NTNU
Norwegian University of
Science and Technology

Design and characterisation of patterned surfaces for bacterial microarray fabrication

Kertu Liis Krigul

Biotechnology
Submission date: May 2018
Supervisor: Marit Sletmoen, IBT

Norwegian University of Science and Technology
Department of Biotechnology and Food Science

Abstract

Bacterial populations are known to be heterogenous. It is therefore important to look at the bacteria at a single cell level to obtain information which can be masked when looking at population averages. Various approaches for single cell analysis exist, however, simple, cheap, and reproducible live-cell methods which can also provide sufficient time resolution are needed. The method proposed by N.B. Arnfinnsdottir should follow these criteria and was therefore tested in this master thesis.

The aim of this master project was to prepare a master mould and Poly(dimethyl)siloxane (PDMS) stamp which could be used for fabricating arrays with chemicals that facilitate bacterial adhesion. These arrays would then be used to adhere bacteria, thereby fabricating bacterial microarrays. In order to achieve the aim, different experimental techniques were used, including various methods in photolithography and microscopy, as well as microcontact printing. The thesis is a continuation of the work initiated in the master theses of K. Dunker and A. Resell who both used the method proposed by N. B. Arnfinnsdottir.

In this thesis, the protocols for fabricating master moulds and PDMS stamps were optimized. Fabricated PDMS stamps were then tested and successfully used to microcontact print fluorescent molecule PLL-FITC. Polydopamine (PDA), poly-L-lysine (PLL) and mucin arrays were prepared. The bacterial microarrays were, however, not reproduced. Future perspectives include optimization of the protocol to deposit PDA, PLL and mucin arrays or finding new chemicals which can successfully be used to fabricate bacterial microarrays

Preface and acknowledgements

This is the final thesis of the two-year Master of Science program in Biotechnology at the Norwegian University of Science and Technology (NTNU). The research was conducted at the Department of Biotechnology from February 2017 until May 2018 under the supervision of Associate Professor Marit Sletmoen. The majority of the research was carried out at the NTNU Nanolab.

First and foremost, I want to thank my supervisor Marit Sletmoen for trusting me with this multifaceted topic. Your comments and positive attitude always helped me to steer in the right direction. In addition, I want to show gratitude to my unofficial co-supervisor Karen Dunker for fruitful discussions during lunch time and for encouragements during the darker hours of writing the thesis. Moreover, without the continuous support from Nanolab engineers, especially Mark Chiappa and Mathilde Barriet, the experiments in the lab would have been a chaos. I hope you will find a person who can take my role as “the problem-finder”. I also want to thank Nina Bjørk Arnfinnsdottir and Marija Tomic from Physics for always helping me to characterize my not-so-perfect samples. Alex, you are exceptional for always being able to provide me with the cells. I also want to thank other students in my lab group, especially all the “potentially dangerous Nanolab particles”, who understand the confusion which we had when starting our work in Nanolab. Last but not least, the support from my roommates and family has been important beyond words. This thesis got done under the sunshine you brought to my life. Thank you all.

Kertu Liis Krigul Trondheim, May 2018

Abbreviations

AFM	Atomic force microscope
EBL	Electron Beam Lithography
FITC	Fluorescein isothiocyanate
Gal	Galactose
GlcNAc	N-acetylglucosamine
IPA	Isopropanol
LB	Lysogeny broth
MLA	Maskless Aligner
N_2	Nitrogen gas
nm	nanometre
NTNU	Norwegian University of Science and Technology
PBS	Phosphate-buffered saline
PDA	Polydopamine
PDMS	Poly(dimethyl)siloxane
PEB	Post Exposure Bake
PEG	Polyethylene glycol
PLL	poly-L-lysine
rpm/s	Revolution per minute per second
SEM	Scanning Electron Microscope
TRIS	Tris(hydroxymethyl)aminomethane
μCP	Microcontact printing

Table of Contents

List of Figures

1 Introduction

1.1 Background

Traditionally, microbiology has concentrated on studying bacteria at the population level. However, population of bacteria can be heterogenous in terms of its physiology, biochemistry, behavior, and genetic composition. The study of this heterogeneity is important to understand how bacteria adapt to the environment when exposed to antibiotics and biocides. Commonly used cell population study methods, e.g. agar-based phenotyping of a bacterial strain, provide average data values from multiple cells without identifying the distinct cells subpopulations within the whole population. These cultures of a single “pure” species can actually have cells with different substrains, serotypes, and phagotypes, all contributing to the population heterogeneity. Developing cheap and reproducible methods to isolate and study single cells is important to understand cells at an individual level, compare them to the average cell population, and identify their contribution to the whole population phenotype. [3, 4]

Among the possible methods to study single cells are cellular microarrays. Cellular microarrays are printed patterns which contain a high number of small-scale patches arranged in an orderly manner on a flat substrate. These patches can be fabricated via microcontact printing (μCP) of different cell-adhering materials, e.g. proteins or antibodies, which can capture living cells on specific spots. Solutions containing eukaryotic or bacterial cells can be applied directly on top of the surface of patterned chemicals, enabling them to capture the cells. Unbound cells will be washed off, which will result in an array of cells

immobilized on specific spots. The spots are separated by distance to avoid cross-talk between the arrayed cells, and the sizes of the spots can be adjusted to fit the size of the cells of interest enabling single cell capture. The cellular array can be coupled with other methods, e.g. fluorescence microscopy or atomic force microscopy. Unlike DNA or protein microarrays which use non-viable cell lysate samples, cellular microarrays probe living cells. [5] These arrays are appealing because they allow high-throughput analyses with small sample volumes as used features are in micron-scale. They can also be used to observe a specific single cell over a period of time, which is an advantage compared to another commonly used high-throughput single cell analysis method called flow cytometry. [6] A subtype of cellular arrays, bacterial microarrays, can be used for detection of bacteria differing from the average population. For example, they have been useful for discovering persister cells which can occur at low frequency in an antibiotic-sensitive population and survive in the presence of antibiotics despite having the same genotype as the sensitive bacteria. The cells can also be isolated with a micropipette for further analysis [7].

Photo- and soft lithographic techniques characterised in the following chapter can be used to create master mould and stamps for microcontact printing and for bacterial microarray production. There are other methods to fabricate bacterial microarrays, e.g. dip-pen nanolithography or producing topographic patterns, but these techniques are beyond the scope of this thesis.

1.2 Fabrication of microarrays

1.2.1 Photolithography

Photolithography is a microfabrication process, which transfers a pre-designed pattern from a mask onto a wafer covered with a light-sensitive organic polymer – a photoresist. The light is exposed on the resist-covered wafer through a pattern created on a metal, quartz or glass photomask, which lets the light only pass through specific regions, thus generating area-selective polymerization or degradation (dependent on the resist). The uncured polymer is removed during development step resulting in a pattern on the silicon surface. [8]

The photoresists are photon-sensitive macromolecule polymers used in photolithography, which can be optically defined into patterns of structures. Although resists sensitive to electrons, ions, or X-ray exist, photon-sensitive resists are most commonly used. The resists can be either positive or negative depending on the polarity of the patterns after photon exposure. In positive resist, the long chain of molecules will be broken into short chains by photon energy, making them soluble in the developer. In negative resist, however, the photon energy will be absorbed resulting in short chains of molecules joining together to form long chains which are insoluble in the developer (Figure 1). [9, 10]

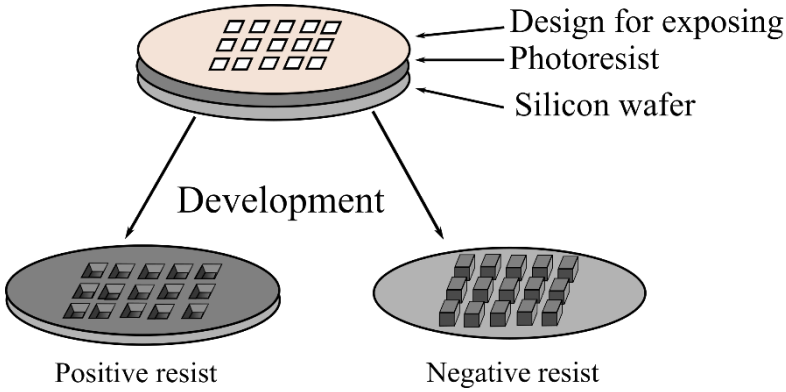

Figure 1. Development of positive and negative resist. With positive resist the exposed regions are soluble in developer solution due to light induced polymer chain cutting and will be removed. With negative resist the exposed regions are insoluble due to cross-linking of polymer, so the unexposed areas will be removed by the developer.

The majority of unexposed photoresists absorb light ranging from 440 nm to near UV spectrum, which makes them sensitive to Mercury (Hg) lamps in Mask Aligner as well as laser beams in Maskless Aligner (MLA) and causes many resists to have a typical reddish-brownish colour. Photolithography is done in an area with yellow light to prevent unwanted exposure of the resits. [9, 10] Choosing a suitable photoresist can be challenging and the following factors need to be considered:

- the cost and easiness to work with the resist,
- required film thickness,
- aspect ratio of patterned structures,
- potential need to strip the resist structures off afterwards. [3]

The final decision of which resist to use is usually made using the data from the manufacturer's protocols and in collaboration with the cleanroom engineers. The choice of resist can also be linked to which instrument is preferred to be used for exposing, e.g. if MaskAligner, Maskless Aligner (MLA) or Electron Beam Lithography (EBL) is the instrument of choice. EBL uses electrons to produce structures in nano-scale that cannot be made using conventional photolithography. However, using EBL can be time consuming and restricted to handful resist types and thicknesses. For larger structures, MaskAligner can be used. MaskAligner aligns a photomask with the photoresist-covered substrate to control the exposure of light. Mask can be either in direct physical contact (hard contact), in proximity over the substrate (soft contact) or projected on to the substrate. To use the Mask Aligner, a mask needs to be ordered and produced, which can be expensive and time consuming. It can be vexing to order such an expensive mask when the experiments are in the testing phase with no certain pattern identified yet This limitation can be overcome by using MLA which uses non-contact exposure to generate the pattern directly onto the substrate without the need of photomask. During an exposure in MLA, lasers are used to illuminate an optical engine containing millions small mirrors to project and de-magnify the image to the substrate. The substrate is fixed on a stage which moves under optical engine to generate the stripe of pattern. Complete pattern is formed from all stripes stitched together. Features down to 1 µm can be exposed using this instrument. [11]

To match our research purposes, a choice was made to use mr-DWL 5, which can be used for exposing the patterns in MLA. *mr-**DWL 5*** is a negative tone photoresist from mr-DWL photoresist series, which can

be used for exposing wavelengths above 400 nm. The developed features of the resist have excellent thermal stability. A wavelength of 405 nm is used for exposing mr-DWL resists in MLA. Film thickness between 3-12 µm can be achieved when using mr-DWL 5. According to the manufacturer's protocol, spin coating the resist at 3000 rpm for 30 seconds should result in the resist thickness of 5 µm (Figure 2). [1]

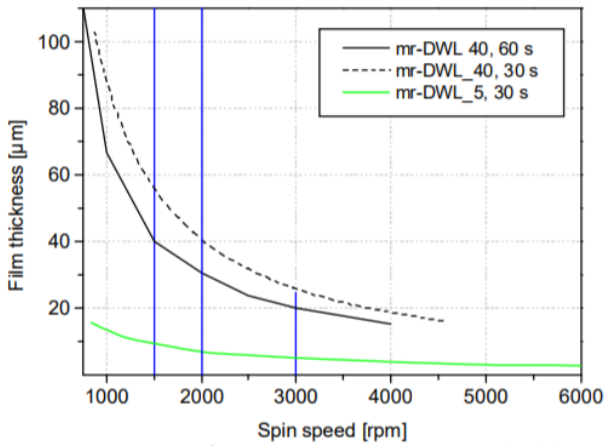

Figure 2. Spin curves of mr-DWL. Resist thickness of 5 µm can be obtained if mr-DWL 5 (green line) is spin coated at 3000 rpm for 30 seconds. Figure from [1].

The major steps in photolithography involve spin coating the resists, exposure to specific wavelengths of light and film developing. However, additional steps must often be included. The steps included in a photolithography process are described below.

In the substrate preparation step, the wafer is cleaned to remove any surface contamination and moisture. For cleaning, organic solvents like acetone, isopropanol and ethanol can be used. In addition, ozone

cleaning can help to degrade and remove organic contaminants from the surface. The cleaning steps are then followed by a dehydration bake step which involves high temperature treatment (180-200 °C) in an oven or on top of a hot plate to remove leftover moisture from the surface of the wafer. The drying process is especially important to promote adhesion between the wafer and the photoresist. If the wafer is not dry, the resist may adhere insufficiently and peel off at later stages of the process. The clean wafer is then spin coated with the resist. [1, 10]

Spin coating is a technique for depositing a layer of resist on a substrate such as a wafer. A typical spin process contains:

1) dispense or low spin speed step with the aim of dispensing and spreading the resist across the wafer
2) high speed spin step with the aim of reaching the desired thickness

To begin with, the substrate is placed onto a vacuum holder or chuck inside a spinner to hold the sample position. For the following dispense step, either static or dynamic dispense method can be used. The static dispense method includes simply depositing a puddle of resist in the middle of the substrate. The volume depends on the viscosity, as resists with higher viscosity need larger amounts to ensure full coverage during the spin step. When using the dynamic dispense method, the wafer is spun at low speed (usually around 500 rpm) to spread the resist over the substrate. With this method, less resist needs to be deposited on the surface to cover the entire surface, which will reduce the amount of waste. As resists can be quite expensive, it can be reasonable to reduce

the amount used to as small as possible by optimizing the parameters. [12]

After dispensing, the sample is spun at high speed (typically around 3000 rpm), which causes the excess resist to spin off, leaving a thin layer on top of the sample. The thickness of the spun layer depends on the parameters chosen for spin coating (e.g. spin speed and time), and resist characteristics (e.g. the viscosity, drying rate, the volume of solids it contains, surface tension, etc.). In general, the higher the spin speed and the longer the spin time, the thinner the resist layer. As 50% of the solvents in the resist can evaporate in the first few seconds of the spin cycle in some processes, it is also important to control the acceleration of the substrate, which affects the drying rate. A slower drying rate results in more uniform thickness of the film. [7]

Ideally, the spun layer should be homogenous over the entire sample. However, various artefacts can occur due to different reasons, e.g. trapped air bubbles or particle contamination (Figure 3).[7] In addition, edge bead, i.e. a build-up of resist at the edge, and coating the underside of the wafer can occur when using thick resists, causing difficulties in exposing and variable dimension levels after development [13]. These problems can be solved by optimizing the photolithography protocol, e.g. by adjusting the cleaning procedure, handling the resist more carefully and/or introducing an edge bead removal step [14].

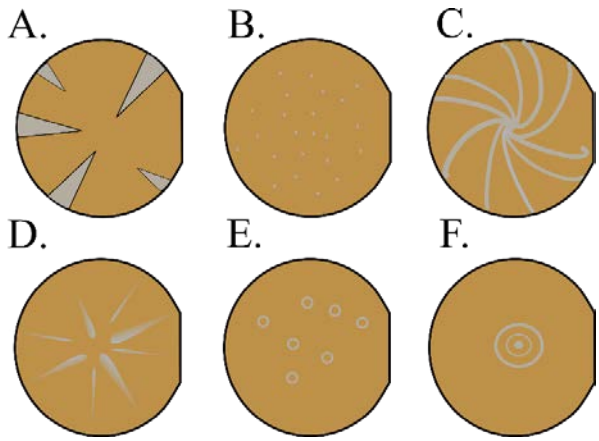

Figure 3. Defects from spin coating process. A. Uncoated areas can occur if the volume of resist added to the substrate is too low. B. Pinholes can occur due to air bubbles or particles in the resist. C. Swirl can occur if the spin speed of the resist is too high. D. Comet streaks can be caused by particles or wrong settings in spin speeds. E. Bubbles can be caused by the air that has gotten trapped when applying the resist. F. Chuck mark can occur if the substrate is slightly bent by the vacuum chuck.

