THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Antibacterial surface coatings for biomedical applications

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CHALMERS UNIVERSITY OF TECHNOLOGY

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Cover: SEM images and flourecent microscopy images of S.epidermidis formed on ELP substrates before and after AMP modification.for SEM images (top row), The bacteria were seeded on ELP coated glass coverslips, fixated using formaldehyde and gold sputtered prior to imaging. For florencent microscopy images (bottom row), The bacteria were stained with SYTO 9 and propidium iodide.

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Abstract

Over the past decades there has been a significant increase in the diversity and function of biomaterials. Today, medical practices utilize a large number of biomaterials in the form of medical devices and implants. However, one major obstacle that limits the efficiency of biomaterials is their susceptibility to develop infection. Biomaterials associated infection caused by adherent biofilms is usually difficult to hinder by means of systemic antibiotic therapy. In addition, the rapid emergence and growth of antibiotic resistant bacterial strains is further limiting the potency of available antibiotics used to treat infections. To address these limitations, different surface modifications are considered to be an effective solution to improve antibacterial performance of biomaterials by hindering biofilm formation.

In this thesis, two different strategies to create surfaces with pronounced antibacterial properties have been developed and evaluated. In both methods, the antibacterial modification has been deposited onto bioactive surface coatings to further improve their future clinical performance. In the first approach, mesoporous titania (MPT) surface coatings were used as antibiotic carriers to investigate the effect of local delivery of the antibiotics Vancomycin, Gentamicin and Daptomycin on the attachment and growth of *S. aureus* and *P. aeruginosa*. MPT thin films with pore diameters of 4, 6, and 7 nm were formed using the evaporation-induced self-assembly method. Reduced bacterial adhesion was observed on the antibiotic loaded surfaces. It was also shown that presence of the pores alone had a desired hampering effect on bacterial colonization. In the second approach, an antimicrobial peptide (AMP), RRPRPRPRPWWWW-NH2 (RRP9W4N), was used to covalently modify elastin like polypeptide (ELP) surface coatings having cell adhesive sequences in its structure. RRP9W4N was immobilized onto ELP surfaces using EDC-NHS coupling chemistry. It was shown that the AMP could retain its antibacterial activity against *S. epidermidis, S. aureus* and *P. aeruginosa* when covalently bonded to ELP. RRP9W4N stability in human blood serum was studied and the results suggested that the AMP could preserve its antibacterial activity up to 24 hours.

The overall results from both surface modification techniques developed in this thesis suggest that they can be considered as promising candidates for the development of antimicrobial surfaces for future biomedical applications.

Keywords: Antimicrobial surfaces, mesoporous titania, antibiotic delivery, antimicrobial peptide, elastin-like polypeptide

List of publications

1. Antibacterial performance of mesoporous titania thin films- Role of pore size, hydrophobicity and antibiotic release
   Saba Atefyekta, Batur Ercan, Johan Karlsson, Erik Taylor, Stanley Chung, Thomas J Webster and Martin Andersson
   Int. J. Nano Med., 2015, 1; 977-990.
2. Antibacterial elastin-like polypeptides coatings- Functionality and stability
   Saba Atefyekta, Maria Pihl and Martin Andersson
   Submitted

Contribution report to the listed publications:

1. Performed all experimental work and wrote the manuscript.

2. Performed all experimental work including elastin-like protein expression and purification, and wrote the manuscript.

Other publications not included in this thesis

1. Controlling drug delivery kinetics from mesoporous titania thin films by pore size and surface energy
   Johan Karlsson, Saba Atefyekta and Martin andersson
   Int. J. Nano Med., 2015, 10; 4425-4436

2. Modulation of nanometer pore size improves magnesium adsorption into mesoporous titania coatings and promote bone morphogenic protein 4 expression in adhering osteoblasts
   Francesca Cecchinato, Saba Atefyekta, Ann Wennerberg, Martin Andersson, Julia Davies, Ryo Jimbo
   Dental Mater., 2016, 32; 148-158

3. Stem cell homing using local delivery of plerixafor and stromal derived factor-1 alpha for improved bone regeneration around Ti implants
   Johan Karlsson, Necati Harmankaya, Anders Palmquist, Saba Atefyekta, Omar Omar, Mats Halvarsson, Pentti Tengval and Martin Andersson
   J. Biomed. Mater. Res. A, 2016, 104A; 2466-2475

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1. Introduction

Despite considerable progress in the development of biomaterials into the healthcare system, the attachment of pathogenic bacteria and subsequent biofilm formation is a significant limitation that undermines biomaterial's clinical performance.[1–3] Today, the post-operative antibiotic therapy is the most common treatment for biomaterial associated infection (BAI). This is while over many years, such antibiotic therapy routines have not only been shown to fail in the treatment of BAI, but has also resulted in a rapid increase in infections caused by bacteria having antiobiotic resistance.[4–7] Moreover, the eradication of impenetrable and resistant biofilms with antibiotics increases the systemic side effects of antibiotics due to long term therapy sections and high dosage. That in turn can further accelerate the amount of antibacterial resistance and subsequent complications for patients.[8–10]

Use of antibacterial surfaces to prevent initial bacterial colonization and biofilm formation is a practical strategy to fight BAI and to replace the need for conventional antibiotic therapies. Research on developing antibacterial surfaces is mainly focused on producing materials loaded with antibacterial agents, antibacterial immobilized surface coatings and surfaces with nanostructures.[11]

The motivation behind my thesis is to design simple and applicable approaches to create antibacterial surfaces to prevent BAI. Today we live in an aging society with increased demand for use of biomaterials. However, very few of them have been designed to be effective against BAI. Moreover, antibiotic resistance is a growing global concern and according to World health organization in 2015, patients with infections caused by drug-resistant bacteria are generally at increased risk of worse clinical outcomes and death.

In part 1 of the thesis, as demonstrated in Figure 1a, local antibiotic delivery from mesoporous titania thin films and the effect of nanoroughness of such thin films on bacteria adhesion were investigated and discussed (Paper 1).

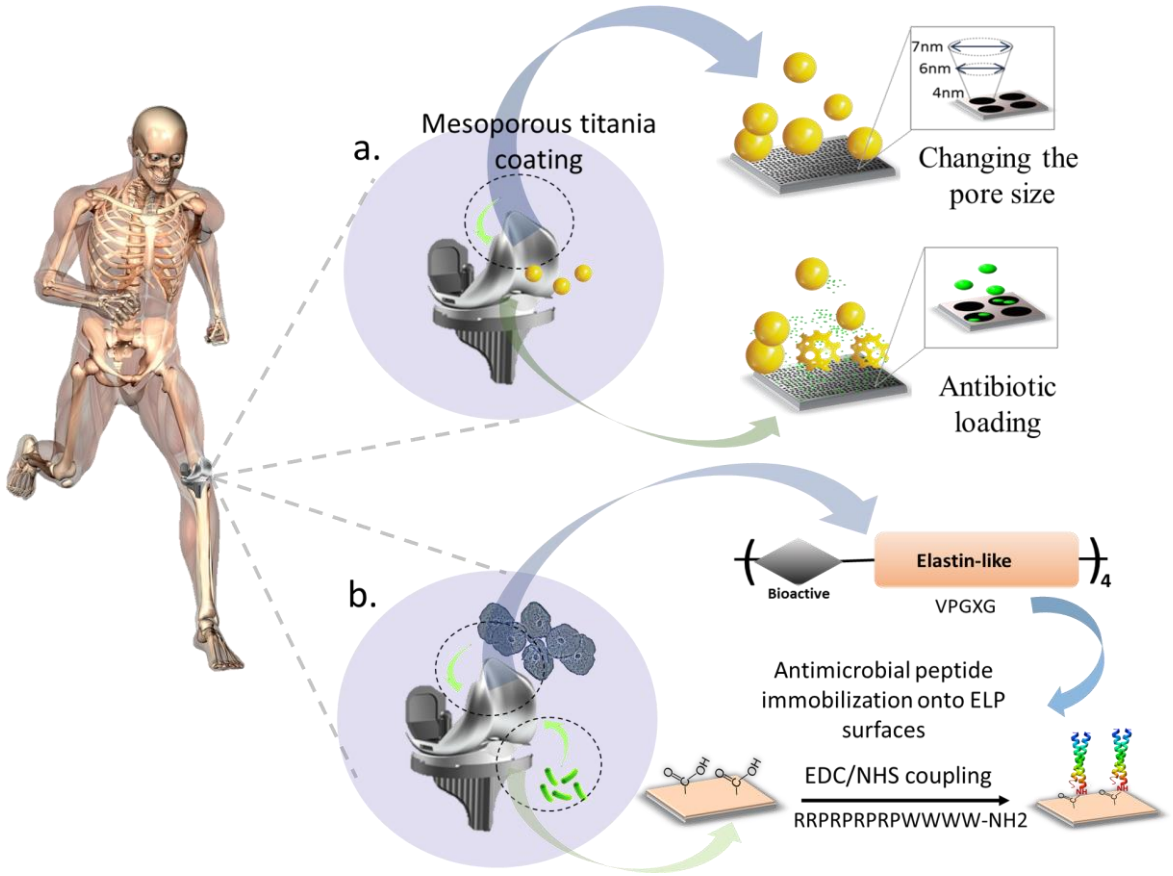

Figure 1. Schematic demonstrating the objective of this thesis **a,** part 1: Antibiotic delivery from mesoporous titania coating and **b,** part 2: Antimicrobial modification of ELP surface coatings by covalent immobilization of RRP9W4N.

The second part of this thesis, as illustrated in Figure 1b, is focused on the use of antimicrobial peptides (AMPs) as antibacterial agents. More specifically, RRP9W4N was covalently attached onto a bioactive surface coating made by recombinant elastin like peptide (ELP) and its effect on the biofilm formation was investigated (Paper 2). Due to low propensity of bacteria to develop resistance to AMPs, they are believed to be promising candidates to replace antibiotics.

