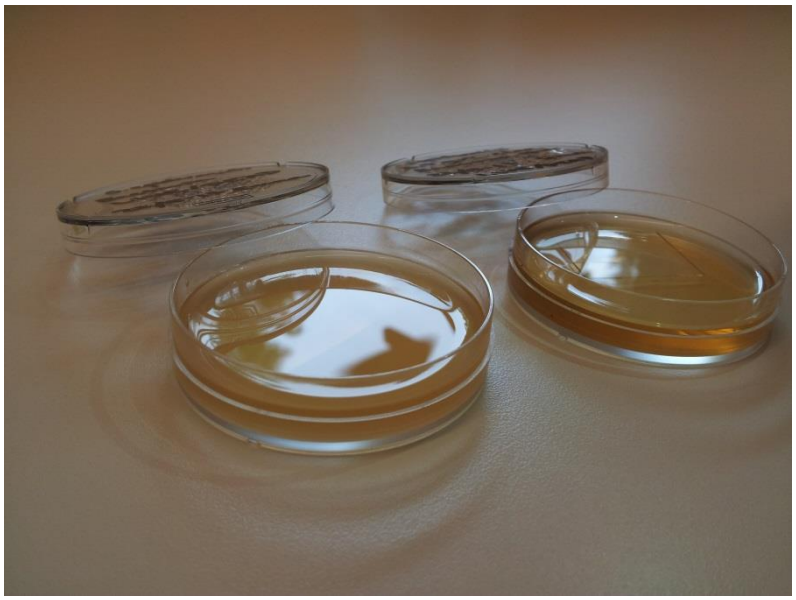
CHALMERS

Preventing biofilm formation using antibacterial loaded mesoporous surfaces



Master of Science Thesis

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Abstract

Infections caused by microbial biofilms are a large problem related to implants. Biofilms are responsible for 80 % of all infections in the world. When treating patients with implant related infections using antibiotics the drug reaches the biofilm from above, which may lead to eradication of bacteria only at the top of the biofilm, leaving viable bacteria in the lower part. One of the aims of this project was to test an option where the antibiotic was fed locally from the surface of the implant. Another aim was to try to integrate an antimicrobial peptide within the surface and see if it had the same or even better killing effect as the antibiotic. Mesoporous titania was spin coated on top of glass slides and charged with antimicrobial substance (Cloxacillin or RRP9W4N) and the bacterium *Staphylococcus epidermidis*. The samples were analyzed with QCM-D, SEM, light microscopy, UV-Vis and ESCA. The study was performed at Chalmers University of Technology at the department of Applied chemistry. The QCM-D and ESCA measurements on Cloxacillin indicate that Cloxacillin enters the pores of the mesoporous titania. The SEM images showed a mesoporous structure where RRP9W4N had the best effect. The UV-Vis spectra gave the same results, RRP9W4N was the best antimicrobial substance, but the light microscopy images favored Cloxacillin.

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1. Introduction

Infections caused by microbial biofilms are a large problem related to implants. Biofilms are responsible for 80 % of all infections in the world [2]. Their resistance to antibiotics can be even a thousand times more than the corresponding planktonic counterparts which makes them problematic to eradicate [3]. These biofilms consist of bacteria living on surfaces embedded in a matrix of polysaccharides, proteins and DNA among other things [2]. When treating patients with implant related infections using antibiotics the drug reaches the biofilm from above which may lead to eradication of bacteria only at the top of the biofilm, leaving viable bacteria in the lower part.

Usually antibiotics are given preventively in association with implant surgery but instead of giving a systematic prophylactic feed one of the aims of this project was to test an option where the antibiotics are fed locally. A disadvantage when using antibiotics is that the bacteria can develop resistance towards the antibiotics. That is why another aim was to try to integrate antimicrobial peptides into the implant surface and compare its bactericidal effect with the effect of the antibiotic. The antibiotic used in this project was Cloxacillin, a commercially available drug. The antimicrobial peptide used was RRPRPRPRPWWWW-NH_2, which has shown high bactericidal effect. The bacterium that was cultured is *Staphylococcus epidermidis*, a common skin bacterium. As this work is only one part of a larger project there will be no animal testing included in this thesis.

2. Background

A brief introduction to the materials and methods used will be presented in the sections below.

2.1 Mesoporous titania

Mesoporous materials can, due to their high surface area and rich porosity, work as a local drug delivery system. Recent studies have indicated that in the biomedical field sol-gel-derived titania coatings on Ti alloys are potentially applicable. Mesoporous titania films (MTF) have shown a higher strength than conventional titania films for two reasons; there is a low amount of polymer and carbon left in the MTF after heat treatment which increases the bonding with the substrate, and there are a lot of Ti-OH groups in the MTF with the ability to work as binders providing strength [4]. Mesoporous titania ($MpTiO_2$) has been evaluated as coating on titania implants. The results showed more bone formation and increased tissue integration to the coated implants [5]. The advantage of using $MpTiO_2$ is its low toxicity, bio-stability, large specific surface area and a pore system with tunable drug loading and release rate [6].

2.2 *Staphylococcus epidermidis*

Staphylococcus epidermidis, or *S. epidermidis*, is a bacterium situated in the skin and mucous membranes of the human body. As it belongs to the normal human bacterial flora, a predisposed host is necessary for it to become a cause infection. It is a common pathogen causing hospital acquired infections in bloodstream, eye, ear, nose and throat as well as cardiovascular infections. *S. epidermidis* often leads to subacute or chronic infections due to lack of tissue-damaging toxins. Another type of infection, that is very common, caused by *S. epidermidis*, is related to foreign bodies like indwelling catheters and implanted devices. *S. epidermidis* forms a biofilm on the indwelling device which makes the bacterium difficult to eliminate since the biofilm decreases the penetration of antibiotics to the target along with a decreased immune response. Therefore, the device often needs to be reinserted [7].

2.3 Cloxacillin

Cloxacillin is a semi-synthetic penicillin commonly used in human and veterinary medicine. The antimicrobial property comes from its beta-lactam ring [8]. Due to this beta-lactam it has a time-dependent bactericidal activity which means that an increase in the concentration past a certain point does not improve the bactericidal activity significantly. How effective these antibiotics are depends on the time that the bacteria are exposed to the penicillin, as the mechanism is to inhibit the construction of the bacterial wall. Cloxacillin can diffuse into the skin, soft tissue and even to fetal blood [9]. The structure of Cloxacillin is illustrated in Figure 1.

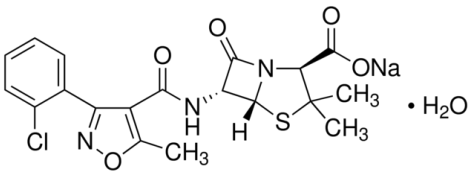

Figure 1. The chemical structure of Cloxacillin [1].

2.4 Antimicrobial peptide

The peptide RRPRPRPRPWWWW-NH_2 (RRP9W4N) originates from derivatives of peptides from the protein proline arginine-rich end leucine-rich repeat protein (PRELP). However, these derivatives have been modified by attachment of a hydrophobic tail of the amino acid tryptophan. This results in a peptide with low toxicity towards human cells and high proteolytic stability. The peptide has a high bactericidal effect, especially at the skin and wound surface [10].

3. Experimental

In the following sections the synthesis steps and preparation of media as well as analysis methods are described. The calculations are found in Appendix.

3.1 Synthesis of mesoporous titania

Pluronic-123 (P123) (Aldrich) and ethanol (EtOH) (Solveco) were mixed in a vial, 0.5 and 8.5 g respectively. In another vial 1.6 g of 37% hydrochloric acid (HCl) (Sigma-Aldrich) were placed and 2.1 g of titanium(IV) tetraethoxide (TEOT) (Aldrich) were quickly added, since TEOT rapidly turns into titania when exposed to air. The reaction is exothermic and when shaken it becomes warm. The two solutions were left to stir for approximately three hours (until the later solution was transparent yellowish). The P123+ethanol solution was poured into the other reaction and the mixture, which had become transparent and colorless, was left to stir over night. The obtained mesoporous titania precursor solution was then used for making thin films on glass slides.

3.2 Thin film preparation

Spin coating is a technique used to spread a liquid on top of a sample using rotation to create a uniform film. Microscope slides (VWR) were cut in somewhat uniform pieces. On each piece, 100 µL of mesoporous titania solution was spin coated (Spin 150) at 7000 rpm for 60 seconds. When spin coating, the sample was attached to the sample holder by vacuum suction. As the spin coater was started the sample was rotated and the liquid was added during the first seconds. Depending on the wanted film thickness the revolutions per minute (rpm) and time can be varied. The samples were then left to age for about a day. This allowed the structure to form. After the aging the samples were placed in an oven for calcination, the oven used was a Nabertherm. The temperature was increased

to 350°C at a rate of 1°C/minute and this temperature was maintained for four hours. During the calcination, Pluronic was removed and cross-linking had occurred. After the samples had cooled they were stored in sterile 99.7% ethanol at room temperature.