A soft bake step must be included after the spin coating in order to drive off solvents from the resist. During this process, the wafer is heated to densify the resist film in order to make it sufficient to support later processing. The adhesion between the resist and the wafer is improved, so the resist would not stick to the mask during exposure. An oven or a hot plate can be used for heating the resist coated wafer and the temperature should be chosen according to the manufacturer's protocols. Resist thickness and solvent content affects the baking time and temperatures. Hot plates have proven to be most useful, as they provide the best temperature control, although overshooting might sometimes occur, if the hot plate controller is not well designed. [15]

In the exposure step, the cured wafer with resist can be exposed by photons to induce chemical change in the resist, which alters the solubility of the exposed areas, so the soluble areas can be washed away with the developer. By selectively exposing the resist in some regions of the wafer via specially designed mask, a pattern can be created. The pattern can be exposed by using either a Hg lamp or laser beam as a light source. Historically, the necessary exposures in optical lithography were obtained using visible light similarly to copying photos in a photographic workshop. However, as the feature sizes shrank, shorter wavelengths were needed. Hg lamps with wavelength emissions at g-line (λ = 436 nm), h-line (λ = 405 nm) and i-line (λ = 365 nm) became the illumination source of choice for exposing photoresists. Normally, a photoresist is designed to work only at specific wavelengths, therefore i-line exposure (365 nm) cannot be used for g-line photoresists (435 nm), nor can g-line and i-line photoresists be exposed with deep UV.[9, 10] Today, Hg lamps are the preferred light sources in Mask Aligners. In Maskless Aligner, the pattern is exposed straight onto the photoresist-covered wafer by using a laser instead of a Hg bulb. Lasers emitting light of a wavelength equal to 405 nm and 375 nm can be used in the MLA available at the NTNU Nanolab.[16, 17]

An appropriate exposure dose (mJ/cm^2) needs to be established. The exposure dose depends on the thickness and sensitivity of the photoresist: the lower the sensitivity of the resist, the higher the exposure dose it needs, and hence the slower the exposure. A too low dose will not completely expose the regions of interest. A too high dose causes light scattering and diffraction in the resist, which results in lower resolution. Generally, exposure series or dose tests can be done where

the dose varies between 50% and 200% of the estimated optimum value of the exposure. From the series of exposure doses, suitable dose can be chosen for all subsequent exposures. [9, 10]

Post exposure bake is a step required by some of the photoresists. It further assists and completes the photoreaction initiated during the exposure. For some resist types it will also improve roughness and profile. [15]

Development is done after the post exposure bake, by placing the wafer in a developer solution, so it dissolves the exposed areas when positive resist has been used, and the unexposed areas when negative photoresist has been used. Three basic development methods exist: immersion, spray, and puddle development. The easiest method is immersion, where the wafer is put into a beaker filled with a developer for a certain time, and then washed to remove the residues of the resist. In spray development, the developer is sprayed onto a spinning wafer. The washing and drying steps are done also while the wafer is spinning. Puddle development method combines immersion and spray method: the wafer is first immersed in a developer and then spun for further spray development, as well as cleaning and drying. Puddle and spray development methods can be more troublesome, as the spray development needs special equipment. A wafer with a developed pattern of photoresist is called the master mould. [10]

Final resist patterns are influenced by various factors during the photolithography process, e.g. spin coating parameters, variations of soft bake and post-exposure baking temperatures, and development

time, etc. Optimal conditions should be identified as part of the method development.

1.2.2 Soft lithography and PDMS stamp fabrication

Photolithography has greatly contributed to the development of biotechnological methods (e.g. microarrays for DNA sequencing, microchips for drug screening, etc), however, it has a number of limitations which make it hard to apply to biological systems. Photolithography can be considered quite expensive, as the equipment it needs was first developed to be used for very demanding processes in the production of microelectronic devices. In addition, it can be very expensive and challenging to set up a cleanroom, which makes the use of the provided methods inaccessible to most life scientists. [18] Furthermore, the methods in photolithography cannot be easily used to pattern non-planar surfaces, because they produce quite rigid inorganic surfaces making it challenging to transfer the pattern to other substrates. Also, these surfaces produced by photolithography do not integrate well with ceramics, plastic, glass or carbon. Thus, soft lithography has been used as a sequential step, because it is relatively cheap and can be done without using a cleanroom.

Soft lithography can be used as an approach for rapid prototyping of structures on planar, curved, flexible and soft substrates, both in micro- and nanoscale [18]. It is possible to use soft lithography to fabricate structures that match the size of bacterial cells and beyond. Soft lithography methods, e.g. replica moulding (REM) and microcontact printing (μCP), are characterised by the usage of elastomeric substance

for transferring a pattern to a target material. [18, 19] Different elastomers can be used, however, poly(dimethyl)siloxane, also known as PDMS, is the most common. PDMS has Si-O backbone and two methyl side-groups for each Si atom (Figure 4).

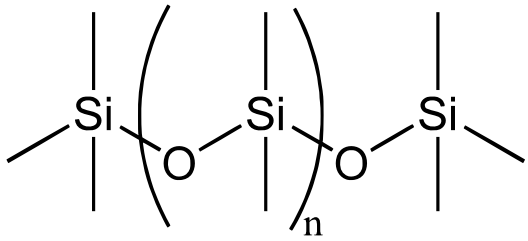

Figure 4. Chemical structure of poly(dimethyl)siloxane (PDMS) (image by Sei, distributed under a CC-BY 2.0 license)

PDMS has multiple features which make it attractive to be used for multiple purposes [18-20]:

- it is a moderately stiff elastomer which can conform to a surface, following its contours and making close contacts;
- it is non-toxic and not very expensive;
- it is hydrophobic but can be modified to be hydrophilic by exposing it to oxygen plasma;
- it is optically transparent down to 300 nm;
- it is durable – the same stamp can be used for several months;
- it is chemically inert;
- it is stable at temperatures which are used for biological procedures (40–95°C).

There are four major steps in the procedure of soft lithography:

1. designing the pattern using computer assisted design (CAD) software,
2. making the master (described in Chapter *"1.2.1 Photolithography"*),
3. fabricating the PDMS stamp,
4. producing micro- and nanoscale structures with the stamp, using printing, moulding, or embossing.

In the design step, the pattern of interest is created. This pattern can be used as the basis for the fabrication of a photomask used in a MaskAligner or as a writing guide for the laser to directly expose the pattern on the photoresist in the Maskless Aligner (MLA). The fabricated master serves as a mould for PDMS stamp preparation. The resulting stamp has the opposite features compared to the designed master – elevated regions of the master correspond to the hollow regions of the stamp. When designing the pattern, it is important to consider the aspect ratios of the final PDMS stamp, as lateral collapse or roof collapse might occur (Figure 5).

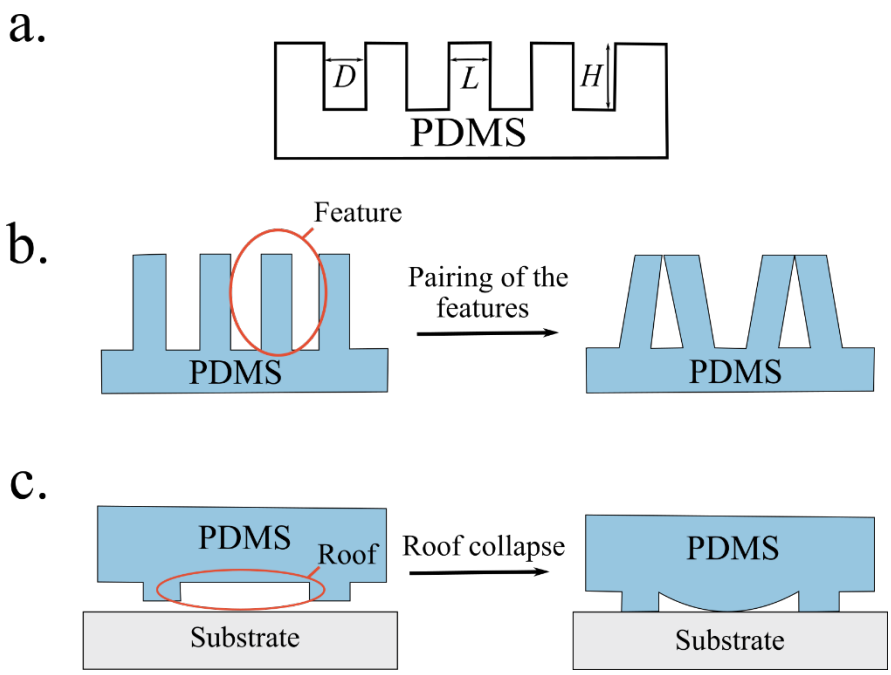

Figure 5. Schematic illustration of PDMS stamp (a) and possible deformations (b, c). In Image a. the parameters for calculating aspect ratios are shown. In Image b. deformation known as lateral collapse or pairing of the features is visualized. This can occur when the aspect ratios for H/L are too high. In Image c. deformation known as roof collapse is visualized. This can occur when the H/D aspect ratios are too low. Optimal aspect ratios are between 0.5 and 5 for H/L, and > 0.05 for H/D.

Lateral collapse, commonly known as pairing of the features, might occur if the aspect ratios for H/L are higher than 5, which results in collapse due to the stress originating from gravity, adhesion and capillary force. Roof collapse can occur if the aspect ratio of H/D is too low (smaller than 0.05) and the features are too widely separated. Roof collapse is due to recessed regions that lose their structure because of the compressive forces generated during stamping. The overall

recommended aspect ratios stay between 0,5 and 5 for H/L and higher than 0.05 for H/D. [18, 19]

In order to produce the PDMS stamp, a mix of elastomer (base) and curing agent (precursor) is poured on top of the master and cured in the oven to cross-link the polymer and solidify it. Commonly used ratio between the curing agent and the elastomer is 1:10. The most common PDMS kit for stamp production is Sylgard 184 (Dow Corning) which can be used to fabricate feature sizes down to 500 nm. As cross-linking the polymer is dependent on temperature, the curing conditions can be varied – Sylgard 184 can cure completely using 10 minutes at 150 °C in the oven or when it is left for 1-2 days at room temperature [21]. Commonly reported temperatures for curing are 65 or 80 degrees. However, PDMS can shrink around 1-2% during elevated temperatures. This shrinking should be taken into consideration when designing the master mould, if the design has dimensions with small tolerances. Shrinking also can be avoided by using room temperatures for curing. [22]

The solid PDMS is peeled off the master after the curing. Sometimes PDMS can get stuck on top of the wafer, there is a danger of breaking the wafer while peeling. In order to avoid that, fluorinated silane can be used to create covalent Si-O-Si bonds on the master, which makes the surfaces more hydrophobic, and thus eases the stripping of PDMS from the wafer extending the mould lifetime. More than 20 copies of PDMS stamps can be made from the master without additional silane treatment. The finished stamp can be used, for example, for patterning surfaces via microcontact printing, as described in the next chapter. [18]

Currently, soft lithography depends on photolithographic methods to generate the master. However, the subsequent fabrication steps can be performed outside a clean room once the master is prepared. Also, master moulds can today be ordered from various companies, thus avoiding the requirement of a clean room. [18]

1.2.3 Microcontact printing (μCP)

Microcontact printing (μCP) is a soft lithographic method used for depositing biomolecules (e.g. proteins and DNA) in a pattern on top of various solid surfaces, using an elastomeric stamp which has micrometre-sized features. One appealing capability of μCP is that it allows the spatial distribution of living cells on a substrate to be controlled. [20] The method was first used to create Self-Assembled Monolayers (SAMs) on gold using alkaneethiols. SAMs are formed by functionalized long-chain organic molecules that adsorb to suitable surfaces by chemisorption and self-organization. [19] As the method is considered relatively cheap and simple, it has also been used for experiments with various materials (e.g. polymers, proteins, and peptides) and surfaces (e.g. glass, tissue culture polystyrene, metal-oxide coated glass wafers) [23, 24].

In order to stamp the pattern, molecular “ink” is first applied to the PDMS layer. The stamp is then dried and used to transfer the pattern to surface of choice. Drying step is important to avoid the ink from spreading during pattern transfer. As PDMS is hydrophobic, depositing water-soluble inks can prove to be quite difficult. However, the

hydrophobicity can be reduced by using oxygen plasma which generates a temporary hydrophilic surface by creating thin polar silica-like layer with silanol functional groups that can bind organic groups, thus making the application of the molecular ink on top of the stamp easier. However, the silica-like layer cracks easily, and thus allows PDMS fragments with low molecular weight to migrate to the cracks and restore the hydrophobic surface. This usually happens within a few hours after the oxidation. The hydrophilicity could, for example, be increased by PDMS surface modifications where silanes are chemically attached to the oxidized stamp surface, keeping the surface hydrophilic for a few days. [25]

After applying the ink on the PDMS surface, it is transferred to the substrate surface (e.g. glass) by bringing the PDMS stamp and substrate into contact with each other. Some pressure needs to be applied while printing. The ink will be transferred onto the surface when the stamp is peeled off. The attachment can be unspecific, i.e. occurring mainly via electrostatic forces (e.g. proteins attached to the glass), or strongly specific (e.g. streptavidin-biotin interaction). The inking time needs to be established, as it might vary for different molecules: shorter time can be used for smaller molecules and longer time for larger molecules, for example proteins and DNA. The μCP can be followed by microscopy, particularly fluorescent microscopy which can be used to visualize patterned surfaces to validate the success of the inking and printing steps. This can be done by labelling the involved cells or proteins with optically responsive dyes and then visualizing them using a fluorescent microscope. [20]

1.2.4 Chemicals used in microarray fabrication

Polymers like poly-L-Lysine (PLL) and polydopamine (PDA) have been used for immobilizing single bacterial cells in an array and were therefore tested in this thesis as bacteria-adhering chemicals [6, 26]. In addition, PLL-FITC and PLL-g-PEG were used to facilitate bacterial microarray fabrication and are described in the following chapter.

Poly-L-Lysine or PLL is a positively charged synthetic polymer made of multiple α-L-lysine amino acid monomers (Figure 6).

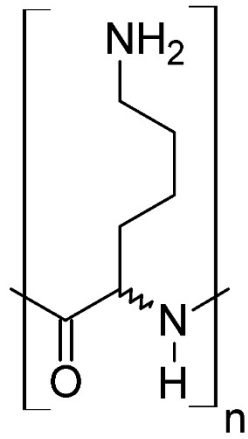

Figure 6. Structure of α-polylysine monomer

PLL can be attached to different types of surfaces and materials, e.g. glass, metals, metallic oxides, and polymers like poly-ethylene glycol [27, 28]. The PLL adsorption has shown to be irreversible but can be affected, for example, by pH. On silica, the thickest adlayers of PLL have been detected with pH 11 where the adhesion has shown to be strongest. Lower pH levels result in significantly lower thickness, as

acidic conditions result in weak PLL adsorption. Weak adsorption also results in rougher and less uniform adlayer compared to strong PLL adsorption, which produces a more uniform and smoother layer. [27]

PLL can be used in biological applications to promote non-specific attachment of cells and proteins to solid surfaces, as it facilitates electrostatic interactions between negatively charged ions of the cell membrane and substrate surface by providing positively charged sites for binding. It has been used to attach both bacteria and eukaryotic cells to different surfaces [29]. There has been some research on PLL's inhibitory and even bactericidal effect in different concentrations depending on the species (e.g. *E. coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*) [30, 31].

PLL-FITC is a graft polymer with PLL attached to fluorescein derivative fluorescein isothiocyanate (FITC, Figure 7) which can be visualized under a fluorescence microscope.