1.1. Biomaterial associated infection (BAI)

1.1.2. Biofilm formation

When biomaterials are inserted into the body, they create a local defect in the immune system and provide a favorable surface for bacterial colonization. Planktonic bacteria are often present around the wound area, or in the blood circulation, and when they attach to the surface of biomaterials, they start to expand and produce a slime of extracellular polymeric substance (ECM). Inside this matrix the population of bacteria grows into a mature 3D structure called a biofilm.[12–14]

A biofilm consists of a hetererogenous population of cells that, despite the same genotype, can vary significantly in phenotype. These include so called persister cells that can enter a dormant state, rendering them unsusceptible to conventional antibiotics.[15] Like many types of chronic infections, the infection associated with biomaterials is generally caused by the formation of biofilms as the biofilm protects the microorganisms from the host's defense and antibiotic therapy. As a consequence, treatment of an infection after formation of a biofilm is almost inpossible without removing the infected implant followed by long-term antibiotic therapy and revision surgery, which is a painful and expensive procedure.[16–19]

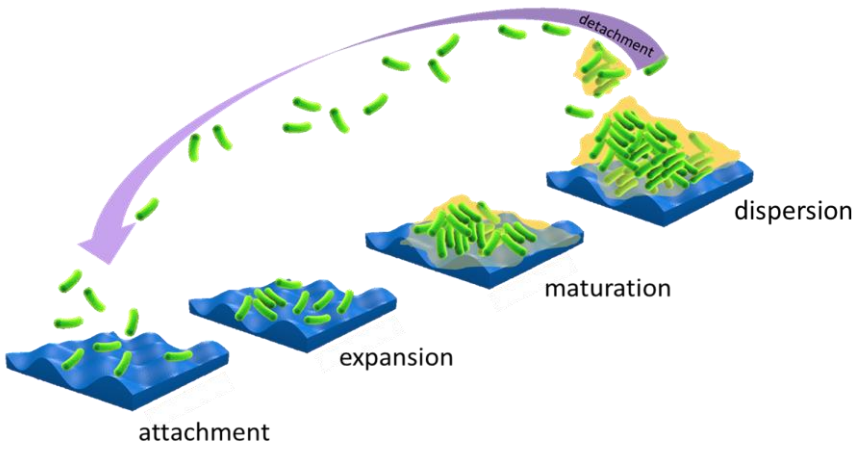

Figure 2. Schematic of biofilm development on a solid surface occurring in four steps.

1.1.3. BAI common pathogens

Most common pathogens responsible for BAI include Gram-positive *Staphylococcus aureus* and *Staphylococcus epidermidis*, Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa* and those from the Proteus group.[20,21] *S. aureus* and *P. aeruginosa* infections usually proceed rapidly and are generally more aggressive than BAI caused by *S. epidermidis*. However, *S. epidermidis* being part of the normal human skin flora, is an opportunistic pathogen with high accessibility to the surgical sites.[22]

1.2. Antibacterial surface coatings

A variety of modification techniques for fabrication of antibacterial surfaces have been developed. Functionalization of bioactive surface coatings is a commonly used approach to create antibacterial surfaces with good cell integration ability. Such surfaces are required to fulfil their function, withstand the complex biological environment and keep their antibacterial properties. Generally, such coatings function as 1) coatings that prevent bacterial adhesion (effect of surface energy and/or roughness), 2) bactericidal coatings, which kill the adherent bacteria (physical killing), 3) coatings that accelerate the adherence of adjacent tissue cells (cell adhesive sequences), 4) coatings that are loaded with or immobilized by antibacterial agents and 5) coatings that combine two or more of the functions.[23–25]

1.2.1. Antibiotic surface modification

Applying surface coatings that release antibiotics from biomaterials is considered as an effective strategy to prevent BAI. Such coatings are loaded with antibiotics and administrate these at the site of implantation and can kill the pathogens before a biofilm has the possibility to form.[26] This approach results in transfer of antibiotics with higher local concentration and decreased systemic side effects compared to the conventional systemic antibiotic therapy via injection or oral routes. However, most of the time, the release kinetics are difficult to control and the drug level will either exceed or remain below the minimal inhibitory concentration.[27,28] Moreover, the duration of the drug release must be restricted to a limited period of time to prevent antibacterial resistance caused by antibiotic therapy. To address these issues, an alternative approach is to covalently bond antibiotics to surfaces.[28] So far a number of common clinical antibiotics, such as penicillin and Vancomycin have been chemically immobilized on biomaterial surfaces. This approach seems to have the advantage of long-lasting antimicrobial activity, lower side effects and non-accumulation in other tissues.[29–32]

1.3. Mesoporous titania thin films

Mesoporous materials have suitable properties such as tunable textural properties, high surface area and pore volume to be applied for drug-delivery purposes.[33–36] The use of mesoporous materials, such as titania that is being used in this thesis, has been highlighted in previous studies to improve osseointegration using local delivery of osteoporosis drugs.[37] Moreover, such materials have the advantage of combining nanoroughness to create low bacterial adhesive materials with the ability of administrating antimicrobial agents. For example, in previous studies sustained release of cephalothin and amoxicillin from mesoporous titania-coated implants *in vitro* have been reported.[38,39]

1.4. Antimicrobial peptides (AMPs)

Increased rate of resistance in human pathogens narrows down the available antibacterial therapeutic options to treat infections. As a consequence, there is a need for an alternative with broad spectrum of activity and minimized potency to develop resistance. Therefore, alternative drugs such as antimicrobial peptides (AMPs) are receiving increased attention.[40–43] Antimicrobial peptides are part of the innate immune system and acts as a first response towards invading pathogens.[43,44] Cationic AMPs are short peptides consisting of less than 50 amino acids with a positive net charge of +2 to +9.

The main mode of AMPs mechanism of action involves interactions with the bacterium membrane leading to its rupturing and breakdown of the bacteria (Figure 3). Due to this, pathogens have less propensity to develop resistance to AMPs compared to traditional antibiotics.[45]

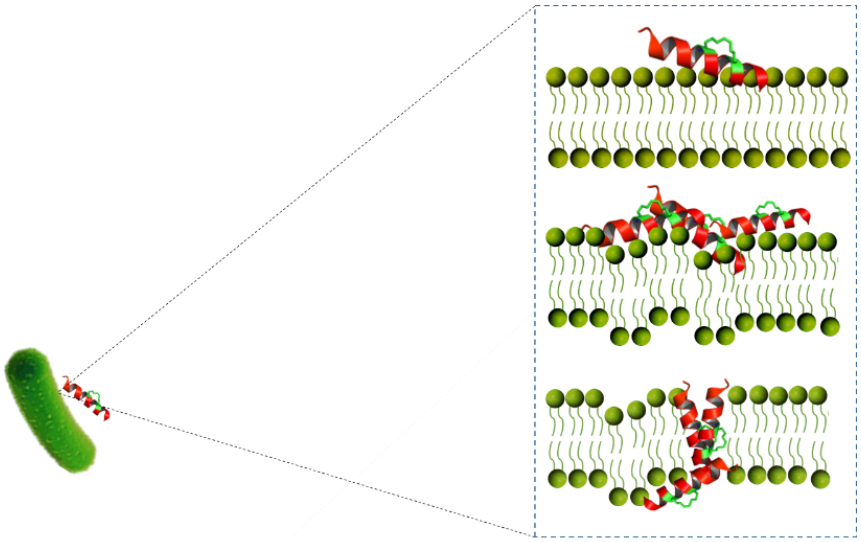

Figure 3. Simplified schematic of antimicrobial peptide mechanism of action through damaging the bacteria cell membrane and succeeding pore formation.

Target specificity is one important consideration in using AMPs. Bacterial membranes mainly consist of anionic phospholipids and are deficient in cholesterol, while human cells consists of zwitterionic phospholipids and are enriched in cholesterol. This makes bacterial membranes negatively charged and more prone to be invaded by positively charged molecules such as AMPs.[46]

1.5. Elastin like polypeptides (ELP)

Elastin is a naturally occurring extracellular matrix protein that can predominantly be found in connective tissues.[47]

Elastin like poly peptides (ELP) are engineered peptides that are synthesized by translating a desired sequence of amino acids encoded into a plasmid by living organisms. ELP can be readily expressed and purified in E-coli culture, which simplifies its large scale purification, Figure 4a.[48] Its structure is composed of a repeating pentapeptide sequence Val–Pro–Gly–Xaa–Gly, where the guest residue (Xaa) can be any naturally occurring amino acid except proline.[49] The protein engineering approach in ELP production, enables a precise control over the properties of the resulting protein. Moreover, it makes it possible to incorporate specific individual amino acids into the peptide sequence to confer chemical modifications. Cell- adhesive RGD domains can be included into the ELP to improve the bioactivity and cell attachment properties, Figure 4b.[50] ELP has recently been considered as a promising material for biomedical applications. It has lately been shown that coatings made of ELP with RGD sequences are favorable for cell interactions, enhance osseointegration and subsequent bone formation.[51]

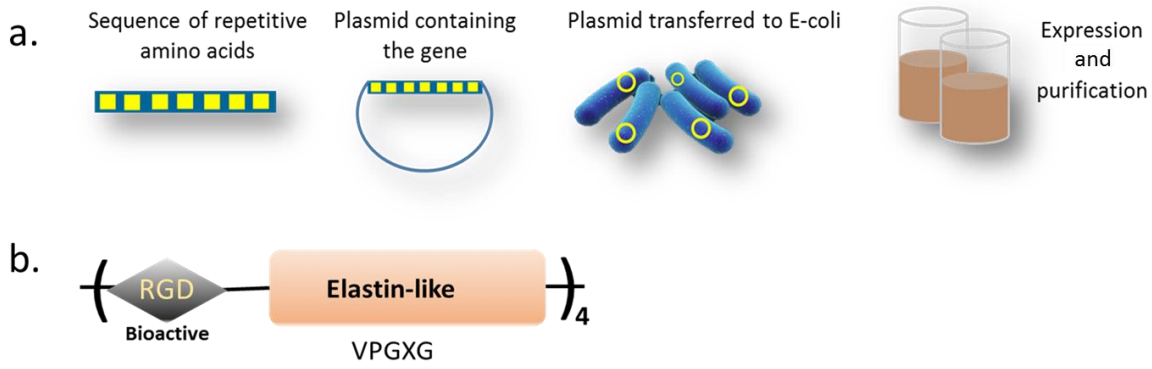

Figure 4. **a,** Schematic of steps in expression and purification of ELP from E-Coli culture. **b,** Proposed ELP structure modified with bioactive RGD sequences.

2. Experimental

The experiments performed and discussed in this work are presented in two sections; the first part is focused on the antibacterial performance of mesoporous titania thin films and the experiments include; 1) synthesis of mesoporous titania thin films with different pore sizes, 2) antibiotic loading/release from mesoporous titania surfaces, 3) the effect of antibiotic release, surface nanoroughness and hydrophobicity on bacterial attachment (Paper 1). The second part of the experimental includes antimicrobial peptide immobilization on ELP substrates and the experiments are focused on 1) expression and purification of elastin like polypeptides (ELP), 2) ELP substrate preparation 3) AMP immobilization onto ELP surfaces and the effect on bacteria (Paper 2).