3.3 Quartz crystal microbalance with dissipation measurements

The Quartz Crystal Microbalance with Dissipation (QCM-D) technique is based on the frequency and energy dissipation response of a freely oscillating sensor [11]. It consists of a thin quartz disc that is connected to two electrodes. Two QCM-D titanium discs (qsense) were spin coated with mesoporous titania solution (60 µL) and calcined in the same way as described in the section above. The samples were loaded in the q-sense E4 and Milli-Q water was passed through the system, at the highest speed of 0.606 mL/minute. The crystal was excited so that it began to oscillate when an AC voltage was applied across the electrodes. A baseline was created with Milli-Q water for four minutes at the speed 50 µL/minute. Then the antimicrobial substance was applied and the time was measured for how long it took for the substance to enter the pores. The flow 50 µL/minute is much faster than the flow in the body. The frequency was measured and as the instrument was running a thin film was attached to the sensor which gave a decrease in the frequency. The energy dissipation indicates the viscoelasticity of the film. Milli-Q water was applied again to measures the release time. Three different Cloxacillin (Sigma-Aldrich) concentrations were run at separate times; 0.5 g/L, 0.5 mg/L and 2.25 mg/L and one RRP9W4N (Biopeptide Co., San Diego, USA) concentration; 200 µM. After finishing the instrument was cleaned with Milli-Q water and SDS (2%) at the highest speed. The QCM-D discs were cleaned in the following way:

1) Sonication in UV for 15 minutes.
2) Suspension in SDS (2%) for 15 minutes.
3) Rinsing in water.
4) Drying with N_2.

3.4 Scanning electron microscope measurements

In a scanning electron microscope (SEM) a focused beam of high-energy electrons is applied to produce a variety of signals at solid specimens' surface. The accelerated electrons contain substantial amounts of kinetic energy that is dissipated as these signals that are provided by the interactions between the electrons and the sample. A few of the obtained signals are secondary electrons, backscattered electrons, photons and visible light. The information obtained is for example external morphology, chemical composition, crystalline structure and orientation of materials in the sample [12]. The two sections below describe the sample preparations for measurements of the mesoporous TiO_2 surface and bacterial growth with/without antimicrobial substance.

3.4.1 Preparation for measurement of the mesoporous surface

Two titanium discs (Alfa Aesar) that had been stored in sterile filtered EtOH were rinsed with sterile filtered Milli-Q water. They were further dried with N_2. Since their size was similar to the size of the QCM-D discs, they were spin coated with the same amount of mesoporous titania solution, 60 µL. The discs were left to age for a day and a half, after which they were calcined in the same way as the QCM-D discs. The discs were stored on the shelf, covered with aluminum foil, before they were analyzed in the SEM. Before analysis the samples were mounted onto the sample holders with double-coated adhesive carbon tape. The instrument used for SEM was a Zeiss Gemini.

3.4.2 Preparation for measurement of the surface with bacteria and antimicrobial substance

The two titanium discs for the previous SEM measurements were reused (sterilized in the autoclave) and a third surface was prepared in the same way. They were stored in sterile filtered EtOH and before use they were rinsed with sterile filtered Milli-Q water. The samples were charged, each in a separate sterile petri dish, with antimicrobial substance; one with 0.5 g/L Cloxacillin, one with 200 µM peptide and the last one is used as control and contains no antimicrobial substance. *S. epidermidis* was then added to the surfaces, 10 mL for the samples with Cloxacillin and the one without any antimicrobial substance, and 2 mL to the sample with peptide. The dishes were then placed in the oven to grow the bacteria for 24h at 37°C. The next day the created biofilms were fixated in the following way:

1) Remove the bacteria with a pipette.
2) Quickly add 5 mL EtOH (50 %) and wait three minutes.
3) Remove the EtOH (50 %), add 5 mL EtOH (80 %) and wait three minutes.
4) Remove the EtOH (80 %), add 5 mL EtOH (99.7 %) and wait three minutes.
5) Remove the EtOH (99.7 %) and let the samples dry in air until they are completely dry.

The samples were then sputtered with gold in a Jeol JFC-1100E Ion Sputter at 10 mA for 90 seconds. They were then studied with the same SEM instrument as in the section above.

3.5 Preparation of cell media

Preparations of the different cell media needed to grow bacteria are presented in the sections below.

3.5.1 Preparation of Todd Hewitt broth

In a beaker, 36.4 g of Todd Hewitt broth (Oxoid CM0189) (TH) were added and a small amount of Milli-Q water, approx. 200 mL was added. The solution was left to stir for a while until the powder had dissolved. The rotation of the stirrer should be slow to avoid the formation of bubbles. Then the

rest of the water (800 mL) was added and it was left to stir for a few minutes. The broth was poured into bottles and placed in the autoclave (Systec DX-65) for 15 minutes at 121°C. The bottles were stored in room temperature.

3.5.2 Preparation of 0.85% NaCl

The solution was prepared in percentage by weight to the wanted volume of 50 mL:

0.85% NaCl (Merck): $0.00085*50*10^{-3} = 4.25*10^{-5}$ L

The density of NaCl is 2165 g/dm^3

$m = 2165*4.25*10^{-5} = 0.0920125$ g

92 mg of NaCl was dissolved in Milli-Q water until the volume measured 50 mL. The solution was autoclaved in smaller vials in volumes of 10 and 1.8 mL and stored in the fridge at -4°C.

3.6 Making agar plates

52 g of Brain Heart Infusion Agar (Fluka) was dissolved in 1 L of Milli-Q water. This was poured into a bottle (together with the stirrer) and the bottle was placed in the autoclave at 121°C for 15 min. When removed from the autoclave the bottle was placed on a stirring plate to keep the agar from falling to the bottom. The agar was quickly transferred with a sterile pipette to petri dishes, 25 mL in each. One pipette is used only for five dishes to keep it sterile. The bubbles were removed with a crème brûlée burner after each ten dishes. This was done fast to avoid burning the agar or the dish. The agar plates were left to cool with the lid open and covered with aluminum foil on the bench until the next day, and then they were stored in the fridge.

3.7 Bacterial growth

Bacterial growths with the two different antimicrobial substances were performed in the ways described in the following sections. Each growth has been done two times in order to get reliable results.

3.7.1 Growth with Cloxacillin

S. epidermidis grown on an agar plate with horse blood was transferred to a clean agar plate using an antiseptic technique. Some bacteria was also transferred to four vials containing 1 mL each of 10% skim milk and these vials were stored in a freezer at -80°C. The agar plate was placed in an oven at 37°C over night and thereafter stored in the fridge. Bacteria from the agar plate was added to 8 small vials containing 5 mL of TH and the vials were put in the oven at 37°C with their lids loosely attached, to allow bacterial growth. The next day the vials were removed from the oven and the solution was

transferred from the vials to larger bottles containing TH; 5 mL of bacterial solution per 100 mL fresh TH. The bottles were placed in the oven for another day with the lid loosely attached to allow the bacteria to grow. On the next day, three glass slides were charged with 2 mL of Cloxacillin (0.5 g/L) in the surface for 2.5 hours (see Section 4.1). In the meantime, bacteria were washed and harvested. Bacteria were harvested by centrifugation at 2500 rpm for 15 min, and the pellets were resuspended in fresh TH and washed (2500 rpm, 10 min). Bacteria were then transferred to a larger bottle giving a total volume of 200 mL. Bacteria and antibiotic were added to 6-well plates according to Table 1 making triplicates of each test/control. 10 mL of bacteria and 0.5 mL of Cloxacillin was added in each well. The plates were put in the oven for 24 h.

Table 1. Addition of bacteria and sterile filtered antibiotic to different surfaces.

Test 1 Bacteria 0.5 g/L Cloxacillin charged in the surface	**Test 2** Bacteria 0.5 g/L Cloxacillin in media	**Test 3** Bacteria 2.25 mg/L Cloxacillin in media	**Control 1** Bacteria No Cloxacillin	**Control 2** Teflon surface Bacteria No Cloxacillin	**Control 3** Non- mesoporous surface Bacteria 2.25 mg/L Cloxacillin in media

In order to know how much bacteria there was in each well the CFU was calculated. Therefore, a dilution series was created where bacteria were diluted in four steps:

1) 100 µL of the centrifuged bacteria was added to a vial containing 10 mL of the NaCl solution. This gives a concentration of $1*10^{-2}$ µL/mL
2) 100 µL of solution 1) was added to a vial containing 10 mL of the NaCl solution. This gave a concentration of $1*10^{-4}$ µL/mL
3) 200 µL of solution 2) was added to a vial containing 1.8 mL of the NaCl solution. This gave a concentration of $1*10^{-5}$ µL/mL
4) 200 µL of solution 3) was added to a vial containing 1.8 mL of the NaCl solution. This gave a concentration of $1*10^{-6}$ µL/mL

Two fresh agar plates were marked on the outside at the bottom by dividing them in half. For each agar plate, 50 µL of a solution was added to one half of the plate and the solution was wiped across the surface. On the other half of the agar plate, the same procedure was performed with the same solution. For each new agar plate a new concentration was used. The agar plates were placed in the oven with the lid opened and tilted so that the solution could dry. When the solution had dried (it took about one hour) the lid was put on and the agar plate was turned with the bottom up. The

plates were left in the oven over night. The next day the plates were taken out and the colonies that had grown during the night were measured. For the solution with concentration 10^{-5} µL/mL the agar plates were divided into quarters to be able to count each colony and then the value were multiplied by 2.