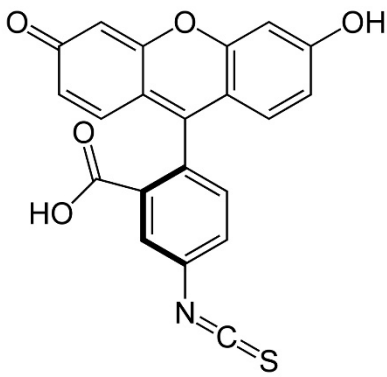

Figure 7. Chemical formula of fluorescein isothiocyanate (FITC)

In biological research, the fluorescein derivatives are commonly used,

as they have good water solubility, excellent quantum yield and high absorptivity. FITC is a green fluorophore which is sensitive to changes in pH and can fade rapidly unless antifade agent is used. If the signal is weak, it can be amplified by using anti-fluorescein antibodies. Its excitation maximum is at 490 nm and emission maximum at 525 nm, giving it the green colour. [32]

PLL-g-PEG is a graft co-polymer with a poly-L-lysine (PLL) backbone and polyethylene glycol (PEG) side chains. PEG is used to prevent the attachment of cells and bacteria to the surface, as well as to reduce protein binding and enhance lubricity, as they form a dense polymeric brush-like layer on top of the substrate. [33] It can be used, for example, in biomaterials technology where protein binding reduction is especially important, since the blood-contacting devices are often made of metals (e.g. titanium or steel) which are covered by oxides that can bind proteins (e.g. fibrinogen) which, in turn, can lead to blood coagulation and thrombosis [28].

The flexible PEG side chains are uncharged and hydrophilic, while PLL backbone has a positive charge which interacts electrostatically with the negatively charged substrate, forming a packed coating on it (Figure 8).

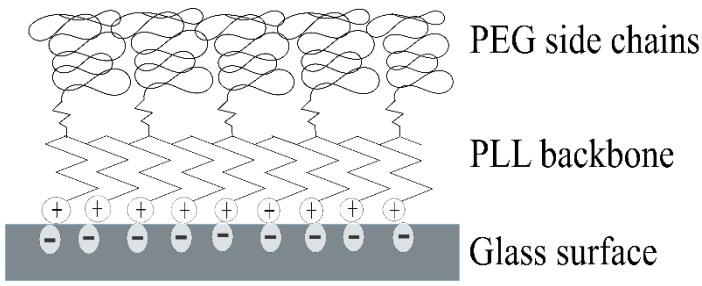

Figure 8. PLL-g-PEG formed structure

In microcontact printing, the PLL-g-PEG can be used to passivate the surface, so the bacterial adhesion is inhibited everywhere, except for the spots where bacteria are set to adhere by depositing adhesive chemicals via microcontact printing [6].

PDA, also known as polydopamine, is a dopamine-derived synthetic polymer, which full structure is still not established. Polydopamine net negative charge can vary in solutions with different pH levels: it is positively charged at pH=3 and negatively charged at pH=7 [34]. At slightly basic pH, PDA is very adhesive and can quite easily deposit onto various types and shapes of surfaces via oxidative self-polymerization of dopamine-hydrochloride. Functional groups, like amino, hydroxyl, and catechol groups as well as π stacking make polydopamine adhesive to various surfaces [35]. Simple immersion of the surface in dopamine solution is enough to achieve the coating. Various coating properties like reaction speed of PDA formation, coating thickness, and surface roughness are affected by different factors like reaction temperature, initial dopamine concentration, solution pH, type of buffer, and oxygen concentration in reaction solution. PDA coatings have been used for patterning various substances like cells, proteins and metal nanoparticles. [36] They have also been used for μCP bacterial cells [6]. Depending on reaction conditions, however, polydopamine may have antimicrobial properties [37].

1.3 Bacterial adhesion

Bacterial adhesion is a crucial step in the colonization of tissues. The adhesion capabilities depend largely on the surface properties of a bacterial cell as well as its ability to adhere to the surfaces of interest. Specific structures present on the bacterial cell surface are needed for adhesion. There are many different strategies for bacteria to adhere to hosts. The adhesion can result from cation bridging, hydrophobic interactions, as well as receptor ligand binding. Cation bridging occurs when the divalent cations help to neutralize the repulsion of the negatively charged bacterial and host surfaces. The most studied adhesion mechanism is via lectins and their corresponding glycosylated receptors. [38]

The adhesion can be facilitated by various substances and structures on the cell surface, e.g. adhesive organelles called pili and flagella, outer membrane proteins, or polysaccharides present on the cell wall. Some of these structures, e.g. pili, can several micrometres long. Many types of pili exist, including P pili and type IV pili, and bacteria can have more than one type at a time. [39] Multiple adhesins and receptors result in multivalent binding which has a higher affinity compared to the single interaction between a ligand and a receptor. The adhesion can affect both bacteria and host cells. The effects on bacteria include stimulation or inhibition of growth and induction of extra adhesive structures necessary for the invasion of host cells (e.g. secretion systems), while the effects on host cells include changes in the cell's morphology and fluid volume, upregulation of cytokines and adhesion molecules, and induction of apoptosis. [40]

Various bacterial adhesins bind to mucosal surfaces. These surfaces secrete mucus which forms a relatively resistant gel that acts as a lubricant, a physical barrier between the organism and its external environment, a trap for microbes, as well as a matrix for a variety of antimicrobial molecules [38]. The mucus layer is a dynamic structure, continuously produced and shed, thus removing the trapped matter, including microbes. The thickness of the layer is estimated by comparing the balance between the rate of generation and the rate of shedding of the mucus. Toxic substances can increase the level of mucus secretion, thus increasing the thickness of the mucus barrier. In addition, mucus production can also be stimulated by the commensal microbiota [41]. It is still unclear whether the binding to mucosal surfaces favours the bacteria or the host. The relationship may be also commensal to both: bacteria can stay in a favourable niche and may even use mucins for their metabolism, as the host can keep the pathogenic bacteria away, since the available niche becomes limited for retention. [38]

The main molecular components of mucus are large glycoproteins called mucins which contain a dense array of O-linked carbohydrates that are attached to repeating amino acid sequences rich in serine and threonine. Mucins are produced by the cells in the epithelium or submucosal glands.[38] As there are several reports about bacterial species adhering to mucins, it is also interesting to use them for the preparation of bacterial microarrays (examples of bacteria adhering to mucins reviewed in [42]).

1.4 Model organisms

1.4.1 *Pseudomonas putida*

Pseudomonas putida is a Gram-negative rod-shaped non-pathogenic bacterium. The size of the organism ranges from 2-4 μm in mid-exponential phase and 1-2 μm in stationary phase. *Pseudomonas putida* is commonly found in soils. [43] *Pseudomonas putida* strain KT2440, commonly used as a host for gene cloning and expression of heterologous genes, was used in this thesis to test the microcontact printing protocol suggested by Nina Bjørk Arnfinnsdottir [6].

1.4.2 *Escherichia coli*

Escherichia coli is a Gram-negative rod-shaped bacterium, which can be easily grown and manipulated, thus making it popular model organism in the field of biotechnology. The cell is around 2 micrometres long and 0.5 μm wide and is frequent in intestines of warm-blooded organisms. *Escherichia coli* strain BL21, commonly used in recombinant protein production, was used in this thesis. *Escherichia coli* was chosen as the organism for bacterial microarray production as it had been successfully used in microarray production before. [26, 35, 44, 45]

1.5 Imaging methods

1.5.1 Light microscopy

Light microscopy, also known as optical microscopy, is a commonly used magnifying technique which uses visible light and a system of lenses to visualize small objects. Typically, the light from a light source is transmitted to the objective lens from the opposite side of the specimen. The light travels through the specimen to the objective lens which is used to magnify the image of the sample, and then continues to the oculars where the image can be viewed (Figure 9).

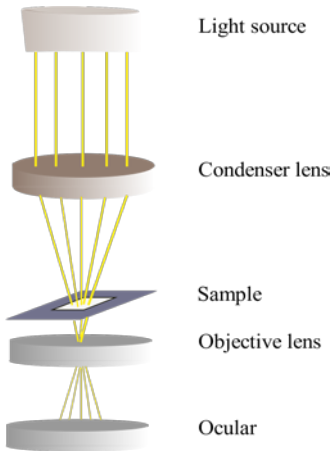

Figure 9. Light microscopy work principle. Light from the source gets focused on the sample by the condenser lens. The light then travels to the objective lens which magnifies the image of the sample. The image can then be viewed via the oculars.

In order to achieve maximum illumination, the light is also passed through a condenser which helps the rays to be focused on the specimen, and thereby achieving proper illumination. The condenser focuses the parallel rays of light on specimen, giving an evenly illuminated field, thus the well-lit image without glare with the sample is minimally heated. The set-up which achieves the best results in specimen illumination and image generation is known as Köhler illumination. [46]

The possible resolving power of a light microscope is approximately 0.2 μm and it is dependent on the numerical aperture of the lenses and the wavelength of the light used: the higher the numerical aperture and shorter the wavelength, the better resolution. The resolving power can be calculated using the following formula:

$$\text{Resolution} = 0.61\ \lambda / n \sin\theta,$$

where λ represents the wavelength of the light used (for white light 0.53 μm is commonly assumed), n is the refractive index of the medium where the specimen is visualized in (usually air or oil), and θ is half of the angular width of the cone of rays collected by the lens. $n \sin\theta$ is also known as the numerical aperture (NA) which characterizes lens light collecting ability – the maximum is 1 for dry lens and 1.4 for oil-immersion lens. The higher the NA, the greater the resolution and brighter the image is. However, this can only be achieved by reducing working distances and depth of field. [46]

Bright field microscopy is the most commonly used light microscopy technique which uses white light to visualize the stained specimen or sample containing inherent contrast or colour. The samples are often

dyed, because most cells have insufficient contrast themselves. The sample is illuminated from below and can be observed from the top, so the light passing through the sample forms the image directly. [46]

1.5.2 Fluorescence microscopy

Fluorescence microscopy is a special type of optical microscopy which tries to display only the target of interest from a black background. In order to be able to image the samples with fluorescence microscopy, the object of interest needs to fluoresce. Fluorescence occurs when molecules absorb light photons of a shorter wavelength and due to this absorption enter an excited state. Within nanoseconds the de-excitation occurs during which the molecules emit light that is characterised by a longer wavelength. The difference between exciting and emitting wavelengths known as the Stokes' shift is used to make fluorescence microscopy so powerful. Based on this, it is possible to illuminate the object of interest at its absorbing wavelength, and then use a filter which will let only the wavelength of emitted light pass (Figure 10).

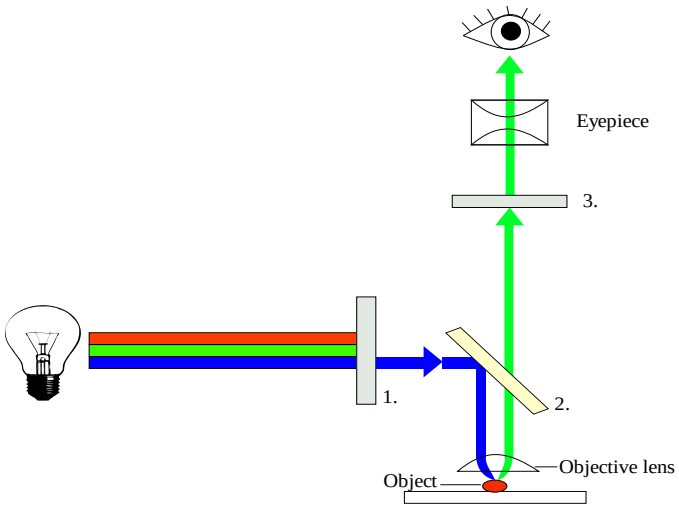

Figure 10. Fluorescence microscope work principle detecting fluorescein. A barrier filter, also known as excitation filter (1.), lets through only blue light with wavelengths between 450-490 nm. Then the beam splitting mirror (2.), also known as dichroic mirror, reflects light which is below 510 nm but lets through the light above 510 nm. Subsequently, the second barrier filter, also known as emission filter (3.), blocks other fluorescent signals besides the specific green fluorescein which is emitted between 520-560 nm. [2]

By filtering out the excitation light, the object of interest can be made to glow against a dark background. Various fluorescent molecules called fluorophores (e.g. FITC) can be used for labelling the objects of interest. However, due to photobleaching the fluorophores lose their fluorescence as they are illuminated and thus can be visualized only for short periods of time by fluorescence microscopy. The imaging periods can be extended by using antifade agents. [47]

1.5.3 Atomic force microscopy

Atomic force microscopy (AFM) is a technique which can be used for manipulation of surfaces and cells, to measure forces between samples,

as well as to image surfaces. The microscope uses a minuscule sharp pointed stylus, also known as the tip, to scan the surface of the sample. This tip is attached to a springy cantilever which itself is mounted to a positioning system that allows precise movement over small distances. The cantilever is brought close to the surface of the sample, which will result in an interaction between the sample and the tip. From this contact, a force is created which causes the cantilever to bend. The laser beam is focused on the end of the cantilever and the reflected light is collected with a photodiode, which allows AFM to detect any bending of the

cantilever from the changed position of the reflected laser spot on the photodiode (Figure 11).

The bending of the cantilever can be controlled with a feedback

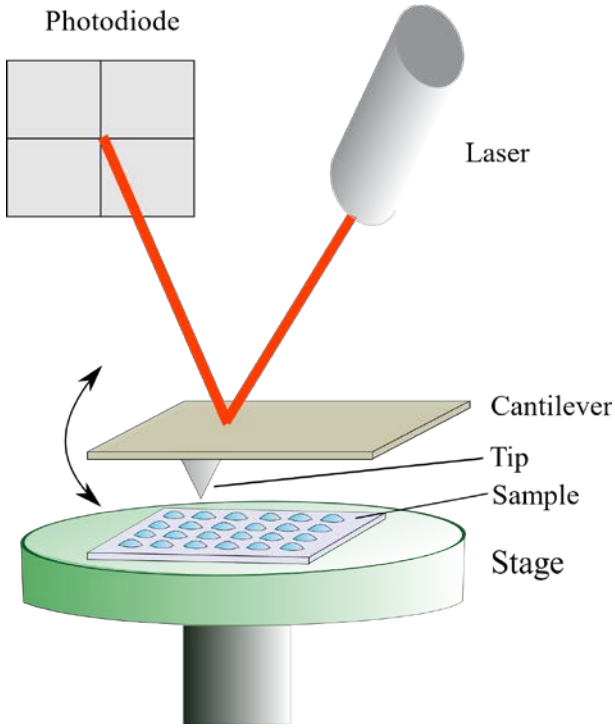

Figure 11. Principle of Atomic Force Microscopy. The sample is scanned with the tip attached to the cantilever which will bend if any forces occur. The bending is detected by a photodiode which will track the position of the laser spot that will change location according to the bending of the cantilever.

algorithm which makes it possible, for example, to create topographic maps of the surface to reveal height differences across the sample surface. In addition, AFM can collect information about a variety of forces including electrostatic, van der Waals and mechanical forces.

AFM can also be used to reveal specific interactions occurring between AFM tips and the sample surface. [2, 48]

1.5.4 Scanning electron microscopy

A scanning electron microscope (SEM) is a type of electron microscope which uses a focused beam of electrons to scan the surfaces of specimen. The scattered or emitted electrons from the surface of the sample are used to form an image. The resolution of scanning electron microscopes is significantly better compared to that of light microscopes due to electrons having a shorter wavelength which allows imaging of specimens with higher resolution.

The source of illumination is an electron gun mounted on top of the cylindrical column which emits the electrons (Figure 12). These emitted electrons are then accelerated by an anode and allowed to pass through a tiny hole, thereby forming an electron beam. Compared to light microscopes in which the lenses are made of glass, the ones used in an electron microscope are magnetic coils. They are placed at intervals along the column and their task is to focus the electron beam.

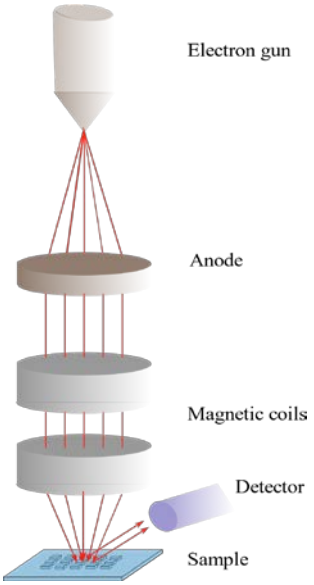

Figure 12. Working principle of scanning electron microscope. The electrons are fired by an electric gun, accelerated by an anode and then focused with magnetic coils to form an electron beam. The electrons hit the sample and are emitted back from the surface of the specimen. The detector captures the electrons and helps to form an image.