Part I

2.1. Antibacterial performance of mesoporous titania thin films

2.1.1. Mesoporous titania thin film preparation

Cubic mesoporous titania with pore sizes of 4, 6, and 7 nm were formed by the evaporation-induced self-assembly method (Figure 5). Pluronic® P123 (triblock copolymer EO20PO70EO20) and cetyltrimethylammonium bromide (CTAB) ($CH_3(CH_2)_{15}N(CH_3)_3Br$), were used as structure directing agents . Larger pores (7 nm) were formed utilizing an organic additive, polypropylene glycol (PPG, Mn ~4,000), which was used as a swelling agent. A titania precursor solution was prepared by adding 2.1 g titanium (IV) ethoxide (20%) to 1.6 g concentrated hydrochloric acid (37%) under vigorous stirring, forming a homogenous solution. The amphiphile (0.5 g) was separately dissolved in 8.5 g ethanol under vigorous stirring followed by mixing with the precursor solution. The final solution was left to stir overnight to achieve a homogenous mixture. To obtain uniform thin films of mesoporous titania, 100 μL of the final solution was spin-coated (7,000 rpm) for 1 minute on glass slides (2×2 cm), titanium discs (8 mm diameter and 3mm thickness) and Ti-coated QCM-D sensors using a Spin150 spin coater. The coated substrates underwent aging for 1 day at room temperature to obtain complete self-assembly. When the swelling agent was used, a moderately humid environment (RH=54%) was provided by a saturated KNO_3 aqueous solution in a refrigerator (T=4°C±1°C) during the aging process. Finally, the films were calcined by heating with a heating ramp of 1°C/min to 350°C, at which temperature they were left for 4 hours to remove the template and to cross-link the titania. Nonporous titania thin films were formed as control samples using the same procedure, but without the addition of amphiphiles.

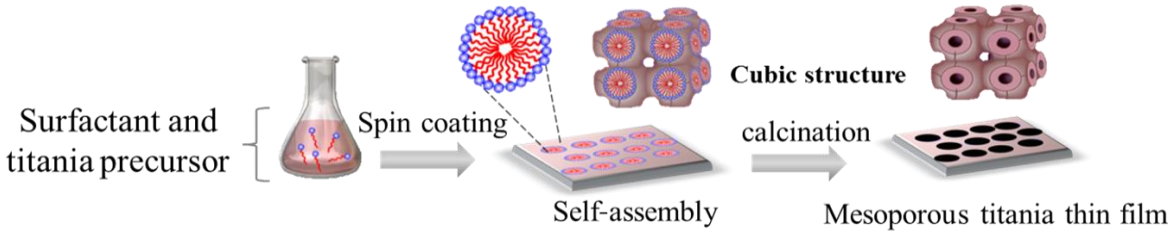

Figure 5. Schematic of the formation procedure to form cubic mesoporous titania thin films using the evaporation induced self-assembly method.

2.1.2. Hydrophobic surface modification

Mesoporous titania thin films were treated in 5% dichlorodimethylsilane (DCDMS) for 1 hour. This step was used to hydrophobically modify the substrates and lower their surface energy. A schematic of surface silanization process is demonstrated in Figure 6. To ensure a sufficient amount of silanol groups on the titania surface, the substrates were pre-treated in water baths at 25°C for 45 minutes, then placed in a DCDMS solution for 15 minutes and thereafter flushed with chloroform to wash off the unreacted silanol groups from the surface, followed by another 45 minutes treatment in DCDMS. The modified films were dried using nitrogen gas.

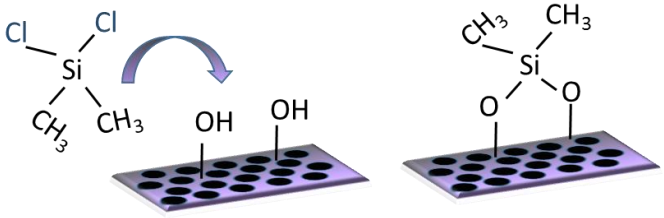

Figure 6. Schematic of silanization of mesoporous titania using DCDMS.

2.1.3. Antibiotic release from mesoporous titania

Substrate immersion was used to load mesopororus titania with antibiotics. The surfaces were immersed in antibiotic solutions for 1 hour. Vancomycin and gentamicin were dissolved in water (1 wt%) and Daptomycin was dissolved in methanol (1 wt%). DCDMS-treated mesoporous thin films were used to load Daptomycin, and the non-modified surfaces were used for Gentamicin and Vancomycin.

2.1.4. Bacteria culture and growth

S. aureus (ATCC 25923) and *P. aeruginosa* (ATCC 35984) were used to study the bacterial attachment and growth on the mesoporous titania with different pore sizes both with and without incorporated antibiotics. One day prior to the experiments, centrifuge tubes of 5 mL tryptic soy broth (30 g/L, Sigma) were inoculated by a single isolated colony from cultured agar plates of each bacterium. The inoculated cells were cultured in a shaking incubator at 200 rpm for 18 hours at 37°C until they reached stationary phase. The optical density of the bacterial suspension was adjusted to 0.52 at 620 nm (estimated to give 109 colonies) using a plate reader (Spectramax M3 Multimode Microplate Reader; Molecular Devices LLC, Sunnyvale, CA, USA).

Each bacterial solution was diluted at a ratio of 1:100 using simulated body fluid supplemented with 1% fetal bovine serum (FBS), and the mesoporous titania samples were seeded with 1 mL of the diluted solution. The samples with bacteria were then cultured for 1 hour in an incubator (ambient air) at 37°C to promote the attachment of the bacteria onto the titania surfaces. After 1 hour of culturing, media containing the unattached planktonic bacteria was aspirated. The samples were rinsed once with PBS and fresh media (simulated body fluid +1% FBS) was placed onto each sample. Samples were cultured for another 47 hours and at the end of this time period, they were rinsed three times with PBS. Each sample was placed inside a sterile 15 mL centrifuge tube filled with 2 mL of PBS. Samples were vortexed at 3,000 rpm for 2 minutes to release adherent bacteria into the PBS solution. Afterward, each bacterial solution was diluted twice, 1:10, and then each dilution was plated as five 10 µL spots onto sterile tryptic soy broth agar plates. Agar plates were placed inside an incubator at 37°C and 5% CO_2 for colonies to grow. The colonies on each plate were counted and the total number of colonies per milliliter of vortexed solution was calculated and converted to colony- forming units/mL.

Part II

2.2. Antimicrobial peptide immobilization onto elastin-like polypeptide thin films

2.2.1. Expression and purification of ELP

ELP containing RGD sequences was expressed recombinantly in Escherichia host. A plasmid encoding the elastin like and RGD sequences was transformed into the E-Coli host and isopropyl β-D-1 thiogalactopyranocide (Sigma) as T7-lac promoter was added to induce the expression. After culturing, the bacteria were lysed and the protein was purified using repetitive centrifuge cycles at above and below the lower critical solution temperature of the ELP (4°C and 37°C). The purified ELP was dialyzed, freeze dried and stored at 4°C.

2.2.2. ELP thin film preparation

A photo reactive heterobifunctional N-hydroxysuccidimide ester-diazirine crosslinker (NHS-diazirine, succinimidyl 4,4'-azipentanoate, Pierce Biotechnology) was dissolved in DMSO (2 mg/mL) and mixed with a solution of ELP in PBS (2 mg/mL). The reaction was incubated for 2 hours and 1M tris buffer was added to the solution to stop the reaction. The diazirine conjugated ELP was dialyzed against DI water, frozen and lypholized. All ELP coated surfaces mentioned subsequently, refers to this photo crosslinkable form of ELP modified with diazirine. Microscope glass coverslips (VWR, 15 mm) were cleaned using basic piranha. The polished titanium discs (grade 4, 8 mm in diameter and 3 mm in thickness) were rinsed with 70% ethanol followed by sonication. All substrates were dried with N_2 gas and stored at 4°C before ELP deposition. A 50 mg/mL (5wt. %) solution of ELP in PBS was prepared at 4°C. The deposition of ELP onto the substrates was perfromed by spin coating. For the glass substrates, 50 μl of the ELP solution and for the Ti discs 30mL of the ELP solution was applied to the center of the surface and spun at 4000 rpm for 90 seconds. The spin coated substrates were treated by UV-light using a 365 nm, 8 W light source for 1 hour for ELP crosslinking. The crosslinked films were rinsed 3 times for 0.5 hour with PBS prior to the experiments to remove any non-crosslinked ELP.

2.2.3. AMP immobilization on ELP surfaces

A solution of antimicrobial peptide RRPRPRPRPWWWW-NH_2 (RRP9W4N, Red Glead Discovery AB, Lund, Sweden) was prepared in sterilized water to a concentration of 200 μM. For covalent attachment of AMP to the ELP surfaces, the ELP coated substrates were submerged into a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (NHS) in MES buffer (PH=6) at a final concentration of 2mg/mL and were allowed to react for 30 minutes slowly shaken at room temperature.

Surfaces were then washed 3 times in PBS (pH 7.4) and suspended in the AMP solution for 2 hours in room temperature. The surfaces were then washed 3 times for 0.5 hour to remove the unreacted peptides from ELP surfaces and used for bacterial assays. The same procedure was used to prepare surfaces on Ti QCM-D discs (QSX 310, Q-sense) for QCM-D anaylsis and Ti discs for SEM analysis.

2.2.4. Bacteria culture

S. epidermidis (ATCC 35984), *S. aureus* (CCUG 56489) and *P. aeruginosa* (CCUG 10778) were used to assess biofilm formation on the surfaces. One day before the experiment a sterilized 10 μL loop was used to withdraw a single colony from cultured agar plates of each bacterium to inoculate a tube of 5mL tryptic soy broth (TSB). The inoculated cells were cultured in an incubator for 6h, diluted in TSB and cultured in the incubator overnight to reach the stationary phase for bacterial growth.

The optical density of the bacteria culture was adjusted to 0.7 at 620 nm (estimated to give 10^9 colonies) using a spectrometer. The bacterial suspension was centrifuged for 10 minutes at 2500 rmp and the formed bacteria pellet was suspended in the fresh TSB media. 2 mL of the suspension was seeded onto the glass substrates, ELP coated substrates, activated ELP substrates and AMP functionalized ELP substrates in a 12 well plate. Bacteria were then cultured for 24 hours and 48 hours under standard culture condition (ambient air at 37°C) to promote biofilm formation onto the surfaces. For the 48 hours time point, after 24 hours of culture, the media was aspirated and replaced with fresh TSB for another 24 hours culture. At the end of each time point, surfaces were rinsed 3 times with fresh PBS to wash off any unattached planktonic bacteria before biofilm analysis.

2.2.5. Evaluation of AMP immobilization on ELP substrates

2.2.5.1. Monitoring by QCM-D

The uptake and grade of attachment of AMP on EDC/NHS activated surfaces were studied by QCM-D (Q-sense E4, Sweden). Two titanium QCM-D discs (QSX 310, Q-sense) were coated with ELP and one of the surfaces activated with EDC/NHS, as described earlier. Pure titanium discs and none-activated titanium discs were used as controls for this test. Milli-Q H_2O was used as the solvent (AMP solution) and rinsing liquid. The mass of adsorbed AMP (ng/cm^2) was calculated from the shifts in the frequency using the Sauerbrey equation (Eq. 1).

$$\Delta m = -\frac{\Delta f \times C}{n} \qquad (Eq.\ 1)$$

, where Δm is the adsorbed mass, Δf the frequency difference, C is the mass sensitivity constant (17.7 ng.Hz-1cm-2) and n the overtone number.