The 6-well plates that were left for growth for 24 h were removed from the oven and the bacterial solutions were removed with a pipette. The next day the wells were stained following the safranin biofilm protocol:

1) The wells were emptied from bacterial solutions and rinsed 5 times with 10 mL of Milli-Q water by filling the wells with fresh water, carefully rotating them and removing the liquid from the wells with a pipette. The pipette was put on the side of the Titania slides so that the biofilm was not destroyed.
2) The biofilm was fixated with 5 mL of absolute methanol (Fluka) for 20 min and after the methanol was removed the slides were left to dry tilted against the wall of the well, approx. one hour.
3) The surfaces were stained with 3 mL of 0.1% safranin (Alfa Aesar) (5mL for the Teflon surfaces since they are so thick) for 15 min. A control was added, a clean well, which was stained in the same way.
4) The stain was removed and the wells were washed 5 times with 10 mL of Milli-Q water in the same way as in 2). The wells were left to dry for another hour with the slides tilted.
5) The samples were analyzed with a light microscope to see how the bacterial growth was affected by the Cloxacillin.

3.7.2 Growth with RRP9W4N

The bacteria were handled in the same way as was done in the section above but the difference being the preparation of the surfaces. Three glass slides were charged with 2 mL of RRP9W4N (200 µM) in the surface for 1 hour (see Section 4.1). The centrifugation was done in the same way and bacteria and peptide were added to 6-well plates according to Table 2 making triplicates of each test/control. 2 mL of bacteria and 0.1 mL of RRP9W4N was used in each well. The plates were put in the oven for 24 h.

Table 2. Addition of bacteria and peptide to different surfaces.

Test 1 Bacteria 200 µM RRP9W4N charged in the surface	**Test 2** Bacteria 200 µM RRP9W4N in media	**Test 3** Bacteria 120 µM RRP9W4N in media	**Control 1** Bacteria No RRP9W4N	**Control 2** Teflon surface Bacteria No RRP9W4N	**Control 3** Non-mesoporous surface Bacteria 120 µM RRP9W4N in media

The dilution series and safranin biofilm protocol were performed as in the section above.

3.8 Ultraviolet-visible spectrophotometer measurements

In the ultraviolet-visible spectrophotometer (UV-Vis) there is a combined deuterium-discharge lamp that works as a radiation source for the ultraviolet (UV) wavelengths and a tungsten lamp for the visible (Vis) and short wave near-infrared (SWNIR) wavelengths. A special rear-access lamp design makes it possible for both light sources to share the same axis to the source lens, creating a single beam of light. This beam travels through the shutter/stray-light correction filter area to the spectrograph lens and slit, passing through the sample. This gives information about all the wavelengths at the same time and this is then displayed in a spectrum [13]. The lamp was turned on approx. 30 minutes before the instrument HP Agilent 8453 was used. 2 mL of 95% EtOH was added to each well containing stained slides including the control well. After 8 min the slides were removed (the time it takes for the biofilm to dissolve). The program “Instrument Online” was started and the prepared method was selected. The cuvettes were blown with N_2 to remove any possible dirt. Each cuvette was charged with 1.4 mL of each solution, respectively. Each sample was run once, then the cuvette was turned upside down and run again (this should not make any difference in the spectrum, should give the same values as the first run). The control well in the safranin biofilm protocol (Section 3.7.1) was used as the blank.

3.9 Electron spectroscopy for chemical analysis

Electron spectroscopy for chemical analysis (ESCA), or X-ray photoelectron spectroscopy (XPS), is an instrument that measures the ejection of an electron from the core level by an X-ray photon with the energy *hv*. An electron spectrometer is used to analyze the energy of the emitted photoelectrons and this is presented in a graph with the electron energy as a function of the intensity (given in counts or counts/s). The important parameters in ESCA are:

$E_B = hv - E_K - W$

where *hv* is the photon energy and W is the spectrometer work function. The quantity experimentally measured is the kinetic energy (E_K) which depends on the photon energy of the applied X-rays. The binding energy (E_B) is specific for electron, both due to the element and atomic energy level. E_B is calculated by the data system and the operator chooses an appropriate binding or kinetic energy scale. The electronic structure of an element is reproduced accurately due to all electrons with a binding energy less than the photon energy appearing in the spectrum. The characteristic peaks in the spectrum come from the electrons that are excited and escape without energy loss. The ones that have inelastic scattering and energy loss are included in the background of the spectrum. The relaxation of the emitted photoelectron results in emission of an X-ray photon (X.-ray fluorescence) [14]. Ten smaller pieces (between 5 and 8 mm from edge to edge) of glass slides were cut out and spin coated with 15 µL of the mesoporous titania solution. They were left to age a day and then calcined in the same way as the other glass slides. The pieces were then stored on the shelf, covered with aluminum foil. Since three of the small glass slides should contain antibiotic when analyzed in ESCA they were charged with 2 mL of the 0.5 g/L Cloxacillin for 2.5 hours. The liquid was then removed and the pieces were stored on the shelf in a petri dish until the analysis. The ESCA used was a Quantum 2000 scanning ESCA microprobe from Physical Electronics and the X-ray source was Al Kα (1486.6 eV). The samples were mounted onto a metal plate where they were attached with a smaller, thin metal plate and two screws per sample. The beam size was 100 µm and the approx. analyzed area was 400*500 µm. The take-off angle was 45° with respect to the sample surface and the information depth was approx. 4-5 nm.

3.10 Colony forming units

The number of microorganisms in or on the surface of a sample can be determined by measuring the colony forming units (CFU). This is done by spreading the prepared sample uniformly on the surface of an agar plate. The agar plate is then incubated for some days at an arbitrary temperature. The CFU is established by counting the colonies and presenting it either as CFU per unit weight, CFU per unit area or CFU per unit volume [15].

4. Results

The sections below present the results from characterization and analysis along with some statistics.

4.1 QCM-D graphs

In order to know how much time is needed for Cloxacillin to enter the pores as well as the time for the release of the drug a QCM-D measurement had to be performed. This is illustrated in Figure 2. The frequency rapidly decreased to a value of about -74 Hz as the drug entered the pores. The

charging time was appreciated to 2.5 hours and this was applied in the preparation of the drug loaded mesoporous surfaces (see Section 3.7.1). The release of the drug (when Milli-Q water was applied) gave a slow increase in frequency and carried on for approx. 13 hours. The dissipation, on the other hand, increased as the drug was entering the pores meaning that the thin drug film created was moving quite a lot. However, as Milli-Q water was applied the dissipation decreased due to less drug film.

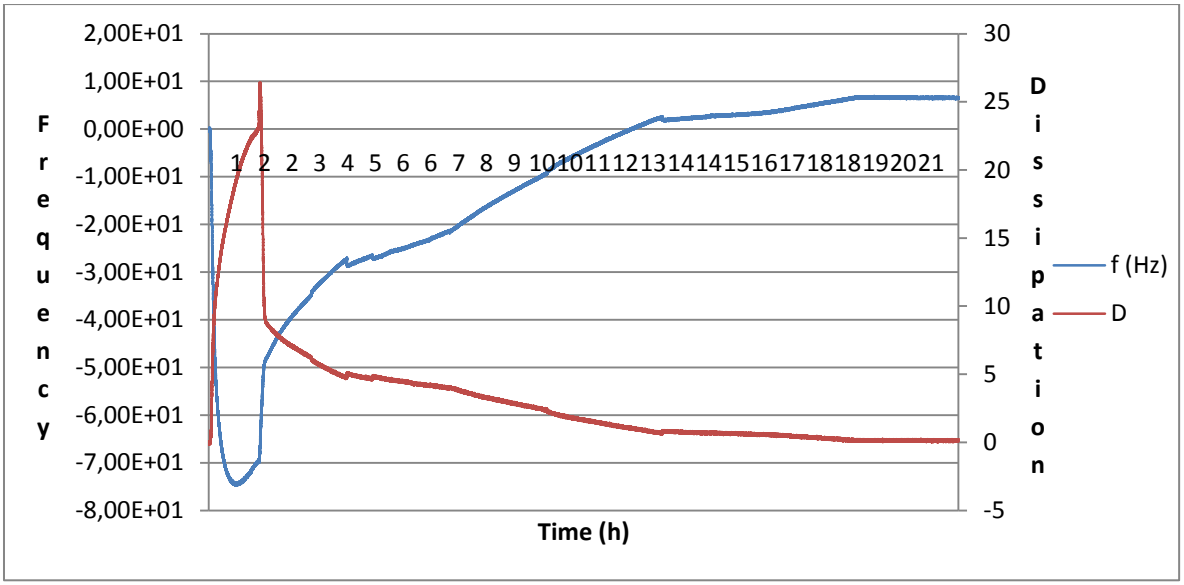

Figure 2. The frequency and dissipation variation with time (hours) in a titania surface using Cloxacillin.

As for the trials with RRP9W4N the same kind of QCM-D measurement was performed, which is illustrated in Figure 3. The frequency decreased rapidly to a value of approx. -12 Hz as the peptide entered the pores. The time needed was appreciated to 1 hour and this was applied in the preparation of the peptide loaded mesoporous surfaces (see Section 3.7.2). As Milli-Q water was applied there was a rather fast release of the drug that stabilized after about 3.5 hours. There was a small decrease in dissipation that stabilized after 1.5 hours meaning that the thin peptide film is barely moving.