The samples which are put into the path of the electron beam are visualized in vacuum, as the electrons can be scattered when colliding with air molecules, resulting in distortion of the surface of the specimen. The samples can be stained with electron dense materials and imaged on a phosphorescent screen or captured with a high-resolution camera. Dense regions look dark due to the increased number of the scattered electrons that hit the detector. This creates images that have three-dimensional appearance. SEM can be used to characterize patterns on masters and stamps in micro- or nanoscale. [2]

1.6 Objectives of the thesis

The main objective of this master project was to develop and characterize patterned surfaces and fabricate bacterial arrays.

The subtasks of this thesis were to

1. optimize the protocol for master mould fabrication which can be used to prepare PDMS stamp,
2. optimize the protocol for PDMS stamp fabrication,
3. use the fabricated PDMS stamp to deposit PLL, PDA and mucins via microcontact printing (μCP), and
4. immobilize bacteria on patterned surfaces.

2 Materials and methods

The research was conducted at the Department of Biotechnology from February 2017 until May 2018. The majority of experiments were carried out at the NTNU Nanolab. The method of fabricating bacterial microarrays included making the master mould for preparing PDMS stamp and then using the stamp for microcontact printing bacteria-adhering chemicals. The bacteria were then adhered to the spots with chemicals making up the bacterial microarray. The overview of the whole method is shown and briefly described in Figure 13.

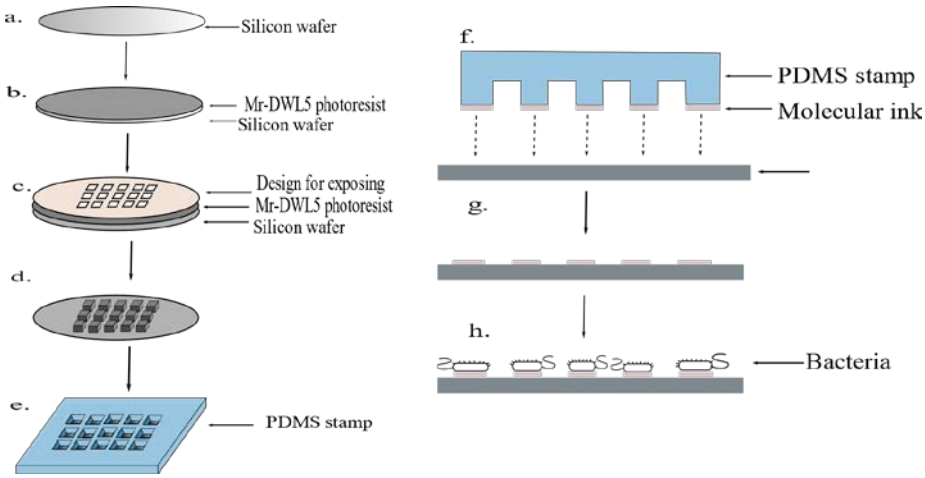

Figure 13. Method overview. a.-e. steps until stamp production, f.-h. bacterial microarray formation**.** Firstly, the photoresist Mr-DWL 5 is spin coated on a silicon wafer (a, b). Then, the desired pattern is exposed in MLA150 and developed (c, d). Subsequently, the PDMS is poured on the developed resist and peeled off, resulting in opposite features of the master (e). The stamp is incubated with molecular ink e.g. PLL, and microcontact printed on glass surface (f). Finally, the patterned glass (g) is incubated with bacteria, which after a wash will result in bacterial adhesion in desired spots, and a bacterial microarray formation (h).

2.1 Fabrication of microarrays

2.1.1 Photolithography

Photolithography was used to produce a master mould for stamp production. First, the pattern which will be exposed on the resist-covered wafer needs to be designed. Second, the wafer has to be cleaned and baked in high temperatures. Third, the wafer needs to be coated with the resist and baked to drive out solvents to enable successful exposure. After this, the exposed wafer needs to be baked again to further assist cross-linking reactions and then developed to remove non-crosslinked resist (workflow summarised in Figure 14). The steps will be further described below.

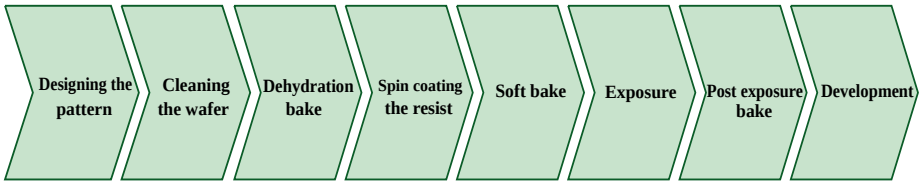

Figure 14. Summary of the workflow to fabricate master mould

2” silicon wafers (University Wafer) were first cleaned in an acetone bath, washed with isopropanol (IPA) and blown dry with nitrogen (N_2). Subsequently, the wafer was placed in an ozone cleaner for 3 minutes at 20 °C to remove organic contaminants from its surface. The cleaning steps were followed by a dehydration bake on a hot plate at 180 °C for 20 minutes to remove all moisture from the surface of the wafer. For

spin coating, around 1 ml of the negative photoresist mr-DWL 5 (micro resist technology GmbH) was added to the wafer which was then spun for 33 seconds at 3000 rpm with an acceleration of 1000 rpm/s. To avoid hot plate overshooting and cracking of the resist, the soft bake was done according to the guidelines from the Nanolab engineers. The following procedure was used:

I. The hot plate was set to 50 °C. When this temperature was reached, the wafer was placed on the hot plate.
II. The set point was increased to 60°C.
III. At about 55°C, the set point was changed to 70°C.
IV. At about 65°C, the set point was changed to 80°C.
V. At about 75°C, the set point was changed to 90°C.
VI. When 90°C were reached, this temperature was kept fix for 2 minutes after which the set point was changed to 50°C.
VII. The wafer cooled on the hot plate until the temperature reached 50°C, after which it was placed on a cleanroom wipe for 10 minutes for relaxation.

The design of patterns to be exposed in Maskless Aligner was made in CleWin. It contained arrays of circles in varying sizes that were surrounded by a square (Figure 15). The following designs were used: 4 μm circles with 10 μm spacing, 5 μm circles with 12 μm spacing, 6 μm circles with 14 μm spacing, and 7 μm circles with 16 μm spacing.

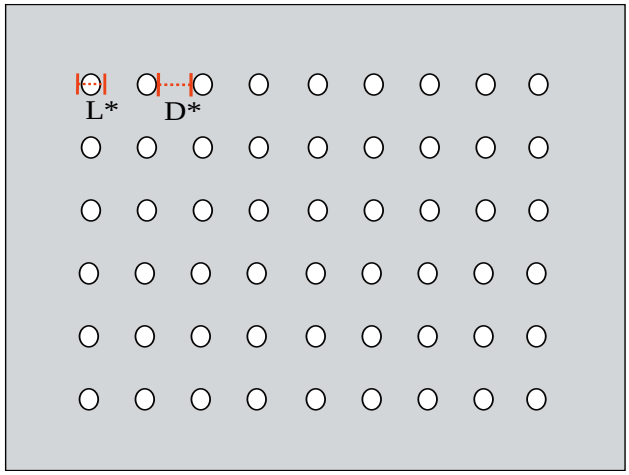

Figure 15. Illustration of design used in the Maskless Aligner. L* (lateral dimension of a feature) was either 4, 5, 6, or 7 μm. D* (distance between features) was 10, 12, 14, 16 μm. There were either 20x20 or 40x40 circles per square.

The design was exposed on the 2" wafer in Maskless Aligner MLA150 (Heidelberg instruments) using UV 405 nm laser. In order to find out the suitable exposure dose, a dose test was performed with exposures from 200 to 690 mJ cm^{-2} (dose step size was 10 mJ cm^{-2}), which resulted in choosing the dose 200 mJ cm^{-2} for all subsequent exposures. The exposure was then followed by post exposure bake (PEB) which was performed the same way as soft bake (previously described). The recommended relaxation period of 1h was followed after PEB.

For development, the wafer was placed on a wafer holder and moved up- and downwards for 1 minute and 45 seconds in mr-DEV 600 (Microchemicals) developer bath. The wafer was then washed with IPA from squirt bottle and placed in an IPA bath for 1 minute. While removing the wafer from the bath, it was rinsed once again with fresh IPA. This was followed by drying the wafer with N_2. The wafer was

visualized using the optical microscope Zeiss Axio Scope A1, and the thickness of the resist was measured using the stylus profilometer.

2.1.2 PDMS stamp fabrication

PDMS stamp fabrication is a multi-step process which includes silanization of the master, mixing the elastomer with the curing agent, stirring the mixture, degassing the mixture to remove bubbles, pouring the mixture on the master mould, curing the master mould and peeling of the cured PDMS stamp (Summary in Figure 16). The procedures are further described below.

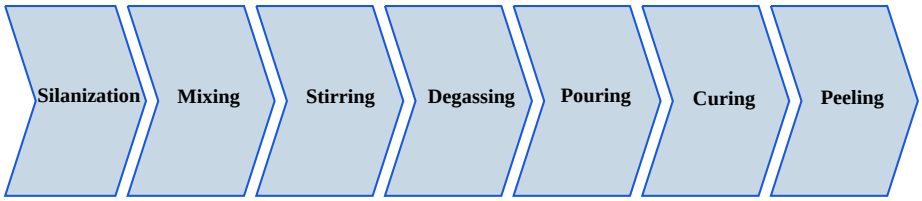

Figure 16. Summary of the workflow to fabricate PDMS stamp.

The wafer was placed in a silanization chamber together with a glass vial containing two to three drops of trichloro(1H,1H,2H,2H-perfluorooctyl)-silane (Sigma-Aldrich) to make the surface more hydrophobic. The house vacuum was turned on for 5 minutes and then switched off for 5 minutes, and this periodical switching was done altogether three times. The wafer was left in the chamber for 1h. Approximately 10 g of PDMS pre-polymer Sylgard-184 base (Dow Corning) was mixed with ~1 g of Sylgard-184 silicone elastomer curing agent (Dow Corning) in a cup with the weight ratio of 1:10 and stirred

slowly using a plastic spoon. In order to degas the mix, a cup was placed in a vacuum chamber for 5 minutes. Meanwhile, the wafer was removed from the silanization chamber and placed in a plastic Petri dish. The mixture was poured on the wafer in the Petri dish and placed in the oven for 3h at 65 °C (also, 4h at 65 °C and 2h at 80 °C were tested). The finished stamp was cut out using a scalpel and peeled off using tweezers.

2.1.3 Microcontact printing (μCP) of PLL FITC

To start with, the glass used for stamping substrate was cleaned in acetone bath, then rinsed with ethanol and MilliQ water and additionally dried with N_2. Thereafter, the stamp was plasma cleaned using either 50% O_2 and 50% generator power or 50% O_2 and 100% generator power for 1 minute. The stamp (usually containing 6 squares, Figure 17) was cut out using a scalpel and placed on a separate glass slide.

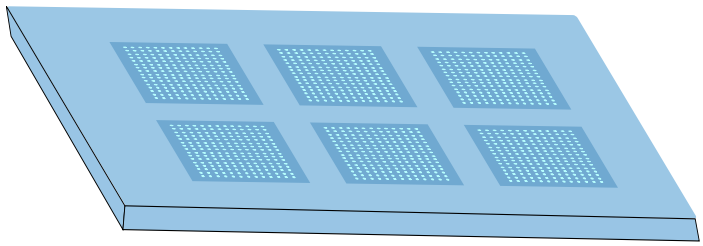

Figure 17. Illustration of PDMS stamp used for microcontact printing.

PLL-FITC (0.5 mg/mL in MilliQ, Sigma Aldrich, mol wt 30,000-70,000) was applied onto the stamp using a pipette, and rubbed around gently with the pipette's tip. The stamp was left to incubate in room temperature for 15-20 minutes. A pipette was used to remove excess liquid from the stamp. The remaining liquid was removed by drying the stamps with N_2. The previously cleaned glasses were put on top of the patterned side of the stamp and pressed carefully with tweezers to remove air. Additional weights of 100 g were put on the stamp and left for 20-30 minutes. A marker was used to draw the outline of the patterned area to make it easier to find it under the microscope. Thereafter, the glass slides were separated from the stamp and patterns were visualized under the fluorescent microscope.

2.2 Bacterial adhesion to functionalized surfaces

In order to fabricate bacterial microarrays, bacteria-adhering chemical was left to incubate on top of the PDMS stamp which was then used to microcontact print the material on PLL-g-PEG covered glass. Thereafter, a solution containing bacteria was left on the patterned substrates. After a predefined period of time, the unattached bacteria were washed off, which should have resulted in an array with bacteria that is adhered to patterned spots. The summary of the method is depicted on Figure 18 and described in detail in the following chapter.

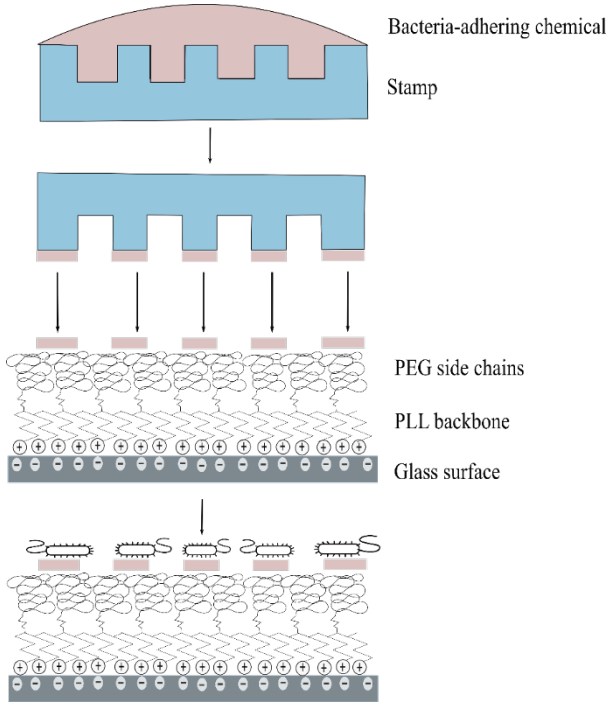

Figure 18. Microarray fabrication on PLL-g-PEG covered glass. Bacterial adhesion promoting chemical is incubated on the PDMS stamp and then transferred onto PLL-g-PEG functionalised glass. Thereafter, bacteria are added and left to incubate for 30 minutes. The unattached bacteria are washed off, which will result in regular bacterial arrays provided the bacteria adhere to bacteria adhering chemicals and not to PEG.

2.2.1 Microcontact printing PDA, PLL or mucin on PEGylated and non-PEGylated surfaces

The main steps for fabricating arrays of bacteria-adhering chemicals include cleaning and passivating (PEGylating) the glasses used as surfaces for microcontact printing, incubating the bacteria-adhering chemical on top of the PDMS stamp, removal of the excessive chemical and drying of the stamp with N_2, using the stamp for μCP the chemical on top of PEGylated surface and visualizing the pattern under the microscope (Summarised in Figure 19). The procedures for each step will further be described in the chapter.

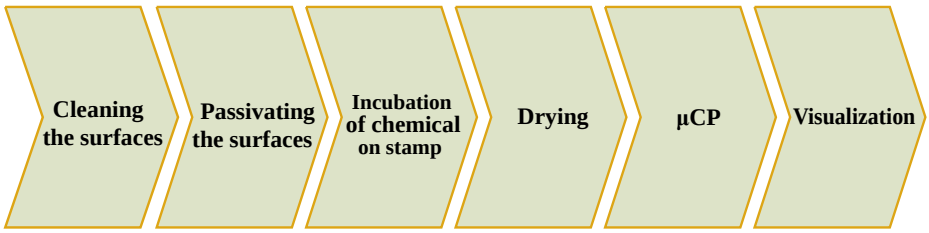

Figure 19. Summary of the workflow to fabricate and visualize patterned surfaces.