2.2.5.2. Stability in serum using a fluorescent tagged AMP

Fluorescent tagged AMP (5(6) carboxyfluorescein-RRPRPRPRPWWWW-NH_2) was used to study the stability of AMP attachment and its distribution on surfaces after various duration of time. The tests were performed by incubating the fluorescent AMP functionalized surfaces in 20% human plasma serum (from 1 hour up to 3 weeks) and imaged using fluorescent microscopy. Bacterial assays were conducted to investigate for how long AMPs could keep their bactericidal functionality against *S. epidermidis*.

2.3. Analytical techniques

2.3.1. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

In scanning electron microscopy (SEM), a beam of focused electrons are scanned over a solid sample to give high resolved images. The highest resolved images are obtained by detecting the signal from the secondary electrons that originates from the surface. In transmission electron microscopy (TEM), an accelerated high energy beam of electrons passes through ultra-thin samples and creates a high resolution image revealing the microstructural information. In part I of the thesis, SEM and TEM were extensively used for imaging the mesoporous titania thin films and powders. In part II of the thesis, SEM was used to image ELP thin films and morphology of the bacteria in biofilms formed on surfaces. All samples in the second part were gold sputtered at a rate of 3nm/min for 1 minute prior to SEM imaging**.**

2.3.2. Small angle X-ray scattering

Long-ranged structural order within materials can be revealed by means of small-angle X-ray scattering (SAXS). The scattered X-ray patterns reveal data about long-range periodicity and mesoscopic dimensions of the samples. In this work, mesoporus titania was grinded into a powder for SAXS measurements. The measurements were performed at MAX-lab, beam station I911 (Lund, Sweden) using synchroton radiation (λ= 0.91 Å) and a two dimensional Mar 165 CCD detector.

2.3.3. X-ray diffraction

X-ray diffraction is a method to determine the crystalline structure of a sample. When the sample is exposed to an X-ray beam, the atomic planes in the crystallites diffract the beam at certain angles with constructive interference of X-ray to fulfil the Bragg condition (Eq. 2).

(Eq. 2) $n\lambda = 2d\sin\theta$

, where d is the lattice spacing, θ is the angle and λ is the wavelength of the incident beam. In part 1 of the thesis, the crystallinity of the mesoporous titania powders were evaluated by XRD. The X-ray diffractometer used was a Bruker D8 advance (Bruker Corporation, Billerica, MA, USA) with a radiation wavelength of 1.5405Å (Cu *K*α1 radiation).

2.3.4. Quartz crystal microbalance with dissipation monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a mass sensitive analytical technique. Upon appliance of an AC voltage, a thin quartz crystal attached to a pair of gold electrodes, starts to oscillate at its acoustic resonance frequency. The mass uptake or release at the sensor surface causes changes in the resonance frequency (Δf) as a function of time. Δf can be converted to the mass adsorbed or desorbed (Δm) by applying the Sauerbrey equation (Eq. 1).

Moreover, an energy dependent property of the surface, the dissipation (ΔD) can be measured by QCM-D. ΔD is correlated to the viscoelastic properties of the adsorbed layer.

In part I of the thesis, QCM-D was used to measure the absorbed mass of the drugs within the mesoporous titania thin films. In addition, the accessible pore volume was calculated from the amount of deuterium oxide (D_2O) that was absorbed. In part II, the adsorption and attachment of antimicrobial peptides onto ELP surfaces before and after EDC/NHS activation were monitored by QCM-D and compared to pure Ti surfaces. The swelling ability of the ELP thin films was also measured by D_2O adsorption tests. The instrument used was a Q-sense E4 and all samples were evalauted using titanium QCM-D discs (QSX 310, Q-sense).

2.3.5. X-ray photoelectron spectroscopy

X-ray photon spectroscopy (XPS) also known as electron spectroscopy for chemical analysis (ESCA) is a surface sensitive analysis technique to determine the chemical composition of a surface. Low energy X-rays irradiate the samples and the binding energy of the photoelectrons leaving the sample allows for quantitative determination of the present surface elements. In part I of the thesis, XPS was used to examine the surface composition of mesoporous titania thin films before and after modification and drug loading. In part II, XPS was used to evaluate the ELP thin films before and after surface activation with EDC and NHS. The equipment used was a Quantum 2000 scanning microscope (Physical Electronics, Inc., Chanhassen, MN, USA) with a 100µm point diameter at a 5nm analysis depth.

2.3.6. Atomic force microscopy

Atomic force microscopy (AFM) is a scanning probe microscopy method to measure local properties of a surface such as its roughness through interactive forces. In this thesis, AFM was used to measure the surface topography of the mesoporous titania films. The measurements were performed in semi-contact or intermittent mode at two different scan sizes (1 and 0.5 µm). A conical tip with a length of 200 nm and a radius of 5nm attached to an NT-RTESPA cantilever was used to scan the samples. The AFM used was an NT-MDT model (Moscow, Russia).

2.3.7. Fluorescence light microscopy

Fluorescence light microcopy (FLM), is used to image samples that are either fluorescently labeled or fluorescing in their natural form (autofluorescence). When fluorescent samples are irradiated with absorbable light of a specific wavelength, they can emit energy detectable as visible light. In this technique, the microscope filters out only the desired wavelength that matches the fluorescing sample. As a result, the electrons in the fluorescing specimen are excited to a higher energy level and when they relax, emit visible light. The emitted light reaches a second barrier filter to eliminate the residual background light from the specimen to pass to the eye or camera. Thus, the fluorescing sample shine out against a dark background with high contrast. FLM was extensively used in part II of this thesis to study the live and dead fractions of biofilms formed on ELP substrates with and without AMP functionalization. The microscope used was a Carl Zeiss GmbH (Jena –Germany) equipped with a HBO 100 microscope illuminating system. Green filter, GFP, 38HE ($\lambda_{Excitation}$ = 470 nm and $\lambda_{Emission}$=525 nm) and red filter , 43 DS Red ($\lambda_{Excitation}$ = 545 nm and $\lambda_{Emission}$=605 nm) were used for imaging. The biofilm were stained using LIVE/DEAD®*Bac*Light™ Bacterial Viability Kit (Molecular probes, Invitrogen). The images were obtained with SYTO® 9 and propidium iodide nucleic acid staining provided in the kit. Live bacteria with intact cell membranes appeared green and dead bacteria with compromised membranes appeared red. The statistical data from live/dead bacteria obtained from red and green fractions of fluorescent microscopy images were presented by the mean value with standard deviation. All the experiments were performed three times with two replicates. 20 images from each surface were used to obtain the image analysis data. FML was also used to image fluorescent tagged antimicrobial peptides to study their stability in serum condition.

3. Results and discussion

The results presented and discussed in this thesis are divided into two parts. In the first part, the formation and evaluation of mesoporous titania thin films, antibiotic delivery and the bacteria response to mesoporous titania surfaces are presented (Paper 1). In the second part, the antimicrobial peptide immobilization onto elastin like poly peptides and bacterial assays are presented (Paper 2).

3.1. Part I

3.1.1. Mesoporous titania thin film evaluation

According to the SEM micrographs, evenly distributed porous titania were formed on titanium substrates (Figure 7). The thicknesses of the films, as obtained by cross-section analysis, was ~200 nm for the ones formed using P123 and CTAB and ~700 nm when the swelling agent PPG was added to P123. The addition of PPG increased the viscosity, which probably resulting in a thicker film upon spin coating.

The average pore size of each film was calculated directly from the SEM and TEM micrographs (Figure 7) and are presented in Table 1. It was shown that mesoporous titania thin films with pore diameters of 4, 6, and 7 nm, accessible on the surface, were successfully formed. The obtained pore diameters correlate well with the size of the templates used in the syntheses.

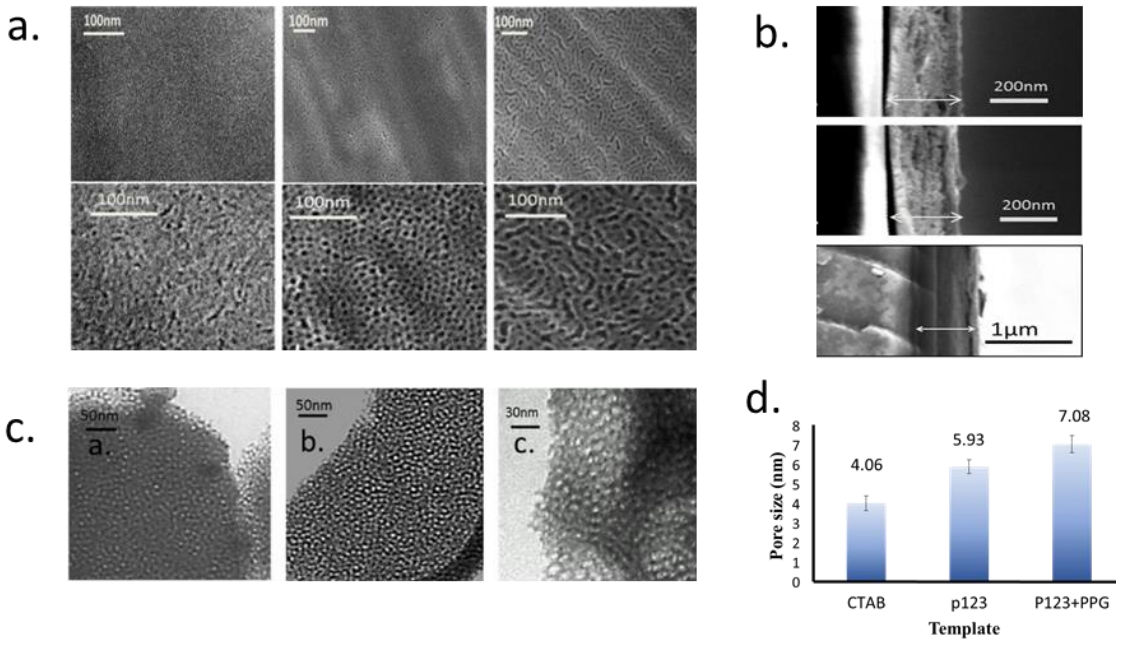

Figure 7. **a,** SEM micrographs of mesporous titania thin films formed using different templates; from left to right CTAB, P123 and P123+PPG (1:1). **b,** SEM images of the cross-section of mesoporous titania coatings, P123, CTAB and P123+PPG (1:1) from top to bottom. **c,** TEM images of mesoporous titania thin films scraped off from the substrates P123, CTAB and P123+PPG (1:1) from left to right. **d**, A graph showing the pore sizes obtained from the TEM micrographs.