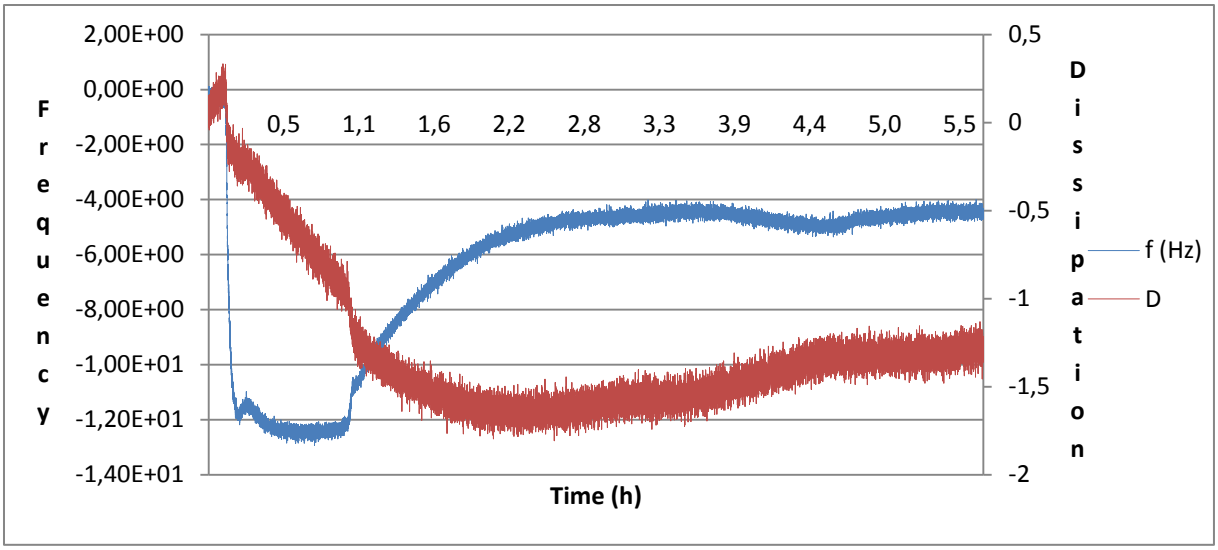

Figure 3. The frequency and dissipation variation with time (hours) in a titania surface using RRP9W4N.

4.2 SEM images

To confirm that the synthesis used gave a mesoporous structure SEM was performed. The structure of the mesoporous titania is shown in Figure 4. As seen the order of the pores (the black dots) was poor. However, the pore size was as expected about 6 nm. From this image it was also clear that the pores were directed towards the surface, which was very important for the application.

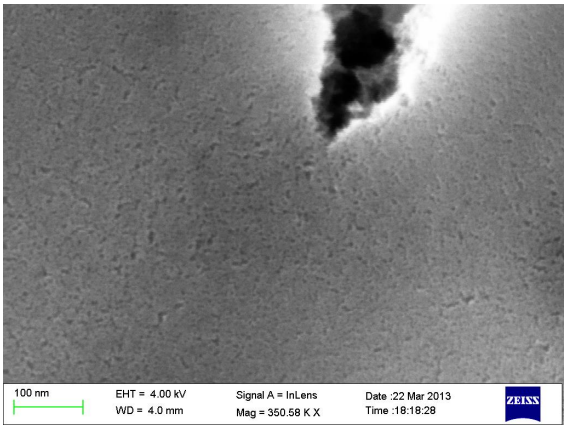

Figure 4. Illustration of the mesoporous structure of TiO_2

To give a clear view of the effect of the antimicrobial substances, and as a complement to the light microscope images, SEM images have been collected (Figure 5). Although it might be difficult to see each bacterium with this magnification (1K) it is clear that the control to the left is covered with much more bacteria (black dots) then the middle image, the sample treated with Cloxacillin. As for the sample treated with RRP9W4N (right image) the image is rather dark but a few bacteria can be seen as granules.

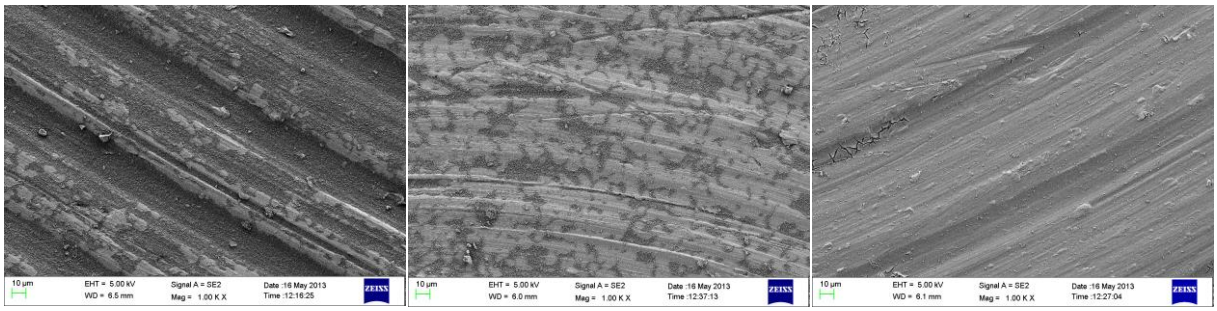

Figure 5. Images of surfaces covered with bacteria (magnification 1 K). Left: Only bacteria; middle: bacteria and surface charged with 0.5 g/L Cloxacillin; right: bacteria and surface charged with 200 µM RRP9W4N.

To clarify the difference Figure 6 is given which shows a larger magnification (Left: 20K; middle: 20K; and right: 27K). Comparing the control (left) and the antibiotic treated (middle) it is evident that Cloxacillin works well as an antimicrobial substance. The morphology also differs; the bacteria in the control are pressed more to the spin coated slide, giving a shape close to the one obtained when frying an egg. The sample treated with antibiotic, on the other hand, has more round shapes like a ball. The RRp9W4N treated sample has very few bacteria that are shaped both as separate and as clusters, where the bacteria in the clusters are connected in a way looking like bridges. The bacteria are uneven in their outer layer (sponge-like) and they have different sizes and shapes.

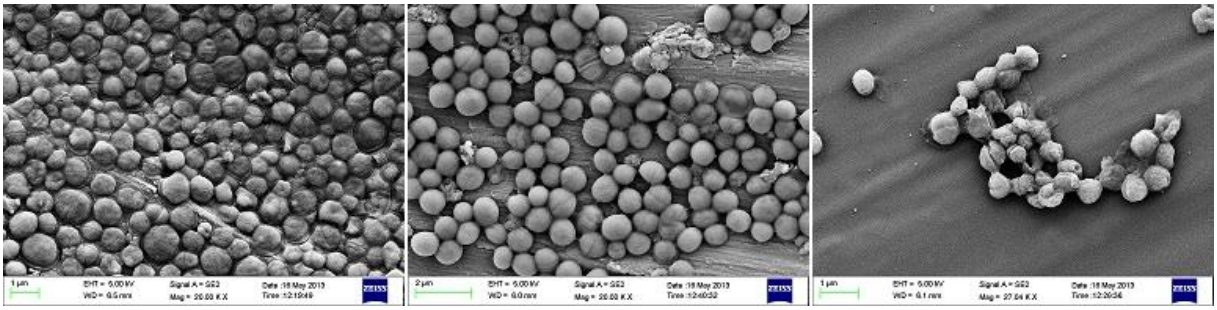

Figure 6. Images of surfaces covered with bacteria (magnification 20 K). Left: Only bacteria; middle: bacteria and surface charged with 0.5 g/L Cloxacillin; right (magnification 27 K): bacteria and surface charged with 200 µM RRP9W4N.

4.3 Light microscopy images of the different surfaces

In the following sections a selection of images is shown.

4.3.1 Light microscopy images of bacterial solutions treated with Cloxacillin

To get some apprehension of how effective Cloxacillin was the controls were compared to the tests. Figure 7 shows the first bacterial growth and when comparing the treated samples (a), b) and c)) with the control (d)) there are a lot less bacteria in the treated samples than the control. The best effect is obtained when Cloxacillin is charged in the surface. The second control, Teflon surface (e)), does not contain as much bacteria as the first control.

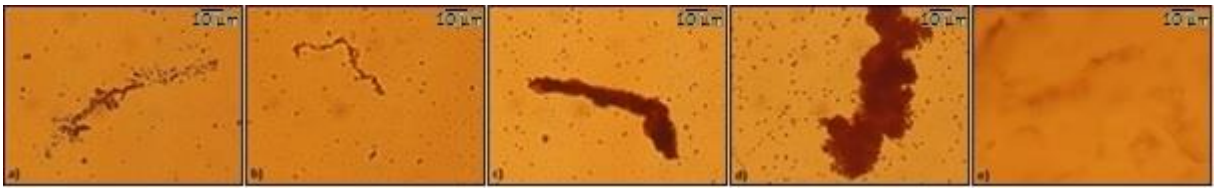

Figure 7. The first bacterial growth trial with antibiotic using a magnification of x50: a) bacteria and 0.5 g/L Cloxacillin charged in the surface, b) bacteria and 0.5 g/L Cloxacillin in media, c) bacteria and 2.25 mg/L Cloxacillin in media, d) bacteria and e) Teflon surface and bacteria.

The images from the second bacterial growth are given in Figure 8. The same effect is seen here (as in Figure 7), the treated samples show fewer bacteria than the controls. However, the effect is better when Cloxacillin is added to the media instead of charged in the surface. There is also a lot more bacteria on the Teflon surface. When comparing the mesoporous and the non-mesoporous samples c) and f) it is evident that a mesoporous surface, giving a lot less bacteria, is necessary for the application.