To begin with, the glass surfaces were cleaned by immersing the glasses in acetone bath, then rinsing them with ethanol and MilliQ water. An alternative cleaning method included immersion of the glasses in freshly prepared HCl-Methanol bath for 30 minutes, which was followed by rinsing and immersion of the glasses in MilliQ water and drying with N_2.

Secondly, some of the glass surfaces were passivated to prevent random binding of bacteria in unwanted locations for experiments with PLL and

PDA (mucin was used on non-PEGylated glasses). For passivation, PLL-g-PEG (1 mg/mL, SuSoS) was applied to the cleaned glass surface and left to incubate for 1h. After incubation the excess liquid was removed, and the glass slide was rinsed with PBS buffer (pH=7.4) and then with MilliQ water, which was followed by drying with a stream of N_2. When glasses without PEGylated surfaces were used, the PLL-g-PEG part of the protocol was skipped.

As the glass surfaces were ready, the bacteria-adhering chemical array had to be fabricated. For that, the protocol largely followed the one described in chapter 2.1.3 Microcontact printing (μCP). However, instead of PLL-FITC, either PDA, PLL or mucin solution was used. PDA was prepared by dissolving 1 mg dopamine hydrochloride (Sigma-Aldrich) in 1 mL of Tris buffer (pH=8.5) to induce polymerisation. The solution was prepared just before applying it to the stamp. PDA was left to polymerize and incubate on the stamp for 30 minutes (Figure 20).

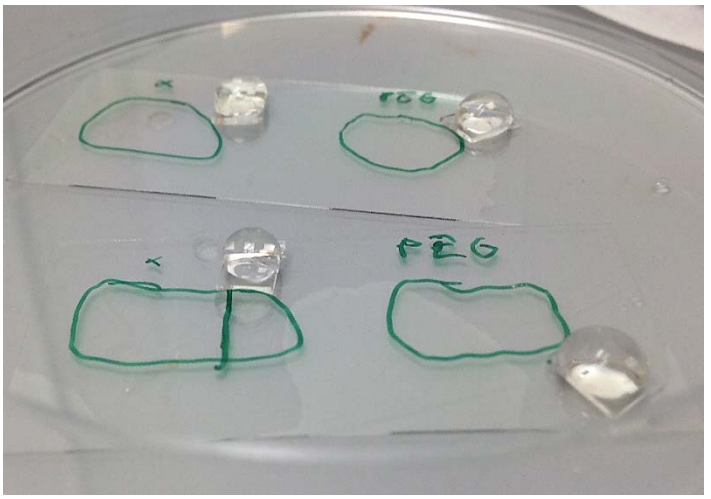

Figure 20. Incubating PDA on top of non-plasma treated stamp.

PLL which was bought as pre-prepared 0.01% solution (Sigma-Aldrich, mol wt 150,000-300,000) was directly applied on the stamp and left to incubate for 15 minutes. Mucin solution which was prepared by dissolving 1 mg of Bovine submaxillary mucin (Sigma-Aldrich) in 1 ml of MilliQ water was applied on the stamp and left to incubate for 15 minutes the same way as PLL. After the incubation step, the excess solution was removed with a pipette and the glass surface was blown dry with N_2, as previously done with PLL-FITC. The passivated glasses covered with PLL-g-PEG were placed on top of the stamp and a 100 g weight was put on top of the glass slide and left for 5, 10 or 15 minutes. The glass slide was then removed and visualized under Zeiss Axiobserver Z.1 microscope to see if any patterns were formed.

2.2.2 Immobilisation of bacteria to PDA, PLL or mucin arrays on PEGylated and non-PEGylated surfaces

The bacteria had to be immobilized on previously fabricated patches. For that, *Pseudomonas putida* strain KT2440 was grown overnight at 30 °C in LB medium (10 g/L tryptone; 5 g/L yeast extract, 5 g/L NaCl), whereas *Escherichia coli* strain BL21 was grown overnight at 37 °C with LB media. Kanamycin was supplied at the final concentration of 50µg/ml when needed. The bacteria were provided by Alex Wong (Department of Biotechnology and Food Science, NTNU) and both bacteria contained the plasmid pHH_SynPromU which constitutively expressed mCherry. 100µl of the bacteria grown overnight was added on the functionalized glass surfaces and incubated for 5 minutes. Afterward the glass was rinsed two to three times with LB medium to

remove unattached bacteria. A small droplet of LB medium was added on top of the glass to reduce the stress to the bacteria. The glass slide was then visualized under Zeiss Axiobserver Z.1 microscope.

3 Results and discussion

3.1 Fabrication of microarrays

3.1.1 Photolithography

Photolithography techniques were used to produce the master mould for PDMS stamp fabrication. Photolithography involves many processes, and several aspects in these can be modified. First, cleaning the wafer can be done in various ways using different solvents and methods. It is essential to choose the suitable photoresist, which will result in desired features and thickness range. Spin coating the resist is another important step, and the parameters used during spinning (acceleration, spin speed and spin time) must be adjusted to achieve the desired thickness without causing artefacts. The optimal soft and post exposure bake ramp rate and cooling temperatures must be identified and used to secure correct curing of the resist and avoid inducing thermal stress. The length of the relaxation period can also affect the resist and should be performed according to the manufacturer's protocols. Various problems, including resist contamination, exposure issues and overshooting hot plate, were noticed when attempting to produce the master and will be further described in this chapter.

The cleaning procedure gave clear wafers that upon visual inspection showed no contaminants. The same protocol was therefore used in all experiments without any significant changes. As several problems, e.g. tidemark of the resist after spin coating, white residue covering the wafer after development, edge bead issues, cracks in the developed resist, etc.,

were encountered when using SU8-5 and SU8 2003.5, a new resist was chosen and used for photolithography (problems encountered with SU8 further described and discussed in [49]). Around the time when the problems were experienced with SU8, a new series of negative tone resist (mr-DWL) and a new easily operable instrument for exposure (MLA) were introduced to Nanolab, which both seemed suitable alternatives for our experiments. The decision to change to mr-DWL 5 instead of SU8 resists was made together with Nanolab engineers based on the thickness range achievable with mr-DWL 5 (3-15 μm) and its suitability for MLA. Exposing the resist in MLA allowed to skip ordering the photomask making the overall production process faster. Compared to SU8, mr-DWL5 was less problematic to work with, as, for example, no tidemark nor white residue on the wafer was detected. Also, the adhesion of the resist to the wafer seemed sufficient, as no detachment of large areas of the resist were observed.

The average thickness around 5.2 μm was constantly achieved using spin coating parameters mentioned in previous chapter (2.1.1 Photolithography). An exposure dose test was done with different exposures to establish a suitable dose. The lowest (200 mJ/cm^2), intermediate (410 mJ/cm^2), and highest (690 mJ/cm^2) exposure doses are depicted in Figure 21. The choice of using 200 mJ/cm^2 was made as the patterns on the resist appeared well exposed. Higher exposure doses seemed to have reduced the size of the features. As with the use of negative resist, the areas around the small features are exposed, higher doses might result in diminution of unexposed areas.

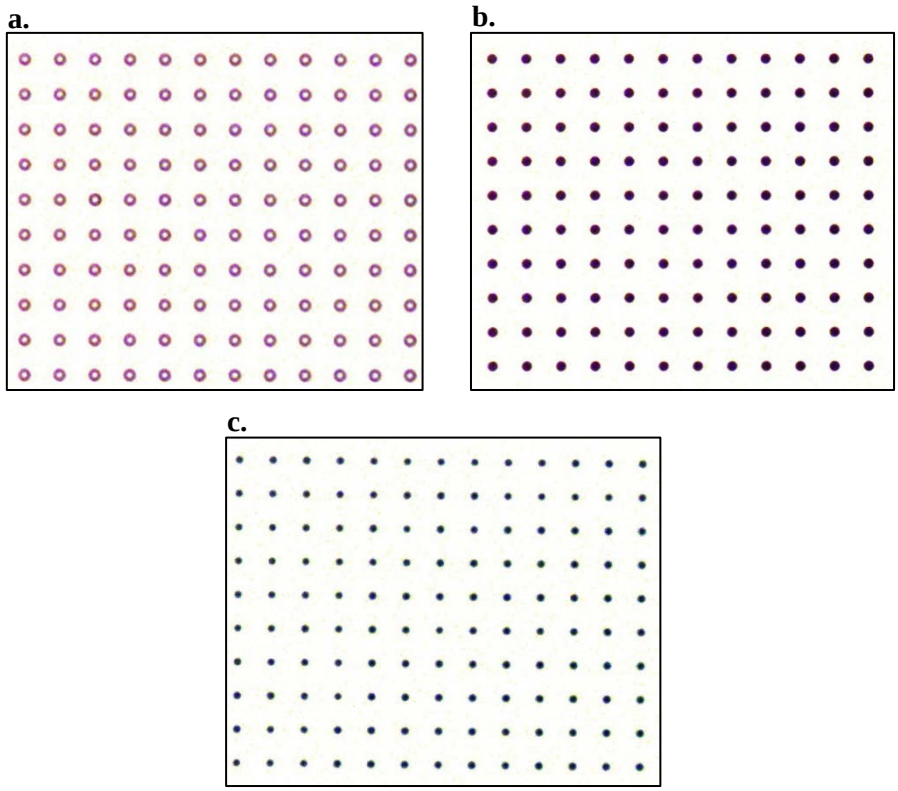

Figure 21. Exposure dose test using a design with 5 µm circles and 12 µm spacing. The images are obtained for the developed wafer. In the image a. the area depicted was exposed with 200 mJ/cm^2. In the image b. the area depicted was exposed with 410 mJ/ cm^2 and in the image c. the area depicted was exposed with 690 mJ/ cm^2. Exposure test was done using MLA150 and samples were imaged with ZEISS Axio Scope A1 using 20x objective.

Around 1 ml of resist was applied to a 2” wafer prior to spin coating. This proved to be sufficient – anything less resulted in uneven coating and starfish pattern of the resist. As there were a few occasions when the spin coater was unevenly positioned on the table, a level was always used prior to spin coating to confirm that the equipment is in balance. This was important to ensure that the resist coating is even throughout the whole wafer.

After spin coating the resist, comets and pinholes were frequently observed (Figure 22). As these artefacts can be caused by particle contamination and a poor resist application process causing the presence of air bubbles, the problem was discussed with Nanolab engineers. The cleaning procedure was considered to be sufficient to remove all the particles and there were no remarks about the application technique of the resist. The decision was made to prepare a new solution of the resist, as presence of contaminants in the resist could not be ruled out. The number of artefacts was reduced after using the new solution of resist, but did not disappear completely. It was advised to blow N_2 on the wafer to remove particles before applying the resist, however, the comets still appeared after using this method. It was still possible to use these wafers for PDMS production and microcontact printing, if avoiding the use of the areas with artefacts during stamping.

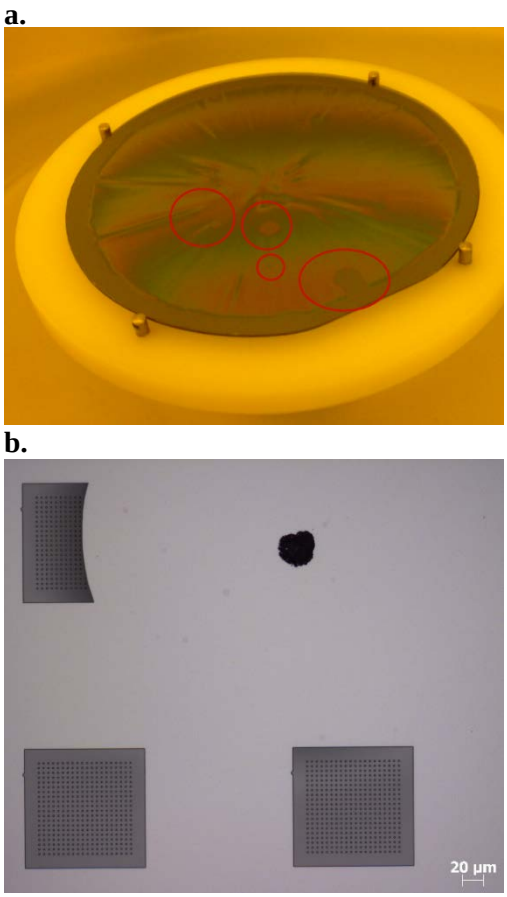

Figure 22. Artefacts on the wafer. In image a. several comets can be observed which may be caused by small particles. The resist coverage is insufficient as the edges of the wafer are left uncovered. The image was made right after spin coating the resist in the spinner. In image b. a particle which caused a comet is visualized with a microscope. Image a. was captured with a portable camera. Image b. was captured with Zeiss Scope A1 microscope

During an exposure which was done after MLA system update, MLA did not completely expose some of the lines of features on the edge of the wafer, leaving the squares “striped” after development (Figure 23, a). The image obtained for the PDMS stamp made from the wafer with exposure issues shows that the problems occurring during the exposure of the wafer will be transferred onwards to PDMS (Figure 23, b). As Nanolab engineers were not exactly sure what caused this exposure problem, the company producing MLA150 (Heidelberg Instruments) was contacted for explanation.

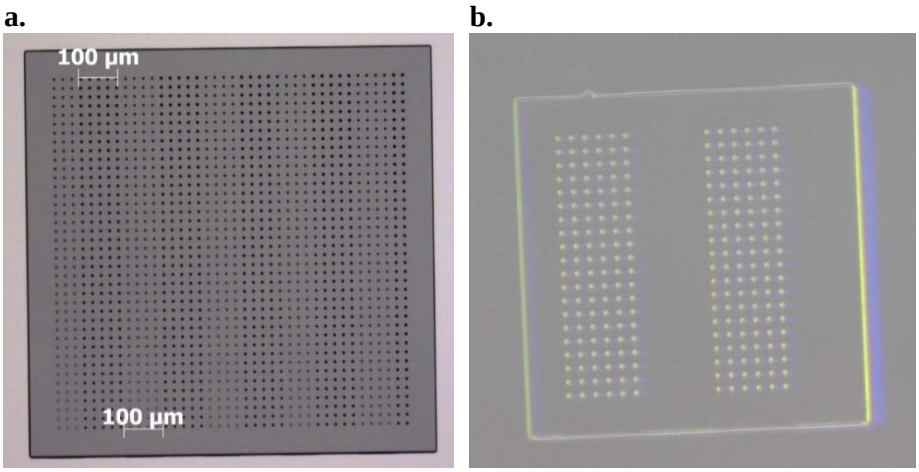

Figure 23. Exposure problem with MLA150. In image a. developed resist is shown. In image b. a PDMS stamp fabricated from the master with development issues is shown. Less exposed lines can be observed in the developed resist. These issues were visible on the squares situated on the edges of the wafer. The design used for making this master mould was 7 µm circles with 16 µm spacing. Image a. was captured with Zeiss Scope A1 microscope using 10x lens and image b. was made using an Olympus SZX10 stereomicroscope.

By the time of handing in this thesis, the response was yet to be received. However, it is important to consider that the problems encountered in photolithography step continue causing issues in soft lithography, it is thus essential to obtain sufficient quality of the master mould before continuing the protocol by preparing PDMS structures.

Soft bake and post exposure bake were done following the manufacturer's protocols and the guidelines from Nanolab engineer Mark Chiappa. Relaxation was kept in accordance with the protocol, as shortening the relaxation period after PEB from one hour to 40 minutes resulted in multiple cracks in the developed features. During a post exposure bake in the experiment, the hot plate overshoot 20 degrees (from 90 to 110 °C). The developed wafer, however, showed no irregularities in the features.