The pore volume of the titania films was calculated from the QCM-D data obtained from H_2O/D_2O exchange experiments using the mass difference between water and heavy water, Table 2. As expected, the volume increased with increasing pore size and increased film thickness.

Table 1. Properties of mesoporous titania thin films including average pore diameter obtained from SEM images, the percentage of pore coverage on the surface calculated from SEM images and the pore volume of the mesoporous titania thin films accessible for water obtained from QCM-D measurements.

Template	**Average pore diameter (nm)**	**Pore surface coverage (±5%)**	**Pore volume (%)**
CTAB	4	18	75
P123	6	25	57
P123 + PPG (1:1)	7	42	55

Clear Bragg peaks appeared in SAXS diffractograms for the mesoporous samples. The peaks appeared at different positions with various intensities while for the nonporous sample no peaks were observed, Figure 8b. The peaks were relatively broad indicating that the degree of order was relatively low, which is probably a result of the spin-coating technique used. Films formed by dip coating instead of spin coating usually show higher degree of order while the films formed by spin coating are more homogenous in thickness.[52]

XRD results showed the formation of crystalline anatase TiO_2. (Mincryst#191). The crystalline structure was the same for all the formed films including the nonporous. In figure 8a, a representative diffractogram for the titania films is shown.

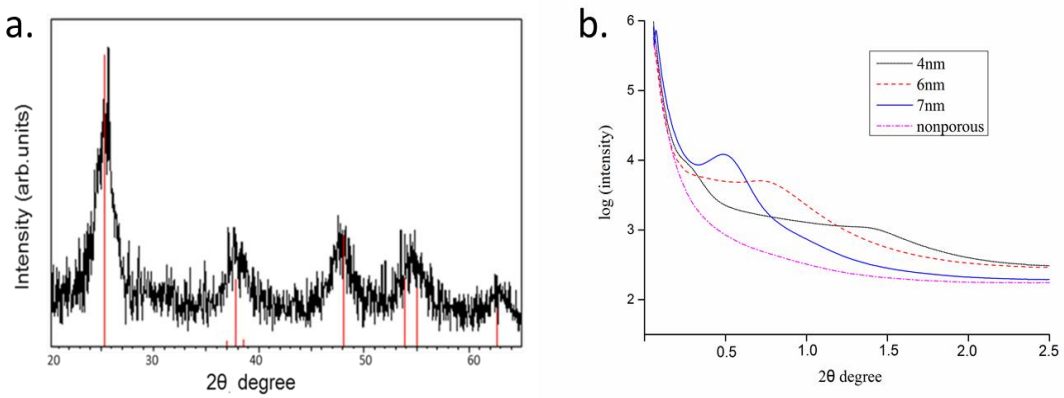

Figure 8. **a,** A representative XRD pattern obtained from mesoporous titania powders. **b,** SAXS diffractograms of mesoporous titania powders, see the graph legend for the sample names.

Results from XPS (Table 2) showed that the surface chemistry was similar on all the formed mesoporous titania films, including the nonporous, and consisted of carbon, oxygen, and titanium, no other impurities were detected. The surface roughness was measured using AFM, Table 3. The only significant difference in surface roughness was observed between the porous and nonporous surfaces; however, all the surfaces were still considered to be smooth on a micrometer length scale.

Table 2. The chemical composition given in atomic percentage (%) of the mesoporous titania thin films measured with XPS.

Template	C1s	O1s	Ti2p
P123+PPG	26.85	53.88	19.03
CTAB	24.79	55.32	19.16
P123	20.7	57.43	21.88

Table 3. Average roughness (Ra) obtained by AFM from mesoporous titania surfaces using two different scan sizes.

Average roughness (Ra) by AFM(nm)		
Pore size (nm)	Scan size 0.5 * 0.5 μm	Scan size 1 *1 μm
7	10.35	14.80
6	19.74	24.74
4	10.17	12.87
nonporous	0.96	1.85

Static contact-angle measurements were performed before and after surface modification with DCDMS. According to Table 4, it shows that the surface energy drastically decreased upon DCDMS functionalisation, forming a more hydrophobic substrate.

Table 4. Results from static contact angle experiments before and after DCDMS modification

Surface	**Contact Angle (°) on pure titania**	**Contact Angle (°) after DCDMS treatment**
4 nm	<5	85
6 nm	<5	80
7 nm	<5	110
nonporous	7	100

From the combined material characterization results it is clearly seen that the physical and chemical properties of the mesoporous titania thin films remain the same independently on pore size, which provide it possible to investigate the effect of pore size alone on drug loading and antibacterial responses.

3.1.2. Drug release from mesoporous titania thin films

The drug loading and release behavior from mesoporous titania thin films were directly correlated to the pore size and volume of the pores as measured by H_2O absorption. (Paper 1)

High loading and fast release rates were observed for the antibiotics according to QCM-D measurements, as is shown in Figure 9.

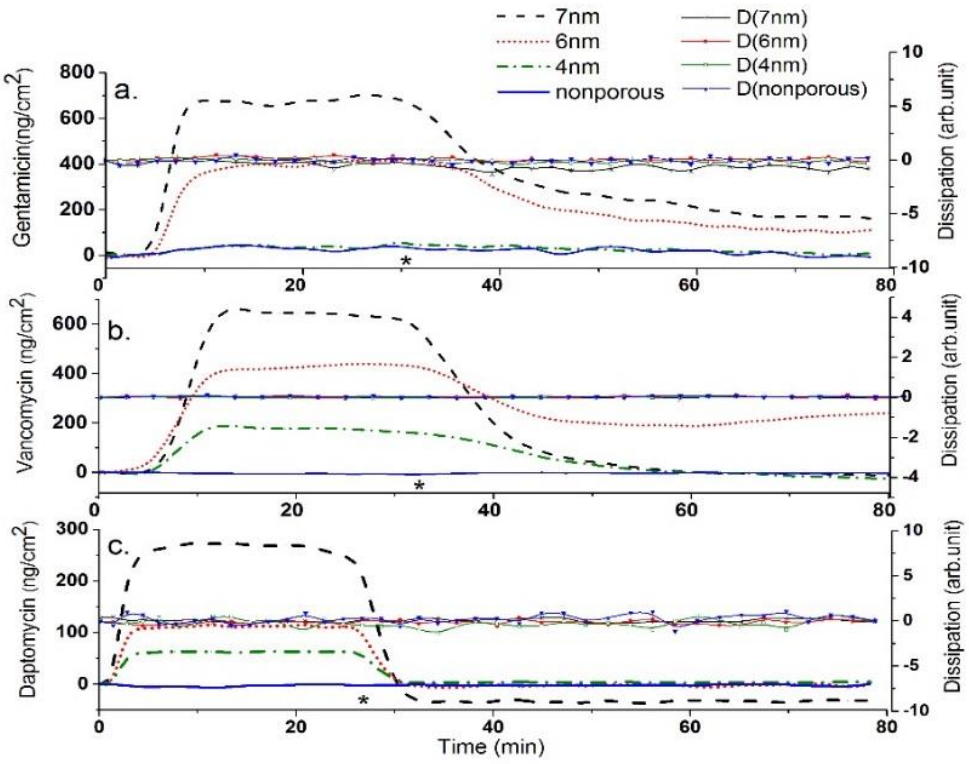

Figure 9. QCM-D recordings obtained from loading and release of the three different antibiotics: a) Gentamicin, b) Vancomycin, and c) Daptomycin on mesoporous titania with variable pore sizes (For Daptomycin DCDMS modified surfaces were used).
* Indicates start of rinsing with solvent to follow the release.

It should be noted that all drug-release studies were monitored upon constant rinsing with the solvent, water or methanol, at a rate of 50 mL/min. This rate is considered to give rise to a much higher release compared to the biological situation. Therefore, the results from the release kinetics cannot be directly transferred to the clinical situation.

The dissipation did not change noticeably during loading of the drugs, indicating that no viscoelastic film was formed onto the mesoporous titania.

3.1.3. Bacterial response

Results from bacterial assays showed that mesoporous titania thin films, apart from their function as hosts for antibiotics, can to some extent hinder bacterial growth. These results indicate that the presence of pores alone can affect the adhesion of bacteria *S. aureus* and *P. aeruginosa* onto surfaces, Figure 10a.

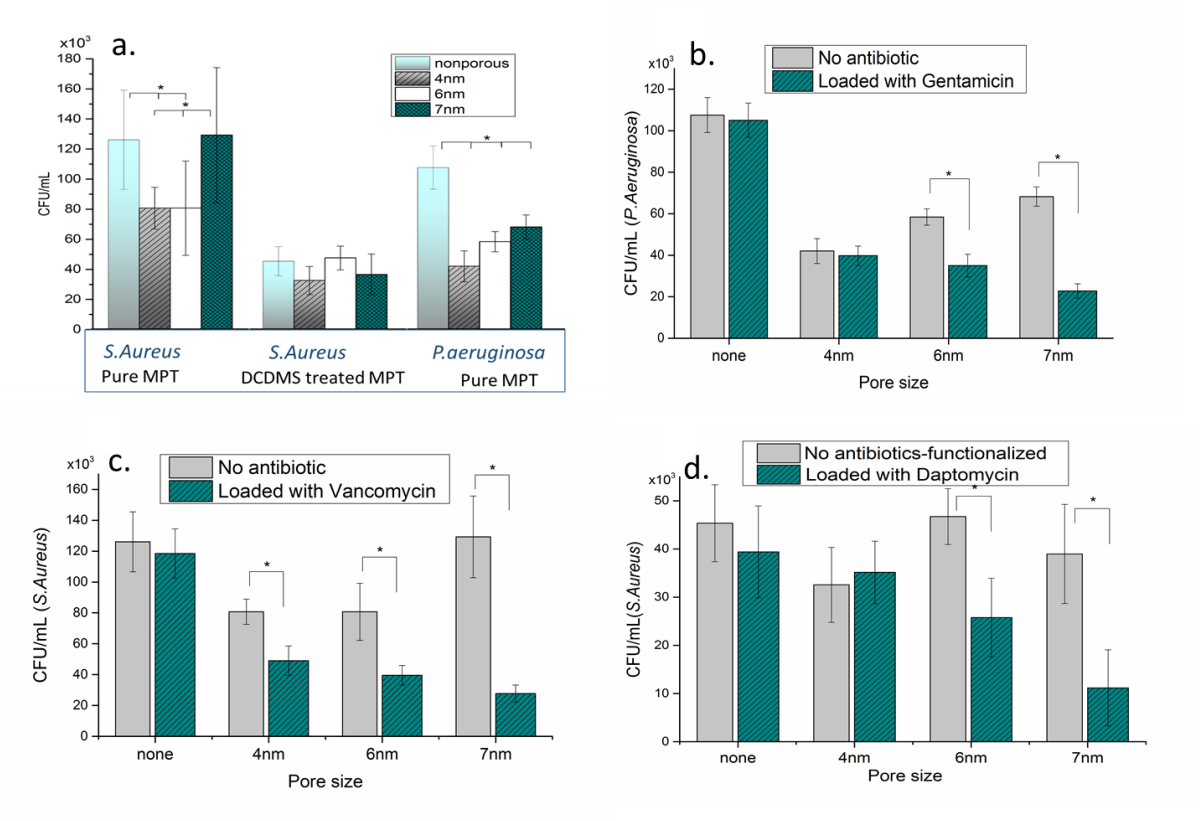

Figure 10. **a,** Data from counting the colony forming units grown on modified and unmodified mesoporous titania **a,** without the presence of antibiotics. **b,** with and without Gentamicin **c,** with and without Vancomycin **d,** with and without Daptomycin. Values are mean ±SD, N=3, **p*<0.05 compared to mesoporous titania surfaces for each bacteria strain. MPT = mesoporous titania.