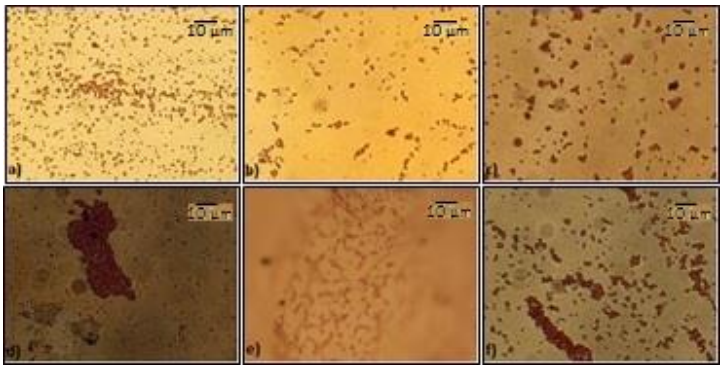

Figure 8. The second bacterial growth trial with antibiotic using a magnification of x50: a) bacteria and 0.5 g/L Cloxacillin charged in the surface, b) bacteria and 0.5 g/L Cloxacillin in media, c) bacteria and 2.25 mg/L Cloxacillin in media, d) bacteria, e) Teflon surface and bacteria and f) non-mesoporous surface and 2.25 mg/L Cloxacillin in media.

4.3.2 Light microscopy images of bacterial solutions treated with RRP9W4N

As in the section above the effectiveness of RRP9W4N is compared and this is illustrated in Figure 9 and 10. In Figure 9 the first bacterial growth is shown and it appears as though the peptide does not affect the bacteria significantly. There is a lot less bacteria in the controls d) and e) than in any of the treated samples (including control 3).

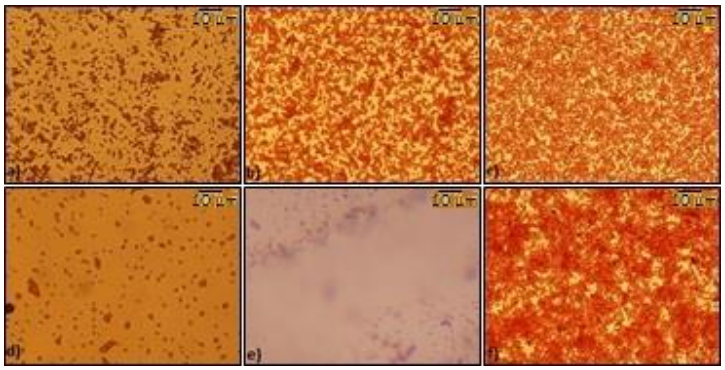

Figure 9. The first bacterial growth trial with peptide using a magnification of x50: a) bacteria and 200 µM RRP9W4N charged in the surface, b) bacteria and 200 µM RRP9W4N in media, c) bacteria and 120 µM RRP9W4N in media, d) bacteria, e) Teflon surface and bacteria and f) non-mesoporous surface and 120 µM RRP9W4N in media.

Figure 10 illustrates the second bacterial growth where the peptide charged surface (a)) shows a really good effect compared to the other treated samples. The mesoporous surface works considerably better than the non-mesoporous surface, comparing image c) with f).

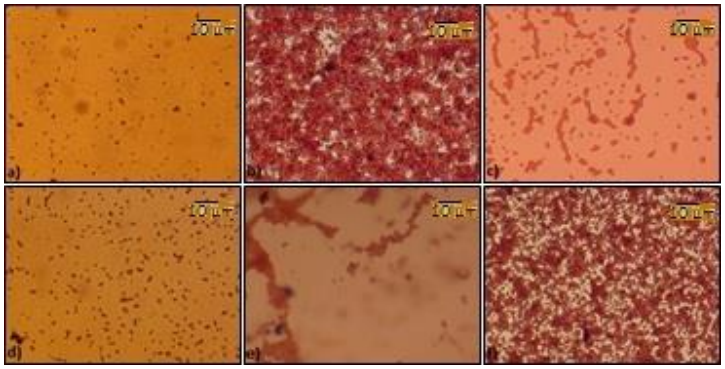

Figure 10. The second bacterial growth trial with peptide using a magnification of x50: a) bacteria and 200 µM RRP9W4N charged in the surface, b) bacteria and 200 µM RRP9W4N in media, c) bacteria and 120 µM RRP9W4N in media, d) bacteria, e) Teflon surface and bacteria and f) non-mesoporous surface and 120 µM RRP9W4N in media.

4.4 UV-Vis

The following sections contain the results of the absorbance measurement for the different trials. The absorbance gives an indication of how much biofilm has been formed on the surface. As described earlier in the safranin biofilm protocol (Section 3.7.1) the slides were washed with Milli-Q water. This was to remove all the dead bacteria. The bacteria that were not washed away (the created biofilm) were fixed with methanol and then colored with safranin. A high absorbance thus means that there were a lot of bacteria on the surface. In addition, some statistics are presented. All samples were analyzed but to provide a cleaner spectrum only the mean values of each sample group is used in the graphs and tables, and the values were taken at 490 nm [16].

4.4.1 Measurements from bacterial growth with Cloxacillin

For the first growth, the UV-Vis spectrum is illustrated in Figure 11. As one can notice there is only two controls instead of three (compared to Table 1 in Section 3.7.1), due to the third control was added after the first trial. The tests lie considerably higher in the spectrum than control 2 but control 1 follows test 1 quite closely. This means that there is less bacteria on control 2 than on the other samples.

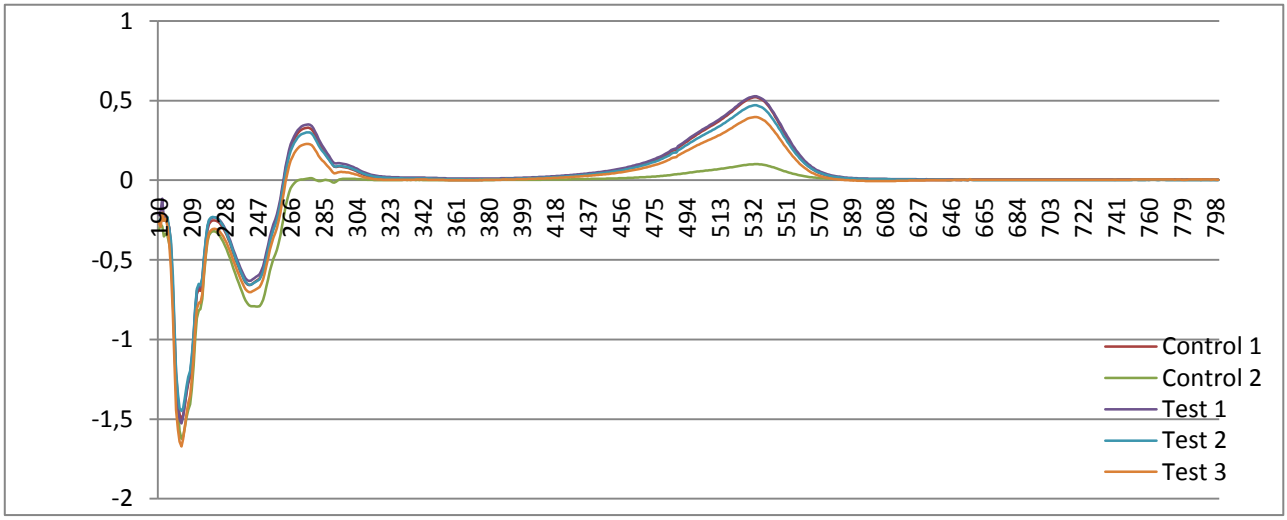

Figure 11. The mean values of the absorbance of the different samples as a function of the wavelength for the first bacterial growth.

Table 3 gives the mean value of the different tests at the absorbance 490 nm and the standard deviation. As one can see the absorbance of the tests lie in the same range. The controls, however, differ large and the standard deviation for control 1 is small.

Table 3. Mean values of the measurements on the first bacterial growth.

Name of sample	Absorbance at 490 nm	Standard deviation
Test 1: 0.5 g/L Cloxacillin charged in the surface	0.221	0.040
Test 2: 0.5 g/L Cloxacillin in media	0.194	0.031
Test 3: 2.25 mg/L Cloxacillin in media	0.161	0.036
Control 1: no Cloxacillin	0.212	0.010
Control 2: no Cloxacillin	0.040	0.056
Control 3: non-mesoporous, 2.25 mg/L Cloxacillin in media	*	*

*Control was added after the first bacterial growth

The UV-Vis spectrum for the second growth is shown in Figure 12. There is a trend for the controls to lie lower than the tests, meaning that the tests contain more bacteria than the controls. Control 1 still follows the tests quite closely indicating that bacteria are sticking to the mesoporous surface.