Development by immersion was used to dissolve the unreactive resist. The wafer was moved laterally in a beaker filled with mr-DEV 600 developer and then in a beaker filled with IPA. Before drying with N_2, fresh IPA was used to clean the wafer after removing it from IPA bath. Using this method still showed some photoresist residues, therefore the wafer was additionally cleaned with fresh IPA after removing it from the developer bath and before placing it in IPA bath. This resulted in cleaner surfaces with reduced number of photoresist particles on the wafer which could successfully be used for PDMS stamp preparation. This additional cleaning step is therefore recommended to be included in the development protocol.

3.1.2 PDMS stamp fabrication and imaging

After the master mould is ready, PDMS stamp can be prepared. Fabricating PDMS stamp has several aspects that can be adjusted, e.g. the ratio between elastomer and curing agent in the mixture, stirring time of the mixture, overall quantity of the elastomer-curing agent mixture, degassing time, curing time and temperature, as well as whether to use silane on the master mould or not.

The ratio of 10:1 between elastomer and curing agent was used. Mixture containing approximately 10 g elastomer and 1 g of curing agent per wafer was identified as a suitable amount. When double the amount was used, the resulting thickness of PDMS was too high making it difficult to cut out pieces of PDMS. Stirring the mixture was done slowly for half a minute using a plastic spoon. The goal was to stir it well while avoiding forming bubbles in the mixture. Degassing time was reduced from 30 minutes to 5 minutes, as there were no visible differences in the number of bubbles when extending the time. Instead, manual air pump was used to remove the bubbles that had remained in the mixture after the degassing step, before pouring it on the master mould.

Various curing conditions were tested – 4h at 65 °C, 3h at 65 °C and 2h at 80 °C with no visible changes in PDMS features. However, with 80 °C, the plastic Petri dish used for curing melted and aluminium foil proved to be difficult to handle as a lot of PDMS flowed under the wafer. So, 65 °C was identified as optimal. Curing 3h with 65 °C was tested due to some of the users in Nanolab using these conditions, however, 4h at 65 °C proved to be the best option, as it was suspected that 3h 65 °C leaves the PDMS fragile and potentially causes the pillars to break off

during microcontact printing (shown in chapter 3.2.2 μCP polydopamine on PEGylated and non-PEGylated surfaces).

As it became challenging to peel the PDMS layer off from the wafer without breaking it, the decision to use silanization was made. This added an extra hour to the protocol, but it proved to be useful as the peeling process was much easier to carry out and no wafers were broken after including the silanization process. In addition, the silanization has a long-term effect, as it is covalently bound to the wafer's surface, thus it is not necessary to repeat the procedure every time PDMS needs to be prepared.

Artefacts, e.g. pairing of the features or roof collapse, might occur due to unsuitable aspect ratios of the PDMS. It is therefore important to design features with optimal sizes to avoid facing problems in the PDMS production and microcontact printing steps. In the pattern design step, the aspect ratios of the planned PDMS stamp can be optimized by changing the lateral dimension of the pillar structure and the distance between the two pillars. The number of structures per stamp can be varied as well. Creating enough space between structured areas is necessary to be able to cut out the stamp without damaging the areas at the side. It is important to acknowledge, that a small volume (around 2 %) is lost during the curing step when elevated temperatures are used. It is relevant to take this into consideration when the design used for the production of PDMS stamp is sensitive to small changes. In addition, the thickness of the resist is not always exactly the same throughout the whole master mould, resulting in reduced or increased height of the final

PDMS pillars (H). It is therefore reasonable to design the master mould with aspect ratios which allow some variability in PDMS features.

In Table 1, the theoretical values of the aspect ratios of the designs used in this master thesis are presented.

Tabel 1. Theoretical aspect ratio values for PDMS stamp design.

Design	H*(µm)	L*(µm)	D*(µm)	H/L	H/D
4 µm ○	5	4	10	1.25	0.5
5 µm ○	5	5	12	1	0.42
6 µm ○	5	6	14	0.83	0.36
6 µm ○	5	6	12	0.83	0.42
7 µm ○	5	7	16	0.71	0.31

H – height of the features; L – lateral dimension of the feature, D - distance between features. * - The shown values are based on the master design, not the experimental measurements of the fabricated PDMS stamps.

From Table 1, it can be visualized that the calculated aspect ratios appear to be optimal staying in between 0.5 and 5 for H/L, and > 0.05 for H/D. The lateral dimensions of the features were designed to be large enough to allow the bacteria to adhere. Therefore, the designs with feature dimensions ranging from 4 to 7 µm were used to enable *Pseudomonas putida* and *Escherichia coli* cells to have area wide enough for stable attachment. The distance between the features were at least double the size of the lateral dimension of the feature to reduce the risk of bacteria forming bridges between the spots [6].

In order to visualize the stamp and to confirm the absence of artefacts, a scanning electron microscope was used. For the stamps fabricated using the proposed procedure, a regular array of pillars was observed. However, the lack of a clearly defined edge of the pillars made the determination of their diameter challenging (Figure 24).

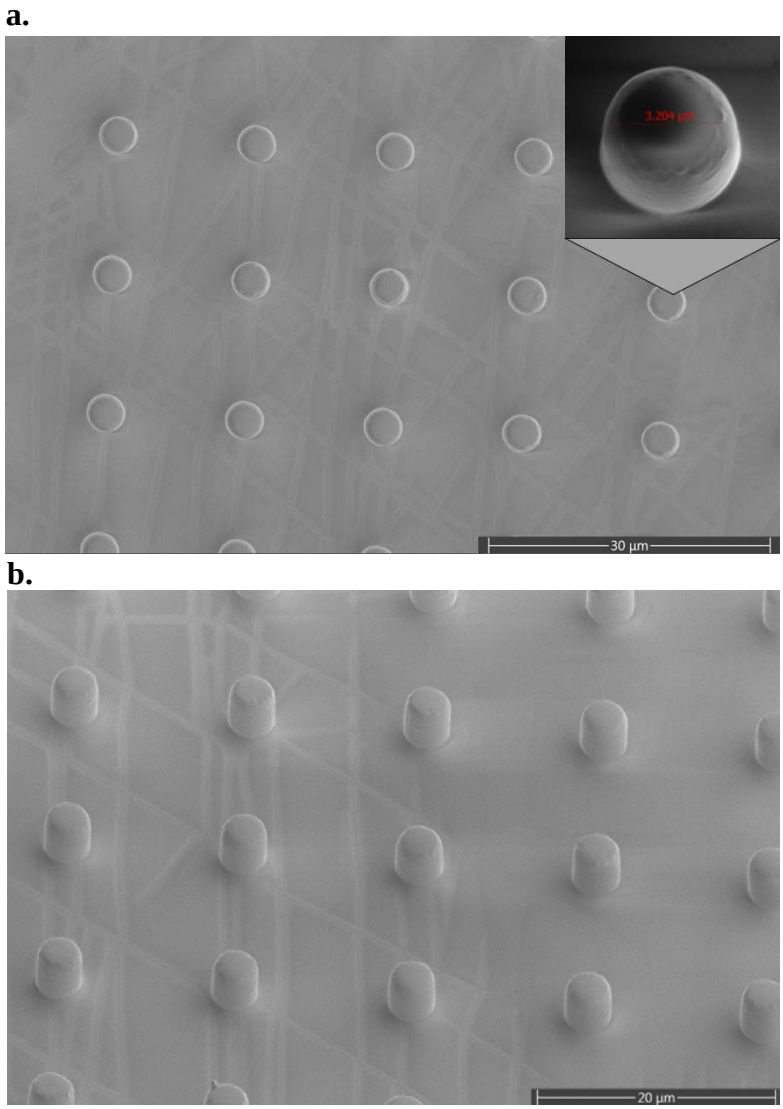

Figure 24. SEM images of a PDMS stamp. The images depict top-down (a.) and tilted view (b.) of gold coated PDMS stamps. The insert in image a. shows a measurement of the width of one pillar (3,204 µm). The design of 5 µm circles with 12 µm spacing was used in photolithography to fabricate master mould for this PDMS stamp. Images were made in Nanolab using SEM Apreo.

3.1.3 Microcontact printing (μCP)

After PDMS stamp is prepared, it can be used for microcontact printing chemicals. Microcontact printing is a multi-step procedure that can be modified by targeting the following questions:

Question 1: What is the optimal procedure for pre-cleaning the surfaces which will be patterned?
Question 2: Should the oxygen plasma treatment be used to alternate chemical properties of the stamp**?**
Question 3: Should the stamp itself be cleaned before usage?
Question 4: How long should the "ink" be incubated on the stamp?
Question 5: How much pressure should be added to secure optimal contact between the stamp and the surface?
Question 6: Is it important to use weights during the stamping?

Visualization of patterned surface can be helpful in determining the success of microcontact printing and understanding how to improve the procedure. Imaging can be done using AFM, as well as fluorescence and light microscopy. In this chapter, the results obtained when addressing the above-mentioned questions will be described.

Before performing the microcontact printing procedure, the glasses had to be cleaned (tackling Question 1). In the beginning, immersion in acetone bath followed by ethanol and MilliQ water rinse was used as the cleaning method. Frequently, the glasses did not often look clean after this procedure, possibly due to dried acetone stains, therefore the cleaning protocol was changed. As an alternative cleaning procedure, the glasses were kept in freshly prepared HCl:Methanol bath for 30

minutes. This was followed by rinsing and immersion of the glasses in MilliQ water and drying with N_2. The glasses looked cleaner after these steps, therefore this was chosen to be the method for cleaning.

PLL-FITC was used to test if the stamp could successfully be used to print patterns on PDMS to glass surfaces. The stamps were also plasma treated to improve the adhesion of PLL-FITC to the stamp and to increase its transfer to glass surface, following the recommendation from Karen Dunker's master thesis [49]. For this purpose, the stamps were treated for 1 minute using 50% O_2 and 50% or 100 % generator power. Printed patterns using plasma treated stamps are depicted in Figure 25, a. and b. As the plasma treatment modifies the surface of the PDMS making it more hydrophilic, it also made applying the ink easier and greatly reduced the amount of ink necessary for covering the surface of the stamp (from 100 µl to 10 µl), as it spread smoothly over the area. Microcontact printing was also tested with stamps that were not plasma treated. This made the application of the ink a bit more complex, as the surface of PDMS is hydrophobic; however, the removal of the ink and the drying of the stamp with N_2 were quicker. The printed pattern using stamp that is not plasma treated is depicted in Figure 25, c. It is worth mentioning that in images b. and c., the solution used for microcontact printing was 3 weeks old. The clearly visible patterns depicted on these two images show that PLL-FITC solution does not need to be freshly prepared. If the solution is kept covered in aluminium foil in a fridge at 4 °C, then it can be re-used for microcontact printing a few weeks after preparation.

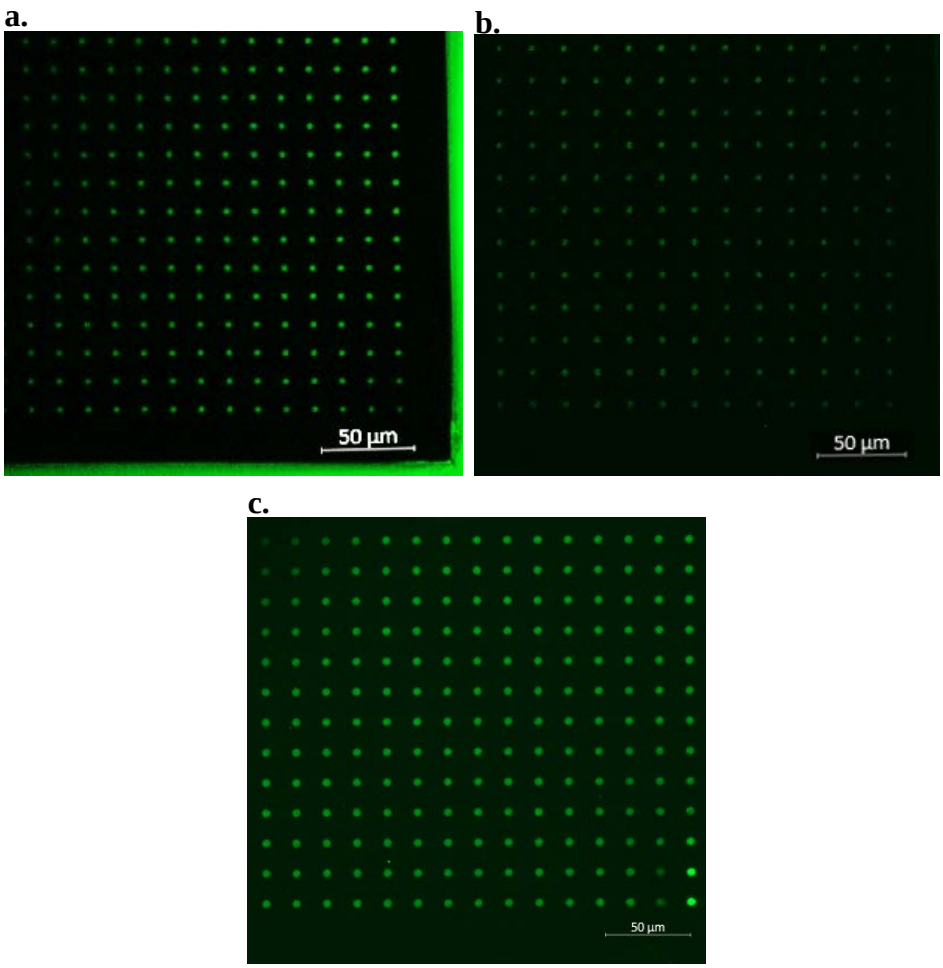

Figure 25. Stamped PLL-FITC using differently plasma treated PDMS stamp. In image a. the stamp (5 µm circles with 12 µm spacing) used for patterning was plasma treated with 50% O_2 and 100% generator power. In image b. the stamp (6 µm circles with 12 µm spacing) was plasma treated with 50% O_2 and 50% generator power and in image c. the stamp (6 µm circles with 12 µm spacing) was not plasma treated. PLL-FITC solution used for b. and c. was three weeks old. Images were obtained using 20x lens and FITC filter on a Zeiss Axiobserver Z.1 microscope.

From these experiments, it was not clear how the plasma treatment difference affects the stamp structure or microcontact printing. Microcontact printed patterns did not seem to differ when plasma treated stamp or non-plasma treated stamp was used (Question 2). In order to evaluate if plasma treating the stamp has any effect on the stamped patches, two types of PLL-FITC patterns were compared with each other. One pattern was made using the untreated PDMS stamp; and the second pattern was created using a stamp which had gone through plasma cleaning 10 times (using 50% O_2 and 100% generator power, Figure 26). 10 times was used to enhance the effect of the treatment.

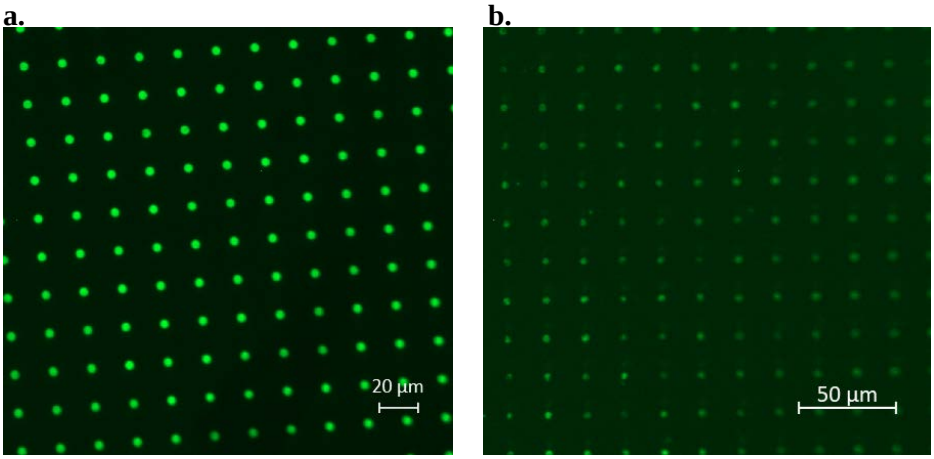

Figure 26. Deposition of PLL-FITC using non-plasma treated (a.) and 10x plasma treated stamp (b.). The design used for making stamp contained variable size circles (4, 5, 6, 7 µm) with variable spacing (10, 12, 14, 16 µm). The stamp area containing 6 µm circles with 14 µm spacing was used to generate the patterns depicted on the images. Images were obtained using 20x lens and FITC filter on a Zeiss Axiobserver Z.1 microscope.