According to the results obtained from counting the colony forming units, the least adhesion of bacteria was observed on the hydrophobic mesoporous titania with 4 nm pore sizes indicating that the bacterial attachment was lower when the surface energy of the substrate decreased, Figure 10a. However, it is not clear whether it is the surface energy and nanotopography of the substrates that control the bacteria adhesion directly or the fact that changing these parameters can affect the adsorption of the fetal bovine serum proteins present in the media, which consequently affect the bacteria attachment onto surfaces.

A predictable pattern was observed when the surfaces were loaded with antibiotics, Figure 10b,c,d. As expected, by increasing the size of the pores and loading higher amounts of antibiotics, the attachment of bacteria on the surfaces was decreased. This result confirms that mesoporous titania films can be considered as efficient drug release coatings on implants to fight bacterial adhesion and consequent biofilm formation. Furthemore, it is possible to regulate the amount of antibiotic loading by changing the pore size and film-thickness.

3.2. Part II

The results discussed in this part, are focused on the antimicrobial peptide immobilization onto ELP thin films and the bacteria response to these surfaces (Paper 2).

3.2.1. ELP thin film evaluation

Photo-crosslinked ELP with cell adhesive RGD sequences was fabricated into thin films as a substrate for immobilization of antimicrobial peptides. ELP coatings have previously been shown to have sufficient mechanical strenght to endure clinically relevant shear forces.[50] Moreover, it has been shown that such coatings are favorable for cell interactions, enhance osseointegration and bone formation in vivo.[51] The crosslinking is performed by conjugation of diarizine molecule to the ELP, which was activated by UV-exposure after spin coating. A reactive carbene intermediate is generated upon UV-exposure to covalently bond to the titanium surface (Figure 11a). SEM images of dried ELP coatings demonstrate a uniform coating on the micro scale with porous structure in the nano scale (Figure 11b). The morphology of the crosslinked films were not changed after extensive washing in physiological conditions (PBS, 37°C) indicating a stable ELP coating.

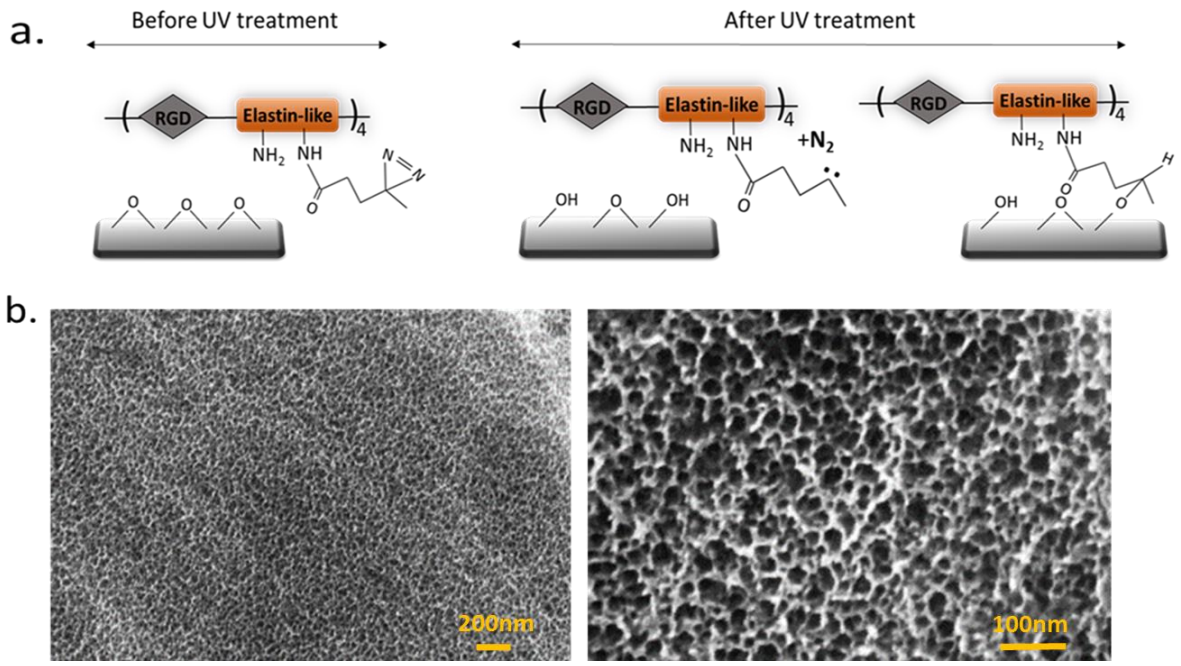

Figure 11: **a**, Proposed schematic model for conjugation of ELP onto Ti substrates using diarizine moiety. **b,** SEM of photocrosslinked ELP onto Ti substrate, the magnification increases from left to right.

QCM-D experiments with H_2O/ D_2O exchange were performed to measure the swelling ability of ELP thin films in water, Figure 12. The density difference between the H_2O and D_2O was used to calculate the amount of absorbed water. It was shown that the ELP thin films swelled ~17 times in water.

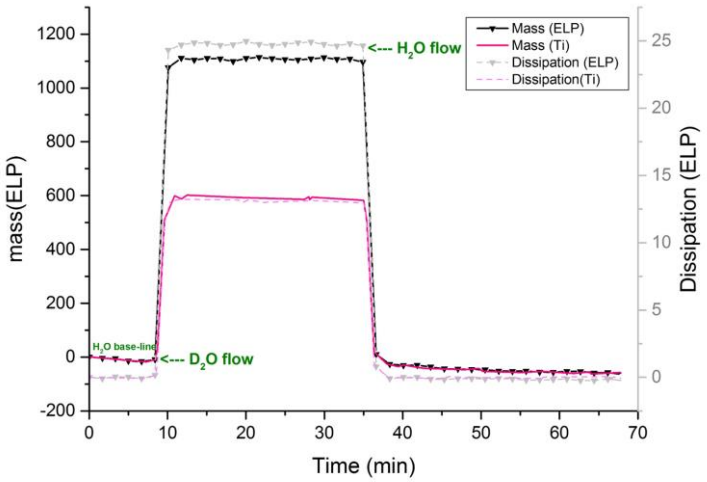

Figure 12. QCM-D recordings showing the amount of D_2O when exchanged with Milli-Q water absorbed onto ELP thin films. A pure Ti surface was used as control.

3.2.2. Antimicrobial peptide immobilization

Immobilization of antimicrobial peptides onto surfaces is suggested to be an effective approach to minimize their cytotoxicity and increase their long-term stability for clinical applications.[28,53–55]

The AMP, RRPRPRPRPWWWW-NH_2, which was used to modify the ELP substrates is a cationic peptide rich in proline and arginine amino acids modified with hydrophobic Tryptophan (W) residues. The RP side chain provides a positive charge to facilitate bonding to negatively charged phospholipid head groups of bacteria membranes. The presence of W limits the toxicity of the AMP to eukaryotic cells since the formed amphipathic structure allows this peptide to only penetrate deep into the interfacial region of negatively charged membranes.[56–59] EDC/NHS coupling chemistry was used to covalently attach the antimicrobial peptide onto ELP surfaces. In this method, EDC activates abundant hydroxyl groups on ELP for direct reaction with primary amine groups present in the AMP structure. NHS is used together with EDC to improve the efficiency and create dry-stable (amine-reactive) intermediates. A schematic of AMP immobilization onto ELP coating via EDC-NHS coupling is shown in Figure 13. According to XPS results, activating surfaces with EDC and NHS did not add any impurites to ELP coatings before and after AMP attachment, see Table 5.

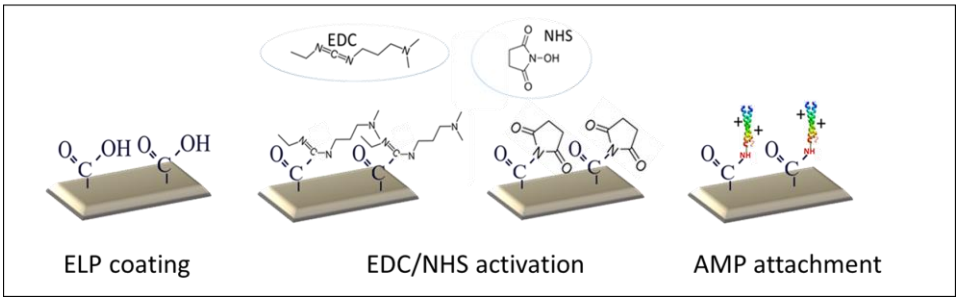

Figure 13. Schematic model for EDC/NHS activation and AMP attachment on ELP coated on Ti surfaces.

Table 5. The atomic percentage (%) of the chemical composition of ELP thin films coated on Ti discs before and after AMP conjugation by EDC/NHS coupling, as measured by XPS.

	C1s	N1s	O1s	Ti2P
Titanium disk	0.75	0.29	55.80	43.16
ELP coated Ti	64.20	15.71	20.09	-
ELP Coated Ti + AMP	51.98	16.88	31.14	

QCM-D measurements were performed to investigate the attachment stability of covalently bonded antimicrobial peptide into ELP surfaces and to compare it with when it is physically adsorbed onto the ELP thin films. It was shown that a higher content of antimicrobial peptide was attached to the EDC/NHS activated ELP surfaces compared to non-activated surfaces, Figure 14. The same behavior was observed on the activated and non-activated Ti surfaces. However, the AMP amount bound to ELP surfaces after EDC/NHS activation is significantly higher. Upon rinsing with Milli-Q water, most of the AMP remained on the activated surface while the whole content of AMPs was released from the other surfaces. These results confirm a stable attachment of AMP onto ELP surfaces via the EDC/NHS coupling approach.

