In addition I speculate that this type of test could investigate amount of bacteria that easily loosen from the device and easily could contribute to a contamination of surrounding tissue.

## 7.4 Is the method good for testing antimicrobial efficiency?

Due to large deviations both within samples and experimental trials, it is not yet possible to tell for sure whether the silver coating exerts an antimicrobial effect in the biomaterial-collagen model. A tendency to lower amount of *P. aeruginosa* was however found on the coated catheter, especially after 48 h of incubation (see figure 7). There also seems to be a tendency to lower amount of *S. aureus* on the coated catheter in experiment 2 (see figure 6). There are no marked differences in bacterial amount between silver and control samples. However the model devices are intended for flowing conditions in the urinary tract and it is unclear whether flowing conditions are needed for the silver ion release. The lumen and outside surface of the product is nonetheless covered with the same hydrogel silver coating.

I speculate that in real life when biomaterials, especially permanent implants, are surrounded by soft tissue there is too strong focus on preventing infections by inhibiting attachment, adhesion and subsequently biofilm formation on the biomaterial surface. It has been suggested that a gradual release of antimicrobial substances from a biomaterial surface is important for preventing late infections (upcoming months to years after surgery) [1]. I do however believe, based on the results of this study and Broekhuizen et al. [12, 14], that gradual release approaches are important also for preventing immediate infections.

The results of this thesis showed that when a biomaterial is contaminated and subsequently put into a collagen matrix, simulating surrounding soft tissue, bacterial colonization is not limited to the biomaterial. The results do on the opposite indicate that high amounts of bacteria/biofilm are also found in the surrounding collagen matrix. Although more development work is needed to make the model more robust, I consider this biomaterial-collagen infection model a promising soft tissue infection model related especially to percutaneous medical devices. With this model it is possible to study the effect of novel antimicrobial concepts related to biomaterials surrounded by soft tissue. The added value when compared to other models is especially the ability to simulate the effect of antimicrobial strategies on bacterial colonization of the soft tissue surrounding the biomaterial.

# 8 Conclusion

- What biomaterials and antimicrobial modifications are possible to test?

I believe that many antimicrobial modified biomaterials would benefit from being tested in this type of model due to the unique ability to investigate bacterial colonization/biofilm formation not only on the biomaterial surface but also in the surrounding tissue, especially important in the case of many percutaneous medical devices. For practical reasons this pilot study was however limited to easy available silver-coated and non-coated silicon urinary catheters.

- How should the biomaterial be inoculated to best mimic a contamination occurring before device insertion in surgical settings?

The devices should be inoculated with a bacterial concentration as low as possible and in a protein-free solution to best mimic a contamination during a surgical procedure. A recommendation is to start at a bacterial concentration of approximately $10^3$ CFU/mL and from there optimize the concentration to different biomaterials and bacterial species.

- How much bacteria will be recovered from the biomaterial and the collagen matrix?

Even small inoculation solution concentrations ($10^3$ cells/mL) resulted in bacterial growth on both the model biomaterial and in the surrounding collagen matrix. In the surrounding media bacterial growth was similar for both materials.

- Is the method good for testing antimicrobial efficiency?

No significant antimicrobial effect was observed for the silver coating although a tendency to lower amount of bacteria was found on the coated catheter. More modified biomaterials must be tested to be able to tell if the amount of bacteria in the model is relevant

More work is of course needed to develop this novel in vitro infection model related to percutaneous medical devices. This first pilot study does however show that bacterial growth is not limited to the biomaterial surface even after a low contamination on an antimicrobial device. It is therefore important for this type of biomaterials and the models used to study them, to also focus on the bacterial growth in the surrounding tissue. It is likely that this in vitro model better mimic the challenges for such material in the clinical situation.

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