It was observed, that the stamp seemed more hydrophilic after 10 plasma treatments, even if the last treatment was done 4 days before applying PLL-FITC. This might indicate that the PDMS hydrophobicity will not be completely restored after multiple treatments. It was also noticed, that the printed patterns were much clearer when non-plasma treated stamp was used. Smudgy undefined patterns obtained with plasma treated stamp were possibly due to the difficulty of establishing a good contact between the glass surface and the stamp. In previously mentioned experiments, using plasma treated stamp also resulted in clearly defined patterns (Figure 25) These stamps were, however, treated only once or twice with plasma. Plasma treating the stamp several times might have a long-term effect. As it is almost impossible to conduct two microcontact printing experiments exactly the same way, results showing undefined patterns might have also been obtained by coincidence. When the treatment is found to be necessary, it is recommended to cut out and separate the part of the stamp which will be subjected to treatment. In the future, SEM could also be used to visualize the plasma treated PDMS to see if any cracks or artefacts can be visualized on the surface of the stamp that might affect microcontact printing.

Sometimes, small dust particles can be observed on top of the PDMS stamp, that might have accumulated after some time. To remove them, it is possible to clean the surfaces with a piece of tape before incubating chemicals on top of the stamps (Question 3). After using this technique, clear PDA patterns were obtained (shown in Figure 32, paragraph 3.2.2 μCP polydopamine on PEGylated and non-PEGylated surfaces).

The incubation times were not systematically studied in this thesis (targeting question Question 4). As 15 minutes showed to be sufficient to deposit PLL-FITC, it was also used for PLL and also initially for PDA. However, it was recommended to extend the time for PDA to 30 minutes to allow it to polymerize longer. [N.B., Arnfinnsdottir, Personal communication, February 2018]

The question how much pressure should be added during microcontact printing to avoid causing roof collapse of the PDMS stamp is important. Usually, the glass was pressed on the stamp with the help of tweezers to remove air and maximize the contact between the features and the glass surface. In addition, the weights were added on top of the glass. As no roof collapse was detected when inspecting the stamps and patterns in light and fluorescent microscope, it is safe to estimate that the pressure added on the glass on top of the stamp was not too high (tackling Question 5). However, during an experiment with the goal of testing if PLL-FITC patterns can be obtained again when using plasma treated stamp, suspicion arose that the initial contact between the stamp and the glass surface might be most important, as one of the stamps which was briefly placed in one spot left a stronger pattern in that area compared to the region where it was placed for 10 minutes under weights (Figure 27).

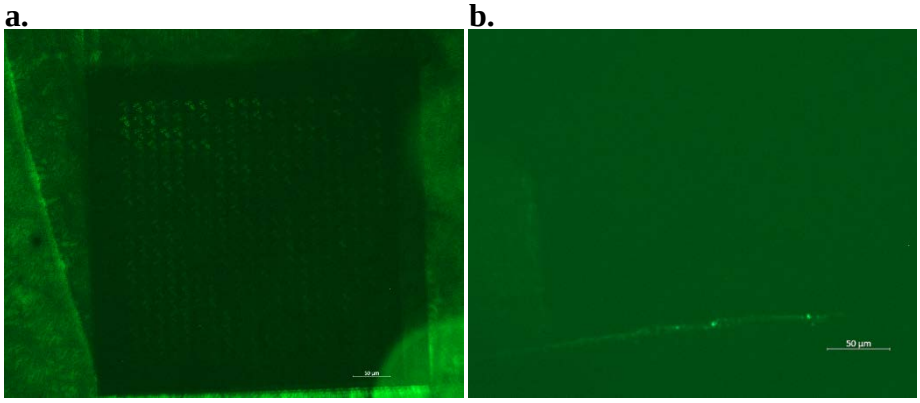

Figure 27. Comparing PLL-FITC patterns regarding the importance of initial contact of the stamp and the glass surface. Image a. shows a pattern that was fabricated when PDMS stamp was only for a short period in the location. Image b. shows the lack of a pattern in the new region using the same stamp**.** Images were obtained using 20x lens and FITC filter on a Zeiss Axiobserver Z.1 microscope.

The stamp moved a lot in the initial contact area, thus the material might have been wiped off to some extent, leaving the pattern weaker in the new location. If the initial contact is important, then placing the stamp on the glass surface needs to be done with extra care, using weights and a PDMS piece that allows the patterning to be stable without the weights tipping over. However, more experiments are needed draw firm conclusions.

During most of the experiments, the practice of using weights was continued following the guidelines from previous studies using microcontact printing for microarray production[49, 50]. However, an experiment depositing PDA with non-plasma treated stamp was done without using the weights. Regular patterns could be detected, indicating that the weights may not be essential (Question 6, Figure 28). It was also

observed that using flat bottomed weights was more successful as they showed reduced tendency to tip over compared to hollow weights.

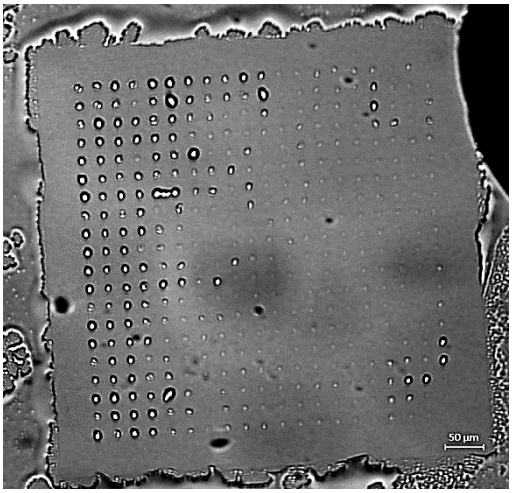

Figure 28. Depositing PDA on glass without using any weights. Image was obtained using 20x lens on a Zeiss Axiobserver Z.1 microscope.

Based on the experiments, it can be said that the microcontact printing results vary quite a lot and there are always areas with variable levels of success during stamping. It seems from PLL-FITC experiments that the first initial contact is most important, resulting in most of the material to be transferred; however, conducing additional experiments is necessary to be certain. In addition, it is worth mentioning that there has not been a single occasion where all the patterns were perfectly transferred throughout the whole stamp. However, there has usually been at least one successful area where the pattern is completely transferred onto the glass surface. Fabricating patterns with PLL-FITC seems to be reproducible, as multiple users in our lab group have successfully obtained clear patterns.

3.2 Bacterial adhesion to functionalized surfaces

3.2.1 PEGylation of glass surfaces

After PLL-FITC patterns were obtained, it was possible to start testing functionalizing surfaces. Firstly, PEGylation of glass was performed to prevent bacterial adhesion to the surface. As PEG chains themselves are not very adhesive, their adhesion to glass surface is enabled by the negative charges of PLL backbone on which the PEG chains are grafted to [33]. PLL-g-PEG was chosen as the anti-adhesive chemical based on previous research, where PEGylation showed to prevent bacterial adhesion more efficiently compared to bovine serum albumin (BSA) and poly(vinyl) alcohol (PVA) [6].

PEGylated surfaces without any further functionalization were visualized under AFM to obtain insight into the surface coverage and potential height differences across the surface (Figure 29). From area to area, the coverage varies, being fairly homogenous in some regions (Figure 29, a and b) and uneven in others (Figure 29, c and d). However, it is worth mentioning that the uneven areas depicted in images c and d were rare, and the surfaces were more dominated by homogenous regions.

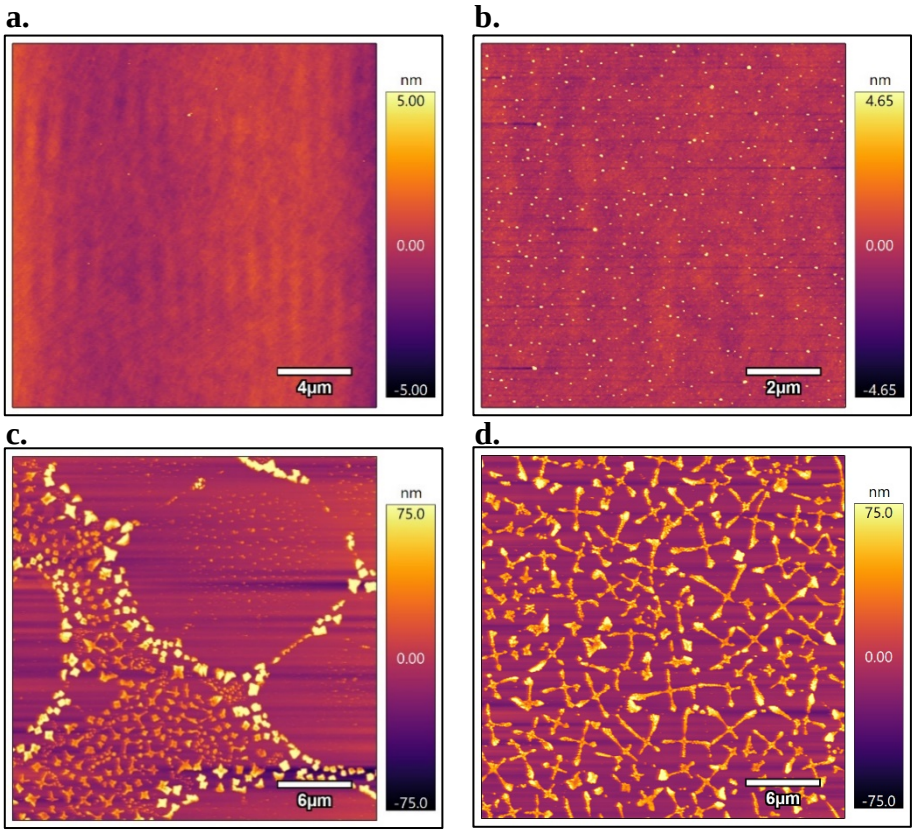

Figure 29. AFM images of PEGylated glass surfaces. AFM results show areas of homogenous surface coverage (a.), regions with small droplets or particles (b.) and areas that seems to show uneven coverage (c., d.). Images captured by Nina Bjørk Arnfinnsdottir using AFM Cypher (Asylum Research).

In order to test if the uneven regions might be due to the washing procedure, cleaned non-PEGylated glasses were also imaged with AFM. These areas showed to be homogenous without any regions containing surfaces similar to the ones depicted in Figure 29 c and d. More similarities with Figure 29 a and b were observed, however, these types of small particles were rarer. Therefore, it was suspected that PBS buffer salt crystals that might have not been washed off with MilliQ might have

been the reason for the patchy areas in the PEG-functionalized surfaces. When using PEGylated surface, the glass was thereafter more carefully cleaned in MilliQ water to avoid creating these non-homogenous areas.

3.2.2 μCP polydopamine on PEGylated and non-PEGylated surfaces

As the surfaces with and without PEGylation were shown to be homogenous, microcontact printing PDA was tested. PDA is an adhesive polymer that has been previously used for bacterial microarray fabrication on top of PEGylated surfaces [6]. When plasma treated stamp (using 50% O_2 and 100% generator power) was used for microcontact printing PDA**,** it was observed that the adhesion between the stamp and the PEGylated glass surface was so strong that the PDMS pillars got stuck on top of the glass (Figure 30), thus breaking them off from the stamp.

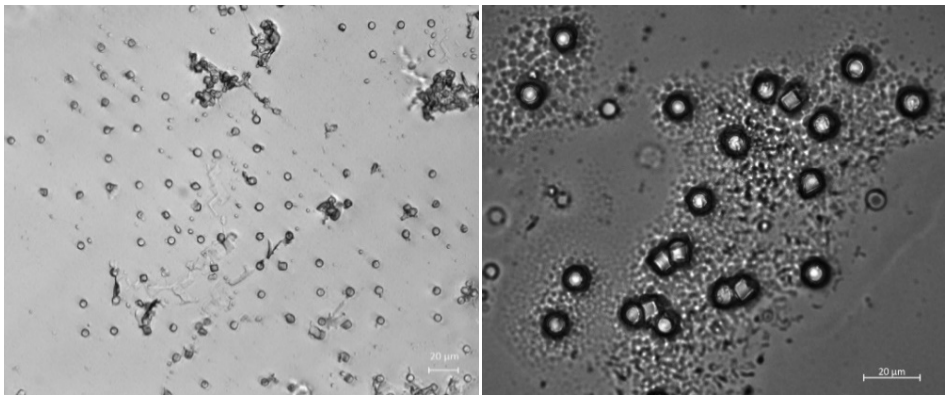

Figure 30. PDMS pillars stuck on PEGylated glass after microcontact printing PDA. PDMS stamp was pre-treated with 50% O_2 and 100% generator power. The image on the left was obtained using 40x lens and the image on the right was obtained using 100x lens on a Zeiss Axiobserver Z.1 microscope.

The microcontact printing PDA was then tried with a PDMS stamp that had been plasma treated with lower generator power (50%). This reduced the number of stuck pillars, however, small parts of the PDMS stamp were still observed in some areas. Suspicion arose that curing PDMS for 3 h at 65 °C might leave it fragile, causing the pillars to break off during the stamping process. Moreover, changing stamping conditions was also tested to see if this improves the patterning and reduces the number of stuck PDMS pillars. Using no weights during the stamping step as well as a shorter contact period between the stamp and the surface (15 minutes instead of 20-30 minutes) was tested. There were no areas with PDMS stuck on the glass surface when using these conditions, even when the stamp was produced using 3 h 65 °C. Additionally, it was once again observed that the patterning seems to work even without the weights (Figure 31, a). However, when PDA was left to incubate for even a shorter period (5 minutes) on the stamp (fabricated using 3 h 65 °C in the curing oven and plasma treated afterwards) and the stamp was placed on PEGylated glass for 10 minutes under weights, there were some pillars of PDMS stuck (Figure 31, b).

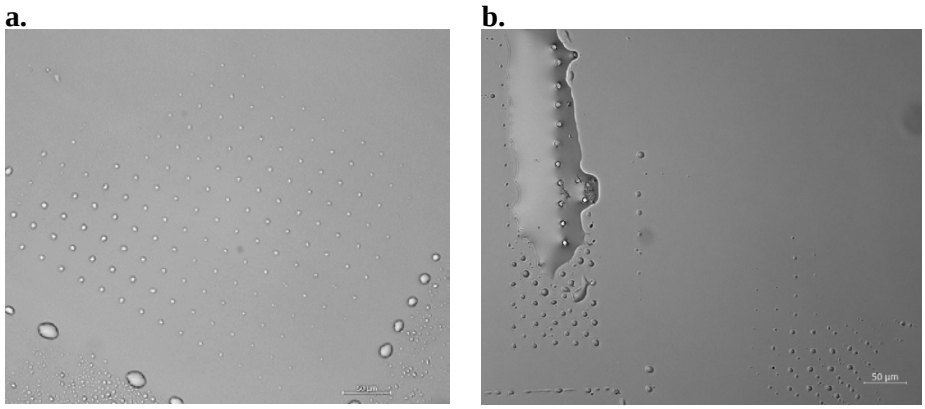

Figure 31. Testing µCP PDA using various conditions. In image a. PDA is patterned on PEGylated glass using no weights during stamping. The stamp has been plasma treated using 100% generator power. PDA was left to incubate on the stamp for 15 minutes. In image b. the stamp was plasma cleaned with 50% generator power, PDA was incubated on the stamp for 5 minutes and the PEGylated glass was placed on the stamp under weights for 10 minutes. Image a. was obtained using 40x lens and image b. was obtained using 20x lens on a Zeiss Axiobserver Z.1 microscope.