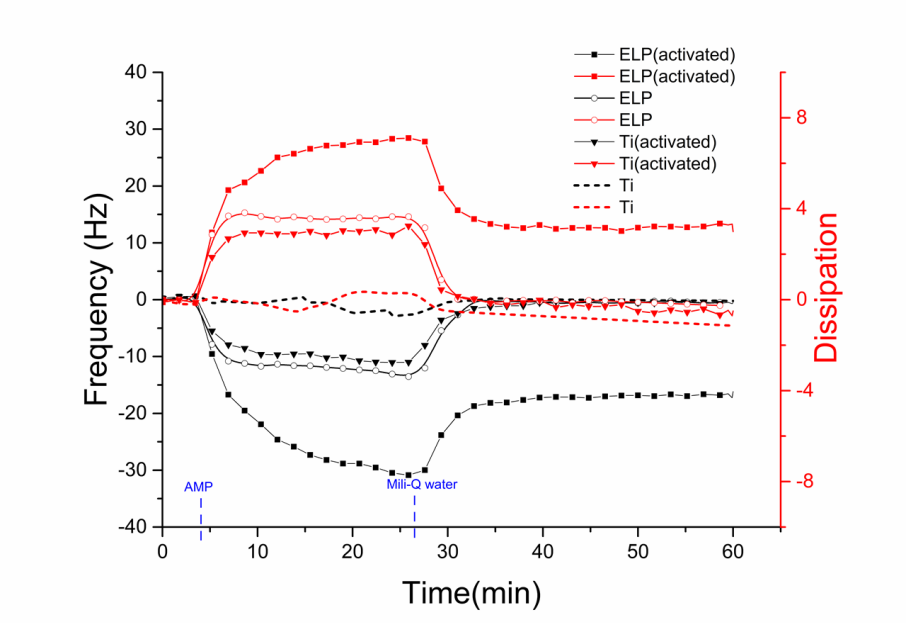

Figure 14. QCM-D results obtained from loading and washing AMP onto ELP surfaces with and without EDC-NHS activation. Pure Ti surfaces were used as control.

According to the results from the bacteria studies, RRPRPRPRPWWWW-NH_2 immobilized onto ELP thin films resulted in antimicrobial coatings. In Figure 15, the results from fluorescent imaging of biofilms with live/dead staining clearly demonstrate a high bactericidal effect on the surfaces modified with AMP. The statistical data obtained from calculating the green and red fractions using the ImageJ software from fluorescent microscopy images demonstrates a significant increase in the amount of dead bacteria on the AMP-functionalized surfaces (Figure 16). Additionally, the total area of the biofilm formed on these surfaces are clearly smaller than those without AMP.

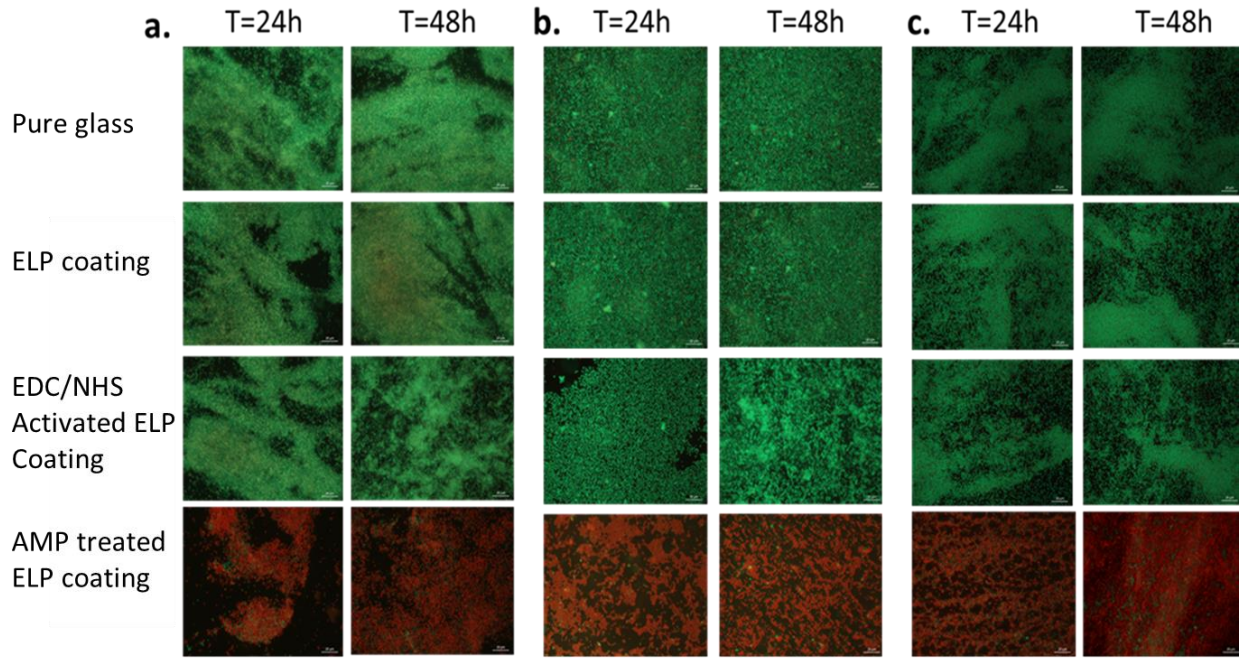

Figure 15. Images of biofilms formed onto ELP thin films with and without AMP. The bacteria are stained with SYTO® 9 and propidium iodide. The live bacteria appear green and the dead bacteria appear red. **a,** *S. epidermidis* **b,** *S. aureus* and **c,** *P. aeruginosa.*

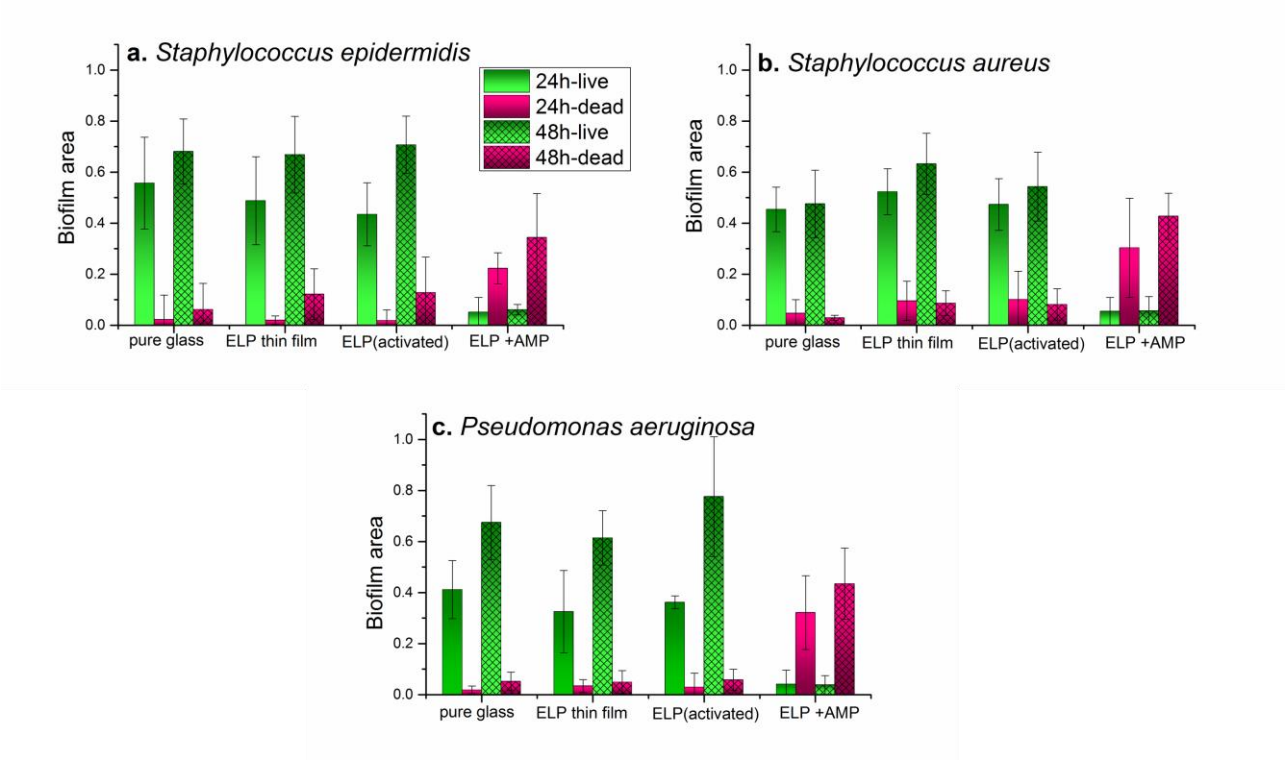

Figure 16. Data from analyzing the live and dead fractions of biofilms formed on ELP surfaces with and without AMP. The values are obtained from analyzing fluorescent images. Values are mean ±SD, N=3.

From the SEM images shown in Figure 17, clear distorstions in bacterial shape can be seen if they have been subjected to AMP modified surfaces. These results suggest that the AMP physically affects the bacterial membrane causing them to deform.