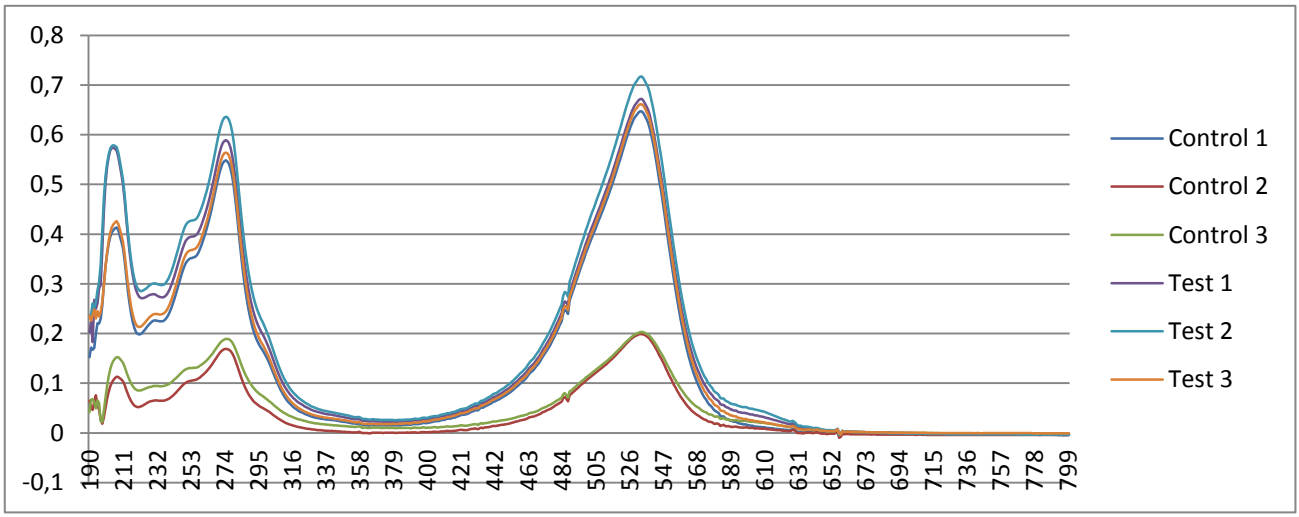

Figure 12. The mean values of the absorbance of the different samples as a function of the wavelength for the second bacterial growth.

The absorbance at 490 nm and the standard deviation are given in Table 4. Compared to the results from the first bacterial trial all the controls lie lower in absorbance than the tests. In fact, controls 2 and 3 lie much lower in absorbance.

Table 4. Mean values of the measurements on the second bacterial growth.

Name of sample	Absorbance at 490 nm	Standard deviation
Test 1: 0.5 g/L Cloxacillin charged in the surface	0.292	0.058
Test 2: 0.5 g/L Cloxacillin in media	0.314	0.077
Test 3: 2.25 mg/L Cloxacillin in media	0.283	0.023
Control 1: no Cloxacillin	0.275	0.050
Control 2: no Cloxacillin	0.078	0.039
Control 3: non-mesoporous, 2.25 mg/L Cloxacillin in media	0.085	0.066

4.4.2 Measurements from bacterial growth with RRP9W4N

Figure 13 illustrates the UV-Vis spectrum for the first bacterial growth and the here then trend is opposite to the second growth with Cloxacillin; the controls lie higher in absorbance. Control 2 is the exception.

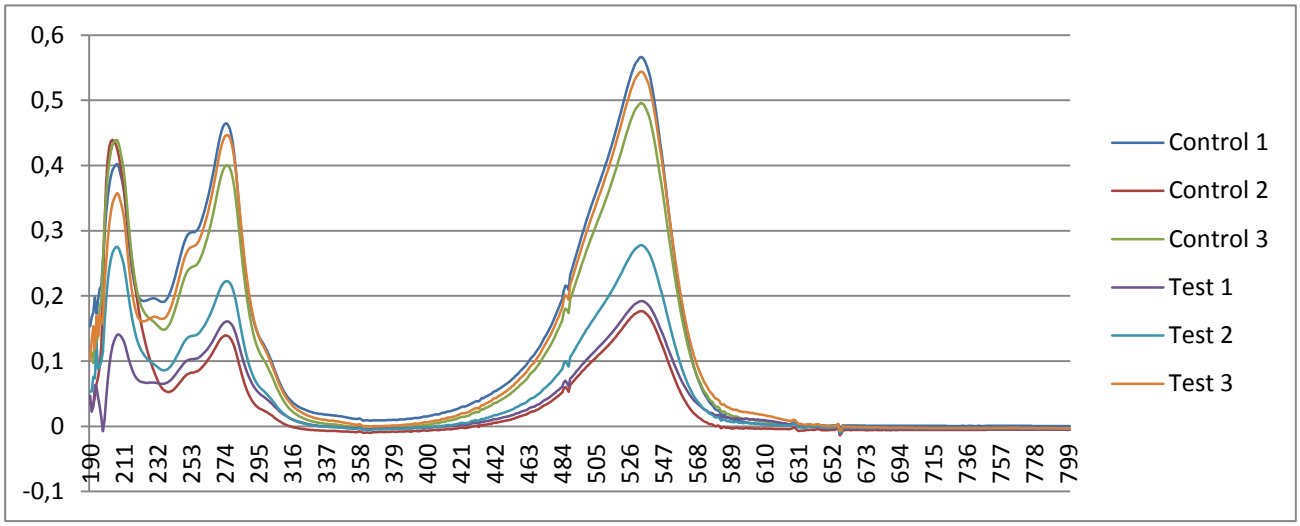

Figure 13. The mean values of the absorbance of the different samples as a function of the wavelength for the first bacterial growth with RRP9W4N.

Table 5 lists the absorbance at 490 nm as well as the standard deviation. As mentioned before, control 2 has a much lower absorbance compared to the other controls. Also, test 3 has a significantly higher absorbance than the other tests.

Table 5. Mean values of the measurements on the first bacterial growth with RRP9W4N.

Name of sample	Absorbance at 490 nm	Standard deviation
Test 1: 200 µM RRP9W4N charged in the surface	0.076	0.014
Test 2: 200 µM RRP9W4N in media	0.110	0.027
Test 3: 120 µM RRP9W4N in media	0.224	0.049
Control 1: no RRP9W4N	0.240	0.034
Control 2: no RRP9W4N	0.066	0.047
Control 3: non-mesoporous, 120 µM RRP9W4N in media	0.202	0.024

In Figure 14 the UV-Vis spectrum for the second bacterial growth is shown. The difference in absorbance seems to be very small, except for control 1 that lies much higher in absorbance. This indicates that there are a similar amount of bacteria on the tests and controls.

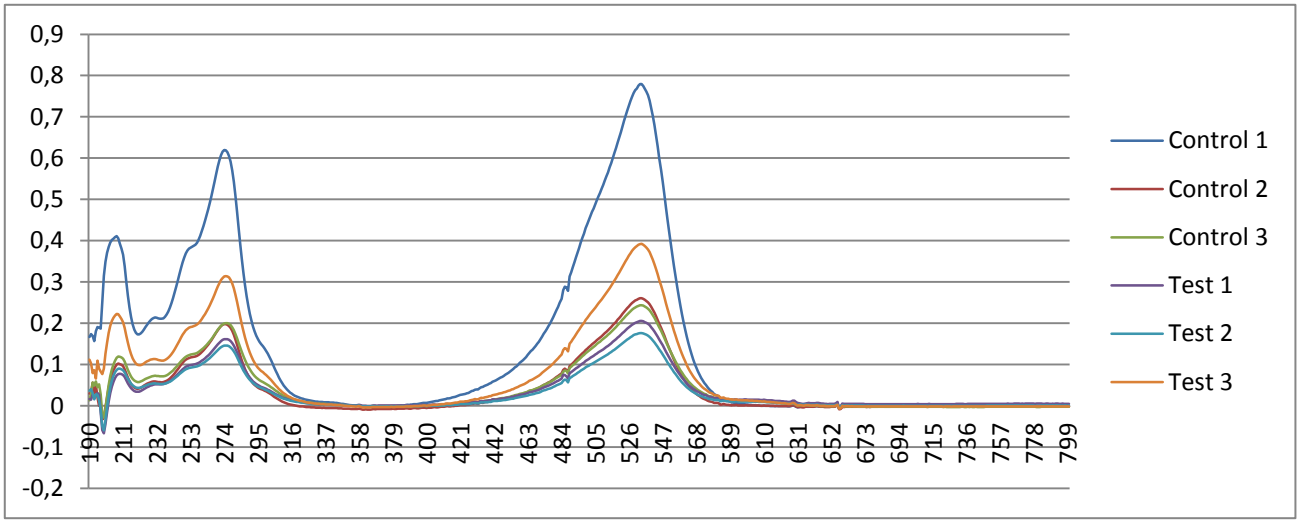

Figure 14. The mean values of the absorbance of the different samples as a function of the wavelength for the second bacterial growth with RRP9W4N.

From Table 6 the absorbance at 490 nm and the standard deviation are given. As stated above, the absorbance values do not differ much between the controls and tests. The samples that deviate are test 3 and control 1. However, looking at the standard deviation one can notice that the values lie closer to each other.

Table 6. Mean values of the measurements on the second bacterial growth with RRP9W4N.

Name of sample	Absorbance at 490 nm	Standard deviation
Test 1: 200 µM RRP9W4N charged in the surface	0.081	0.038
Test 2: 200 µM RRP9W4N in media	0.069	0.034
Test 3: 120 µM RRP9W4N in media	0.155	0.077
Control 1: no RRP9W4N	0.324	0.053
Control 2: no RRP9W4N	0.100	0.104
Control 3: non-mesoporous, 120 µM RRP9W4N in media	0.095	0.035

4.4.3 Statistics

The data collected is presented by using IBM SPSS. Data was analyzed by an Independent samples T-test and the significance level was set to $P < 0.05$. The values are given in Table 7 and this indicates that Cloxacillin is the better antimicrobial substance. The value for the first bacterial growth with RRP9W4N is so large that it seems as though the peptide has a very low antimicrobial effect.

Table 7. Student's T-test comparing the controls with the tests for the different bacterial growths.

Sample	T-test	Significance
First bacterial growth with Cloxacillin	0.003	The values for the tests are significantly larger than the values for the controls
Second bacterial growth with Cloxacillin	0.021	The values for the tests are significantly larger than the values for the controls
First bacterial growth with RRP9W4N	0.631	The values for the tests are not significantly smaller than the values for the controls
Second bacterial growth with RRP9W4N	0.031	The values for the tests are significantly smaller than the values for the controls

4.5 ESCA

The ESCA measurements were performed to confirm that the mesoporous surface consisted of titania and to show that Cloxacillin in fact entered the pores, as a complement to QCM-D. Values from the ESCA measurements are given in Table 8. Except for the elements that should be in the titania samples (such as O, Ti and C) there are also others like Na, Ca and quite a large amount of Si. As for the samples with Cloxacillin, as illustrated in Figure 1, the antibiotic contains N, O, Na and S, which are all found in Table 8.

Table 8. The atomic concentration for three samples with mesoporous Titania and three samples with mesoporous titania surface charged with 0.5 g/L Cloxacillin.

Sample	C1s	O1s	Na1s	Ti2p	Si2p	Cl2p	Ca2p	N1s
	[0.314]	[0.733]	[1.102]	[2.077]	[0.368]	[0.954]	[1.927]	[0.499]
TiO2 1	20.12	53.11	5.44	21.33	-	-	-	
TiO2 2	14.95	44.55	17.63	6.44	7.11	8.52	0.80	
TiO2 3	17.04	50.74	9.44	11.79	7.27	2.59	1.12	
TiO2 Cloxacillin 1	21.91	53.05	3.41	21.07	-	-	-	0.56
TiO2 Cloxacillin 2	21.77	53.02	3.40	21.22	-	-	-	0.59
TiO2 Cloxacillin 3	21.65	53.56	3.59	21.20	-	-	-	-

4.6 CFU

The CFU is calculated by using the following equation:

$$\frac{Number\ of\ bacteria * dilution}{Volume\ of\ added\ bacteria}$$

The obtained values are presented in Table 9 and the values are in the same range. This means that the results for the different trials should be comparable since they contain similar amount of bacteria.

Table 9. The calculated CFU/mL for the four trials.

Sample	Dilution 1:100000	Dilution 1:1000000
First bacterial growth with Cloxacillin	$5.26*10^8$	$7.7*10^8$
Second bacterial growth with Cloxacillin, First bacterial growth with RRP9W4N*	$4.54*10^8$	$6*10^8$
Second bacterial growth with RRP9W4N	$4.18*10^8$	$5*10^8$

*the same bacterium suspension is used for the two trials

5. Discussion

As the QCM-D measurement on Cloxacillin indicates, since there is a rapid decrease in frequency, the antibiotic enters the pores of the MpTiO_2. The increase in dissipation is due to the movement of the created antibiotic film. The slow increase in frequency is good for the application because it means that the dose will last for a longer time, having a larger impact on the biofilm. The plateau in frequency after approx. two hours may be due to the fact that Cloxacillin has a tendency to stick to the walls and as the Milli-Q water is applied the first antibiotic molecules to be washed away are the ones in the center of the pores and after a while the rest will wash away too. In comparison, the peptide charged surface shows a frequency decrease which is rather small and the release rate is quite fast. However, the dissipation is very small which means that RRP9W4N creates a stable film. The ESCA values confirm that the antibiotic enters the pores of the MpTiO_2, which is given by looking at how much of the elements in Cloxacillin are actually detected at the surface. The values for Na are even higher for the TiO_2 samples than the samples charged with the antibiotic. Why other elements like Si, Cl and Ca are detected in the TiO_2 might be due to the fact that the samples were stored in a glove on an open shelf. The exposure to air may have introduced new elements to the surface.

The SEM image of the mesoporous titania shows, as mentioned before, that the pore size is around 6 nm. Though the order is poor it does not affect the application since the most important features of the mesoporous surface are the pore size and how the pores are directed. The image clearly illustrates that the pores are directed towards the surface which makes it possible for Cloxacillin to enter as it is added on top of the surface. When comparing the SEM images for the three different surfaces (magnification 1K) it is clear that RRP9W4N has a much better effect as an antimicrobial substance. There is much less bacteria on the surface treated with peptide than any of the two other surfaces. Cloxacillin also shows a good antimicrobial effect (more easily viewed due to brighter images) but the difference compared to RRP9W4N is so large that the most determining issue is the cost. The peptide is much more expensive than the antibiotic meaning that every peptide trial needs to be handled with much more care to avoid contamination and errors in characterization measurements.

For the SEM images at the magnification 20K the differences in morphology between the three separate treatments are clearly shown. As mentioned in Section 4.2 the bacteria in the untreated sample have spread out into a shape that resembles a frying egg. They stay close to the mesoporous surface and their round shape is uneven. There are a lot of them and they are stacked closely. The Cloxacillin treated surface, on the other hand, displays bacteria with a rounder shape, larger spaces between the bacteria and a tendency not to spread out on the surface. The surface treated with

RRP9W4N shows a very small amount of bacteria with varying shape and roundness. There seems to be some kind of bonding between the bacteria that is not seen in the other images. There is a larger separation between the bacteria colonies.

From the UV-Vis spectrum (and table) given for the first trial with the antibiotic it appears as though control 1 and the tests lie in the same range of absorbance, control 2 is much lower. For the second trial, however, there is a trend for the controls to lie lower in absorbance than the tests. This means that there are more bacteria in the tests (more safranin due to more biofilm on the surface). Comparing these results with the spectra and tables for the trials with the peptide it seems as though the peptide is a much better antimicrobial substance. The controls lie higher in absorbance than the tests (with the exceptions control 2 and test 3) for the first trial. The results from the second trial show the same trend but the exception is test 3. The light microscopy images indicate that Cloxacillin is the better antimicrobial substance. In the images from the first trial with Cloxacillin there is much less bacteria in the control. This is also the case for the second trial and these images also illustrate how the mesoporous surface has a better effect than the non-mesoporous surface. The images from the first trial with RRP9W4N show that there is less bacteria in the controls then in the treated surfaces and that the mesoporous surface has a better effect than the non-mesoporous surface. It is though uncertain if all the bacteria displayed in the light microscopy images are living or if there is some dead bacteria still left in the biofilm after washing. A LIVE/DEAD staining would be necessary to confirm this. The second growth with peptide shows the opposite of the first, less bacteria in the treated samples than in the control (with the exception of addition of 200 μM peptide in media). The impact of mesoporous versus non-mesoporous surface is though the same.

During the experiment it was also tested if the antimicrobial effect would be better when growing the biofilms for two days instead of one. However, due to contamination of all these trials the original one day growth had to be used. The cause of the contamination is still unclear since other factors, such as addition of an air inlet into the oven, were included in the change.

6. Conclusion

It is still unclear whether Cloxacillin or RRP9W4N is the better antimicrobial substance and more analysis needs to be done. However, the obtained results show that they both affect *S. epidermidis* in the way wanted. Both substances show good results in the application of the mesoporous titania as a drug delivery system for them and their release rates are promising.

7. Future work

It would be interesting to see how the bacteria are affected when growing with antimicrobial substance for two days instead of just one. Since each antimicrobial substance is studied only twice, more trials are needed to be able to show a clear effect although this research gives an indication. It would also be good to do a LIVE/DEAD study on the different surface to clearly show the difference between the antimicrobial substances and the control. Further, when making the decision about which antimicrobial substance to use the possibility of *S. epidermidis* becoming resistant needs to be considered. Finally, when a clear method is obtained it should be tested in animals, such as rats.

8. Acknowledgements

I would like to thank my examiner Associate Professor Martin Andersson for all his help and devotion to the project. I would also like to thank my supervisor Maria Pihl for her help and knowledge when teaching me to handle the bacteria. My family, boyfriend and friends deserve special thanks for their understanding and support during these very interesting but busy months. Finally, I would like to thank the people at Applied Chemistry, especially Anne Bee Hegge.

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10. Appendix

10.1 Calculations of antibiotic solutions

The following calculations are based on the molecular weight of 435.88 g/mol for Cloxacillin.

10.1.1 Cloxacillin concentration 0.5 g/L

The required volume is 40 mL, since 20 mL are going to be used in the QCM-D measurement and 20 mL in test 1.

$c_1*V_1 = c_2*V_2 \rightarrow 0.5\ g/L*0.040\ L = 0.02\ g$

0.02 grams are measured and diluted with Milli-Q water to a volume of 40 mL.

10.1.2 Cloxacillin concentration 0.5 mg/L

The required volume is 20 mL. Since it is such a low concentration the dilution is done in two steps. A stock solution of 5 mg/L is made by dissolving 0.25 mg in Milli-Q water to a volume of 50 mL.

$c_1*V_1 = c_2*V_2 \rightarrow (0.5*10^{-3}*0.020)/5*10^{-3} = 0.002\ L$

2 mL of the stock solution is mixed with Milli-Q water to a volume of 20 mL.