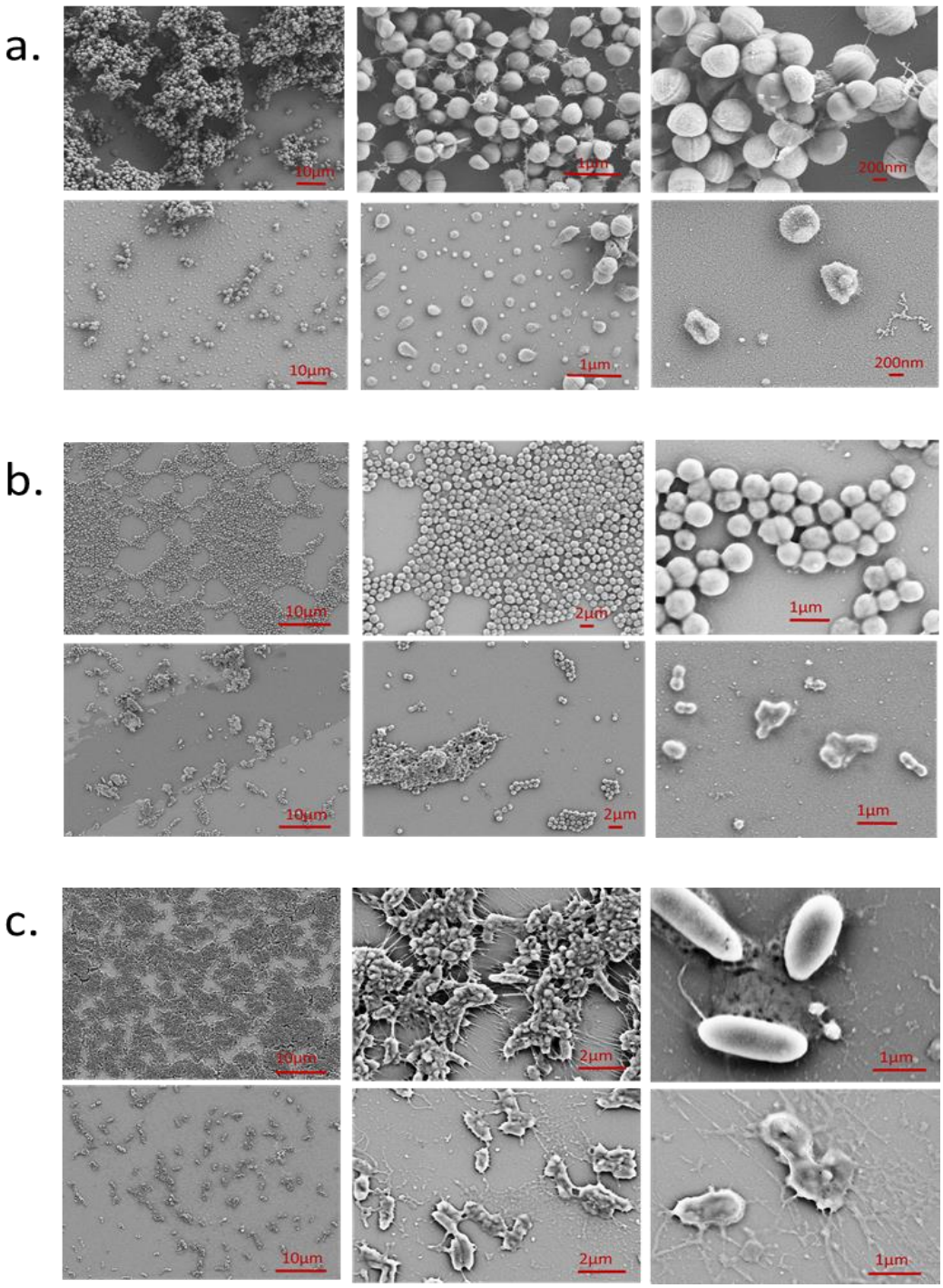

Figure 17. SEM images of bacteria taken after 24h **a,** *S. epidermidis* **b,** *S. aureus* and **c,** *P. aeruginosa.* The magnification increases from left to right. The pictures on the top row are the biofilms formed on ELP surfaces and the bottom row shows the bacteria grown on AMP functionalized ELP surfaces.

The overall results implies that the interaction of AMP with the bacteria outer membranes causes a lethal activity.

3.2.3. AMP stability

Few studies have been performed investigating the long-term stability of antimicrobial peptides in their intended clinical environment. One major challenge in the development of peptide based pharmaceuticals towards the clinic are their inherent instability towards enzymatic degradation in vivo.[60,61]

In this section of the thesis, results from experiments investigating the stability of immobilized AMP on ELP using fluorescent microscopy and bacterial assays are presented and discussed. We have taken the approach to investigate the in vitro stability of peptides in serum, since this has been shown to be a representative model for their in vivo stability.[60]

3.2.3.1. Fluorescent AMP in serum

In Figure 18, microscopy images showing the fluorescently labeled AMP modified ELP in both human blood serum and water as a function of time is presented. As can be seen, the immobilized peptides had started to aggregate after 24 hours when treated in serum, while in water no difference could be observed up to 3 weeks. The bacterial assay on AMP functionalized surfaces at the same duration of time showed that the immobilized antimicrobial peptides started to loose its activity after 24 hours in serum and that the antimicrobial activity was totally lost after 48 hours, Figure 19.

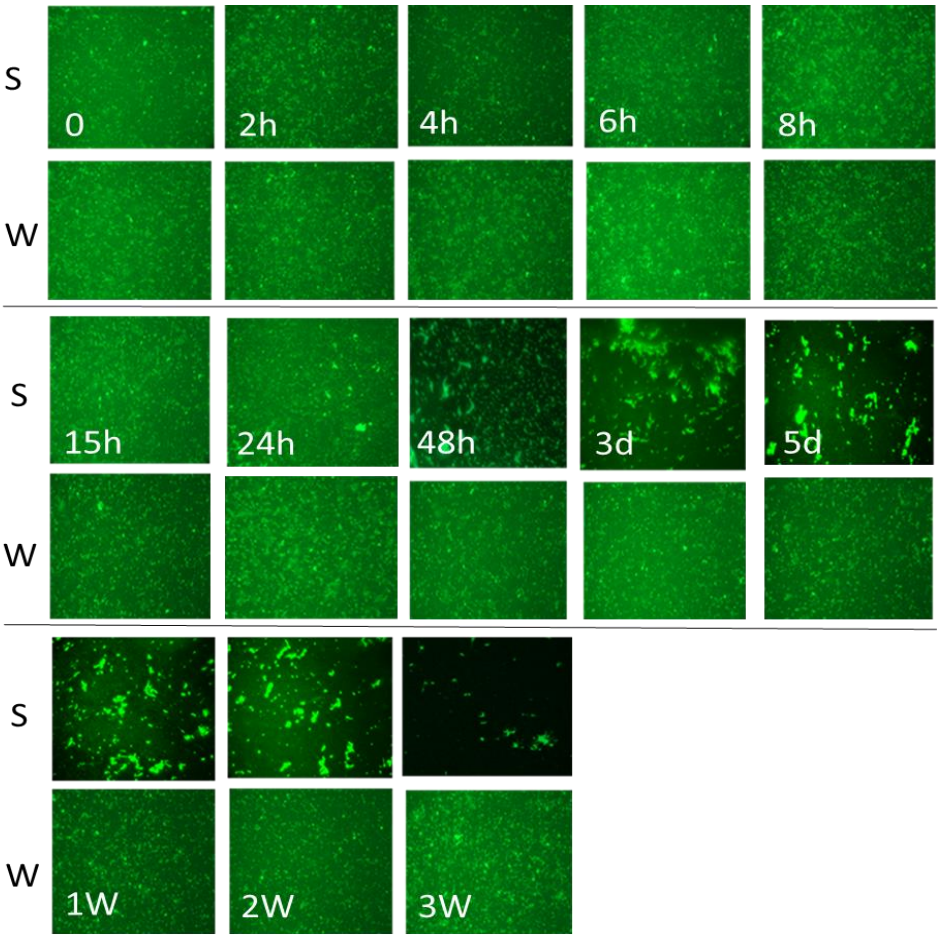

Figure 18. Fluorescent microscopy images of 5(6) carboxyfluorescein tagged RRPRPRPRPWWWW-NH_2 immobilized onto ELP surfaces treated in 20% human blood serum at different duration of time. Milli-Q water treated peptides was used as control.
(S= 20% serum media, W= Milli-Q water media)

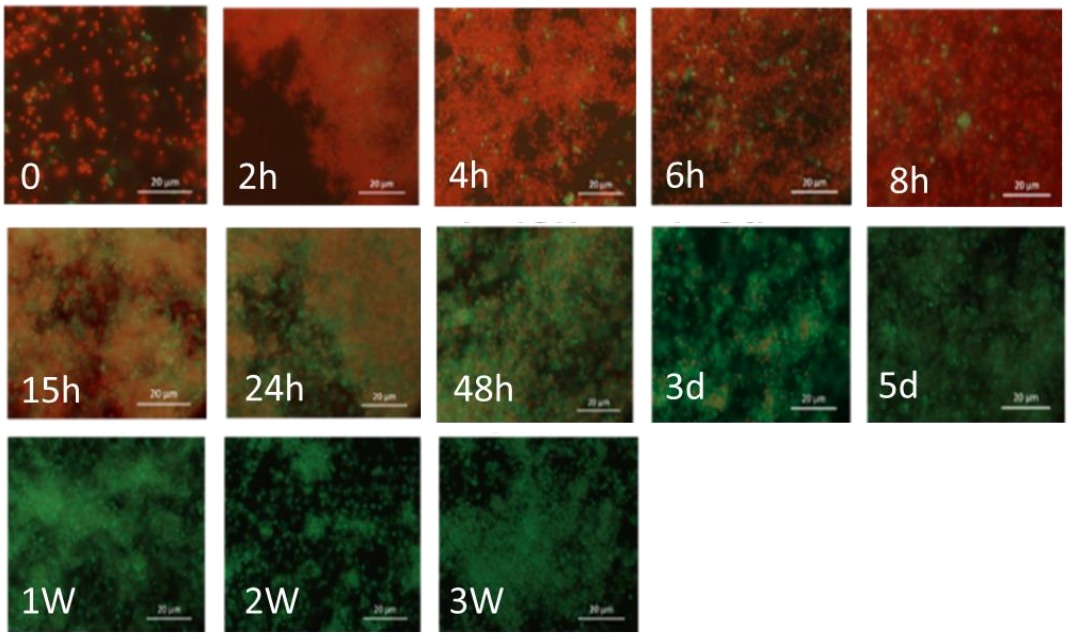

Figure 19. Bacteria (*S. epidermidis*) response to AMP functionalized surfaces treated in 20% serum at different duration of time.

These results suggest that the stability of AMP has been enhanced by the immobilization onto ELP surfaces compared to what has been reported when they are dissolved in serum media. Most evaluated AMPs show a half-life time stability of a only a few minutes to a few hours under physiological salt and serum conditions.[62–64] The reasons for why AMP becomess ineffective in serum is suggested to be caused by an increased ionic strength and the presence of serum proteins, which can lead to change in the peptide conformation and loss of activity due to peptide degredation.

4. Conclusion

The vulnerability to develop infection after surgery is a significant limitation that undermines biomaterial's performance in clinical applications. Creating antimicrobial biomaterials by means of modifying the surface is a promising solution to avoid such biomaterials associated infections.

In this thesis, two approaches to create surfaces that hinder bacterial colonization and growth are presented. One approach is based on the use of mesoporous titania (MPT) with the ability to release antibiotics. Mesoporous titania thin films with pore sizes of 4, 6 and 7 were formed and successfully loaded with three different antibiotics; Vancomycin, Gentamicin and Daptomycin. The antiobiotic loaded MPT films showed a significant reduction in the attachment of *S. aureus* and *P. aureginosa*. It was also shown that a smaller pore size and decreased surface energy of the MPT alone had a desired effect in reducing bacterial attachment without the use of antibiotics.

The other approach examined in the thesis was to create a bactericidal surface with the use of antimicrobial peptides. An antimicrobial peptide (AMP), RRPRPRPRPWWWW-NH_2, was covalently bonded to recombinant elastin protein (ELP) substrates and the bacterial response was evaluated. Engineered ELP having RGD sequences and photo-reactive sites were expressed in *E-Coli* culture followed by purification. Uniform ELP thin films were formed by spin coating, followed by crosslinking using UV radiation. NHS/EDC coupling chemistry was used to covalently bond the AMP to ELP. *S. aureus, S. epidermidis* and *P. aeruginosa* were used to evaluate the functionality and results showed a significant bactericidal effect when the films were functionalized with AMP. The stability of the AMP was studied in serum media and it was shown that the antimicrobial effect lasted up to 24 hours in the presence of human blood serum.

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