10.1.3 Cloxacillin concentration 2.25 mg/L

The required volume is 20 mL in two separate bottles, since 20 mL are going to be used in the QCM-D measurement and 20 mL in other tests. A stock solution of 5 mg/L is made by dissolving 5 mg in Milli-Q water to a volume of 1000 mL.

$c_1*V_1 = c_2*V_2 \rightarrow (2.25*10^{-3}*0.020)/5*10^{-3} = 0.009\ L$

9 mL of the stock solution is mixed with Milli-Q to a volume of 20 mL in each bottle.

10.1.4 Cloxacillin for test 2

The required volume is 10 mL and the solution should have the concentration 0.5 g/L when it is mixed with 10 mL of bacteria.

$c_1*V_1 = c_2*V_2 \rightarrow (0.5*10.5*10^{-3})/0.5*10^{-3} = 10.5\ g/L$

0.21 g of Cloxacillin is dissolved in Milli-Q water to a volume of 20 mL. From this, 10 mL is saved since only 9 mL is needed for the trials.

10.1.5 Cloxacillin for test 3 and control 3

The required volume is 20 mL and the solution should have the concentration 2.25 mg/L when it is mixed with 10 mL of bacteria.

$c_1*V_1 = c_2*V_2 \rightarrow (2.25*10^{-3}*10.5*10^{-3})/0.5*10^{-3} = 0.04725$ g/L

23.63 mg of Cloxacillin is dissolved in Milli-Q water to a volume of 500 mL. From this, 20 mL is saved since only 18 mL is needed for the trials.

10.2 Calculations of peptide solutions

The following calculations are based on the molecular weight of 1931 g/mol for RRP9W4N. Since the available amount of the expensive peptide is so low, a stock solution is made from 50 mg dissolved in 5 mL of Milli-Q water:

$n = m/M = 50*10^{-3}/1931 = 2.58933*10^{-5}$ mol → 25.8933 µmol

$c = n/V = 25.8933/0.005 = 5178.66$ µM

10.2.1 RRP9W4N concentration 200µM

The required volume is 40 mL in two separate bottles, since 20 mL are going to be used in the QCM-D measurement and 20 mL in test 1.

$c_1*V_1 = c_2*V_2 \rightarrow V_1 = 200*40/5178.66 = 1.5448$ mL

1.545 mL of the stock solution is mixed with Milli-Q to a volume of 20 mL in each bottle.

10.2.2 RRP9W4N for test 2

The required volume is 2 mL and the solution should have the concentration 200 µM when it is mixed with 2 mL of bacteria.

$c_2*V_2 = c_3*V_3 \rightarrow (200*10^{-6}*2.1*10^{-3})/0.1*10^{-3} = 0.0042$ mol/L → 4200 µM

$c_1*V_1 = c_2*V_2 \rightarrow 4200*2/5178.66 = 1.622$ mL

1.622 mL of the stock solution is mixed with Milli-Q to a volume of 2 mL.

10.2.3 RRP9W4N for test 3 and control 3

The required volume is 3 mL and the solution should have the concentration 120 µM when it is mixed with 2 mL of bacteria.

$c_2*V_2 = c_3*V_3 \rightarrow (120*10^{-6}*2.1*10^{-3})/0.1*10^{-3} = 0.00252$ mol/L

$c_1*V_1 = c_2*V_2 \rightarrow 2520*3/5178.66 = 1.46$ mL

1.46 mL of the stock solution is mixed with Milli-Q to a volume of 3 mL.

10.3 Making 0.1% safranin solution

A total volume of 250 mL is wanted. The concentration of safranin should be 0.1 percentages by weight.

Milli-Q: m = ρ*V = 1000 g/L*0.250 L = 250 g

Safranin: m = 250 g*0.001 = 0.25 g

Dissolve 0.25 g of safranin in Milli-Q water to a volume of 250 mL.

10.4 Tables with UV-Vis values

Table 7. Values of the measurements on the first bacterial growth with cloxacillin.

Name of sample	Absorbance at 490 nm
Test 1.1: 0.5 g/L Cloxacillin in surface	0.21857
Test 1.2: 0.5 g/L Cloxacillin in surface	0.18217
Test 1.3: 0.5 g/L Cloxacillin in surface	0.26180
Test 2.1: 0.5 g/L Cloxacillin in media	0.19063
Test 2.2: 0.5 g/L Cloxacillin in media	0.16484
Test 2.3: 0.5 g/L Cloxacillin in media	0.22571
Test 3.1: 2.25 mg/L Cloxacillin in media	0.14310
Test 3.2: 2.25 mg/L Cloxacillin in media	0.20219
Test 3.3: 2.25 mg/L Cloxacillin in media	0.13761
Control 1.1: No Cloxacillin	0.22226
Control 1.2: No Cloxacillin	0.20841
Control 1.3: No Cloxacillin	0.20437
Control 2.1: Teflon, no Cloxacillin	$6.2709*10^{-3}$
Control 2.2: Teflon, no Cloxacillin	$8.4052*10^{-3}$
Control 2.3: Teflon, no Cloxacillin	0.10390

Table 8. Values of the measurements on the second bacterial growth with cloxacillin.

Name of sample	Absorbance at 490 nm
Test 1.1: 0.5 g/L Cloxacillin in surface	0.35968
Test 1.2: 0.5 g/L Cloxacillin in surface	0.26198
Test 1.3: 0.5 g/L Cloxacillin in surface	0.25550
Test 2.1: 0.5 g/L Cloxacillin in media	0.28779
Test 2.2: 0.5 g/L Cloxacillin in media	0.25262

Test 2.3: 0.5 g/L Cloxacillin in media	0.40033
Test 3.1: 2.25 mg/L Cloxacillin in media	0.29877
Test 3.2: 2.25 mg/L Cloxacillin in media	0.29377
Test 3.3: 2.25 mg/L Cloxacillin in media	0.25648
Control 1.1: No Cloxacillin	0.32844
Control 1.2: No Cloxacillin	0.22906
Control 1.3: No Cloxacillin	0.26829
Control 2.1: Teflon, no Cloxacillin	0.11650
Control 2.2: Teflon, no Cloxacillin	$7.9110*10^{-2}$
Control 2.3: Teflon, no Cloxacillin	$3.9362*10^{-2}$
Control 3.1: Non-mesoporous, 2.25 mg/L Cloxacillin in media	$3.3602*10^{-2}$
Control 3.2: Non-mesoporous, 2.25 mg/L Cloxacillin in media	0.15939
Control 3.3: Non-mesoporous, 2.25 mg/L Cloxacillin in media	$6.3174*10^{-2}$

Table 6. Values of the measurements on the first bacterial growth with RRP9W4N.

Name of sample	**Absorbance at 490 nm**
Test 1.1: 200 µM RRP9W4N in surface	$5.9336*10^{-2}$
Test 1.2: 200 µM RRP9W4N in surface	$8.5703*10^{-2}$
Test 1.3: 200 µM RRP9W4N in surface	$8.1479*10^{-2}$
Test 2.1: 200 µM RRP9W4N in media	0.13971
Test 2.2: 200 µM RRP9W4N in media	0.10399
Test 2.3: 200 µM RRP9W4N in media	$8.6130*10-^{2}$
Test 3.1: 120 µM RRP9W4N in media	0.28102
Test 3.2: 120 µM RRP9W4N in media	0.19666
Test 3.3: 120 µM RRP9W4N in media	0.19574
Control 1.1: No RRP9W4N	0.23794
Control 1.2: No RRP9W4N	0.27489
Control 1.3: No RRP9W4N	0.20716
Control 2.1: Teflon, no RRP9W4N	$1.5115*10^{-2}$
Control 2.2: Teflon, no RRP9W4N	$7.6390*10^{-2}$

Control 2.3: Teflon, no RRP9W4N	0.10702
Control 3.1: Non-mesoporous, 120 µM RRP9W4N in media	0.22177
Control 3.2: Non-mesoporous, 120 µM RRP9W4N in media	0.20845
Control 3.3: Non-mesoporous, 120 µM RRP9W4N in media	0.17456

Table 7. Values of the measurements on the second bacterial growth with RRP9W4N.

Name of sample	**Absorbance at 490 nm**
Test 1.1: 200 µM RRP9W4N in surface	0.23794
Test 1.2: 200 µM RRP9W4N in surface	0.27489
Test 1.3: 200 µM RRP9W4N in surface	0.20716
Test 2.1: 200 µM RRP9W4N in media	$1.5115*10^{-2}$
Test 2.2: 200 µM RRP9W4N in media	$7.6390*10^{-2}$
Test 2.3: 200 µM RRP9W4N in media	0.10702
Test 3.1: 120 µM RRP9W4N in media	0.22177
Test 3.2: 120 µM RRP9W4N in media	0.20845
Test 3.3: 120 µM RRP9W4N in media	0.17456
Control 1.1: No RRP9W4N	0.28102
Control 1.2: No RRP9W4N	0.19666
Control 1.3: No RRP9W4N	0.19574
Control 2.1: Teflon, no RRP9W4N	0.13971
Control 2.2: Teflon, no RRP9W4N	0.10399
Control 2.3: Teflon, no RRP9W4N	$8.6130*10^{-2}$
Control 3.1: Non-mesoporous, 120 µM RRP9W4N in media	$5.9336*10^{-2}$
Control 3.2: Non-mesoporous, 120 µM RRP9W4N in media	$8.5703*10^{-2}$
Control 3.3: Non-mesoporous, 120 µM RRP9W4N in media	$8.1479*10^{-2}$