It seems that the cause for the pillars to break off the stamp may be resulting from plasma treatment, as stuck PDMS was not observed in the experiments with stamps that did not undergo the treatment. This had not been previously noticed when using PLL-FITC. Possibly when using the fluorescence microscopy, the stuck pillars would not be clearly detectable. However, nice droplet arrays were more often obtained when using plasma treated stamps. If the plasma treatment is considered necessary, it is worth keeping the contact between the stamp and the glass surface shorter and trying the microcontact printing step without using weights. Moreover, it is advisable to follow the recommendation

from chapter 3.1.3 Microcontact printing (μCP) to isolate the stamp area which will be subjected to plasma treatment.

A few optimization steps were introduced to the initial protocol when stamping PDA. Firstly, the PDA incubation time was extended to 30 minutes to allow the PDA to polymerize on the stamp. An additional step to clean the PDMS stamp using tape before applying the polymers was used. Moreover, all subsequent PDMS curing processes were performed at 4 h at 65 °C to be certain of PDMS being cured. After these changes no stuck PDMS pillars were detected on the glasses (even when using plasma treated stamp) and clear patterns of PDA droplets were observed when inspecting the surfaces with AFM (Figure 32). The height maps usually showed approximately the same height as shown in PDA topograph by Arnfinnsdottir, *et al*. (2015), staying around 5-10 nm, indicating that the deposition of chemicals in both experiments was approximately the same [6]. The protocol of stamping PDA was thereby optimized; however, sometimes varying patterns were still observed.

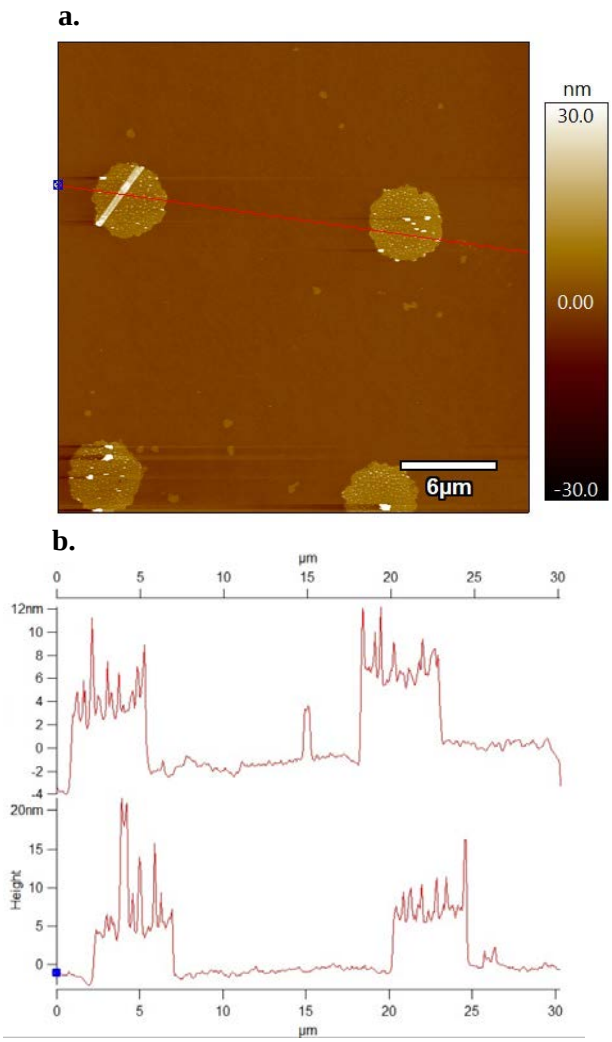

Figure 32. Height map and graph of patterned PDA surfaces in AFM. PDA array was made using non-plasma treated and tape-cleaned stamp. The design of 6 µm circles with 12 µm spacing was used in photolithography to fabricate the master mould for this PDMS stamp. PDMS was cured in 4 h at 65 °C. The top two patches in image a. correspond to the bottom graph in image b. A red line is shown where the height of the features was measured. Image was captured by Nina Bjørk Arnfinnsdottir using AFM Cypher (Asylum Research).

Tris buffer is often reported as the solution of choice for polymerizing dopamine hydrochloride and was therefore also used in this thesis. However, a recently published study indicates that polydopamine polymerized in Tris buffer might have antimicrobial properties against *E. coli* [37]. Alternative solvents, e.g. PBS, NaOH, or $NaHCO_3$, can be used for dopamine polymerization, although all of them also show antimicrobial effect [37]. In short term, this antimicrobial activity of PDA might not be significant, as it has been used to adhere bacteria to AFM tip for an hour without observing any alterations in viability of the bacteria [51]. The antimicrobial effect might also be dependent on the roughness of PDA coatings, as roughened surfaces seem to possess enhanced bactericidal effects [52]. The concentration used in our experiments is smaller compared to the ones used in all above-mentioned publications, possibly resulting in less stress caused to cells. Nevertheless, to avoid potentially harming the bacteria, finding another chemical for microarray fabrication can be considered.

3.2.3 μCP poly-L-lysine on PEGylated and non- PEGylated surfaces

As obtaining regular arrays with PLL-FITC was achieved, PLL without a fluorescent marker was also tested for patterning surfaces. PLL is a polycationic polymer which can adhere to negatively charged glass. PLL can facilitate electrostatic binding of negatively charged bacteria to the surface via its positively-charged sites. [26, 53] In our experiments, PLL with high molecular weight was used (150,000-300,000 Da) which provides more attachment sites per molecule compared to lower weight versions [54].

When conducting experiments with PLL, patterns which looked like outlines of circles were observed (Figure 33).

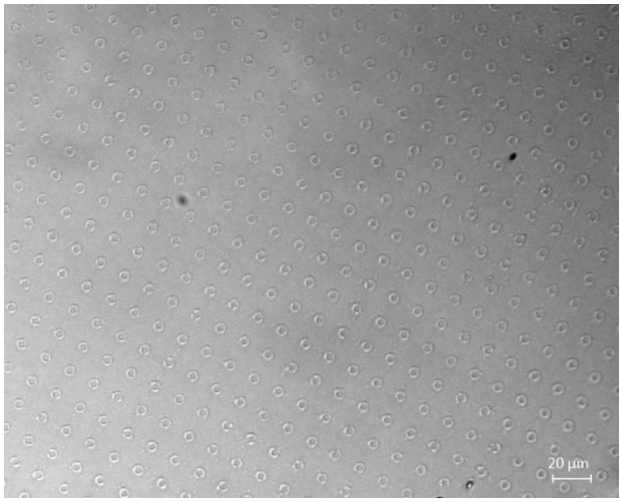

Figure 33. Microcontact printed PLL on PLL-PEG covered glass. Single area of patterns could be observed, as all the rest of PDMS stamp got stuck on the glass. Stuck pillars occurred possibly because the plasma treatment (50% O_2, 100% generator power) was used on the stamp. Image was obtained with 40x objective on a Zeiss Axiobserver Z.1 microscope

These types of circles were also visualized when PDA was used for functionalizing surfaces. The patterns seem to occur regardless of the PEGylation of the surfaces and plasma treatment as with PDA this was visible when using non-plasma treated stamp. The cause of this type of pattern is unknown. Additional observation was made during the same experiment: the stuck pillars from the plasma treated stamp were noticed on the glass. As this was also noticed with PDA, it indicates that the phenomenon probably does not depend on the patterned chemical, but the modifications introduced by the treatment.

Generally, the patterns of PLL were less often visible compared to PDA. Regular droplet patterns were obtained only when plasma treated stamp was used (Figure 34), however, the patterns were very variable in different areas containing a nice small array of droplets in one region, but microdroplet aggregates in the other.

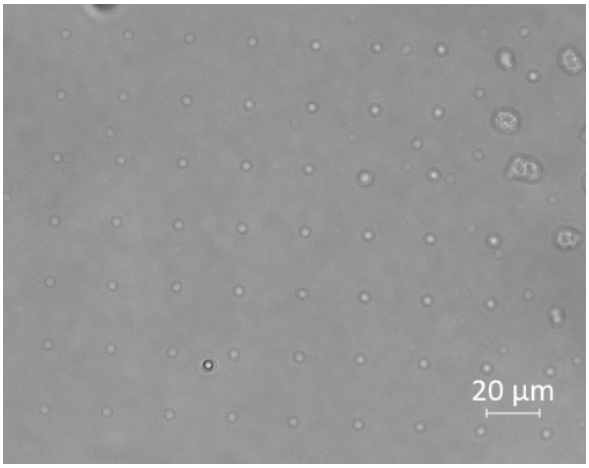

Figure 34. Microcontact printed pattern of PLL. The stamp used for microcontact printing was plasma treated (50% O_2, 100% generator power). The stamp was based on the design containing 7 µm circles with 16 µm spacing. Image was obtained using 20x lens on a Zeiss Axiobserver Z.1 microscope

Overall, visualizing microcontact printed PLL with brightfield microscope was rather challenging as the array of PLL was difficult to find and observe. Based on PLL-FITC experiments, it is probable that PLL is printed, but just not distinguishable with regular light microscope. Using AFM imaging could help to detect the presence of patterns. If PLL is not deposited, then optimization is possible e.g. using

solution with higher pH, e.g. pH=11, which has shown to induce strong adsorption of PLL on various surfaces producing smooth and uniform layers [27] Alternatively, Poly-D-Lysine (PDL) which is also used for promoting cell adhesion to solid substrates could be tested [53]. Considering other chemicals might also be relevant, as PLL's antimicrobial activity has previously been reported [30]. The authors, however, kept PLL in contact with bacteria for 24 hours and used lower molecular weight PLL. Using PLL concentrations that are lower than minimum inhibitory concentration (e.g. lower than 0,153 mg/mL for *E. coli*) might keep the bacteria unharmed. Also, short-term attachment might still plausible, even if higher concentrations are used. It should be investigated if immobilized PLL is equally antimicrobial compared to PLL free in solution. In the article, it was speculated that antimicrobial activity is due to lysine being basic amino acid that enables it to establish electrostatic interactions with acidic microbial cell surfaces disrupting their membrane. The positive charge of the molecule is thought to be important in antimicrobial activity [55]. If this is the case, all positively charged molecules might be imperfect to be used for extensive periods.

3.2.4 μCP mucin on glass surfaces

As the viability of bacteria immobilised onto poly-L-Lysine (PLL) can potentially be reduced and polydopamine might have antimicrobial effects, an alternative to these immobilisation approaches was considered. Mucin was thought as an interesting alternative, since bacteria are known to adhere to mucosal surfaces [42]. However, mucin deposition on glass surfaces proved to be unsuccessful, as only one

small area from the whole stamp was patterned (Figure 35). This might be due to the mucin and glass being both negatively charged creating repulsive forces towards each other. In addition, the peptide backbone of mucin is hydrophobic preferentially adhering to hydrophobic surfaces [56]. However, as the microscopic glass slides are hydrophilic, the adhesion of mucins might be poor [57]. Further experiments should be done to find out if mucin is suitable for microcontact printing. Alternative substrates that have been shown to adhere mucins, e.g. silicon, could be used [56]. However, as glass is cheap and commonly used in labs for microscopy, experiments with an aim to improve mucins' adhesion to glass surfaces should be performed. Improved adhesion can, for example, be achieved by making the glass surface more hydrophobic using specific chemicals, such as fluorosilanes. [57]

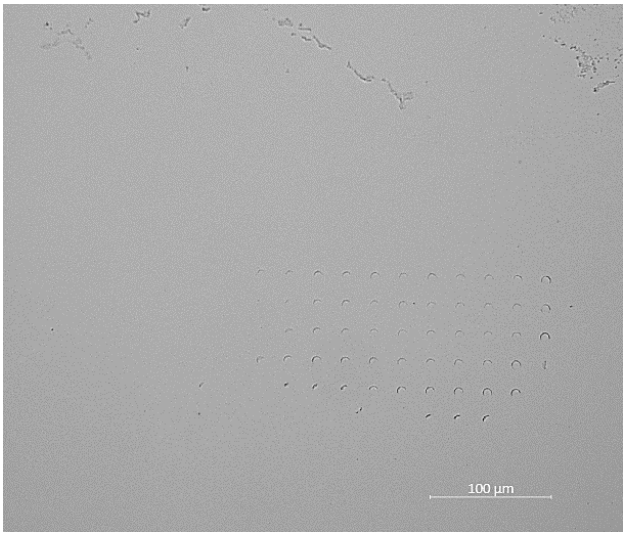

Figure 35. Mucin deposition on glass surface. Vague pattern can be observed right side of the figure. Image was obtained with 20x objective on a Zeiss Axiobserver Z.1 microscope

When bacterial solution was put on top of small patterned surface, no adhesion in an organized manner was observed. Moreover, the pattern previously visualized was lost, possibly due to the washing step removing the patterns. It was discovered, that bovine submaxillary mucin coatings have been actually shown to reduce bacterial adhesion rendering it imperfect for bacterial microarray fabrication [56, 58]. This might occur due to the mucin-bound glycan structures, as de-glycosylated mucins seem to lose their ability to repel bacteria [58]. There are, however, other types of mucins and mucin structures, which facilitate bacterial adhesion instead of preventing it (Examples reviewed in [42]). In the future experiments, microarrays containing specific

components of the mucin that are known to bind bacteria, e.g. Galβ1-3GlcNAc could be used instead of bovine submaxillary mucin to adhere bacteria [42].

3.2.5 Immobilisation of bacteria to functionalized surfaces

In addition to obtaining patterned surfaces with bacteria-adhering chemicals, bacteria must be immobilised on top of the patterns. For this, experiments with different conditions were done with the aim of successfully fabricating bacterial microarrays.

The bacteria grown overnight in LB medium were incubated on top of pattern covered glasses (PEGylated and non-PEGylated). Immobilising *Escherichia coli* or *Pseudomonas putida* on PLL proved to be unsuccessful, as no patterns were visible after incubation for 30 minutes. When immobilising *E. coli* on PDA, small areas of patterns were observed, however, in most of the areas no pattern with bacteria was detected (Figure 36).

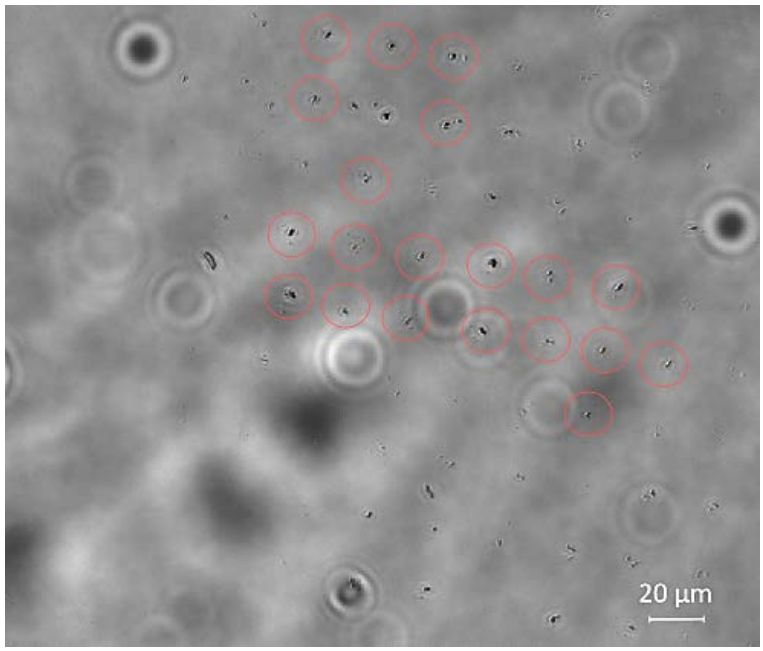

Figure 36. Patterns visible after *Escherichia coli* was immobilized on PDA array. Image was obtained with 20x objective on a Zeiss Axiobserver Z.1 microscope.

Immobilising *Pseudomonas putida* on top of PDA was tested to replicate the results reported by Arnfinnsdottir, *et al.* (2015); however, no arrays were obtained [6]. Sometimes the pattern could be visible under the bacteria, but no pattern-specific attachment was noticeable (Figure 37).

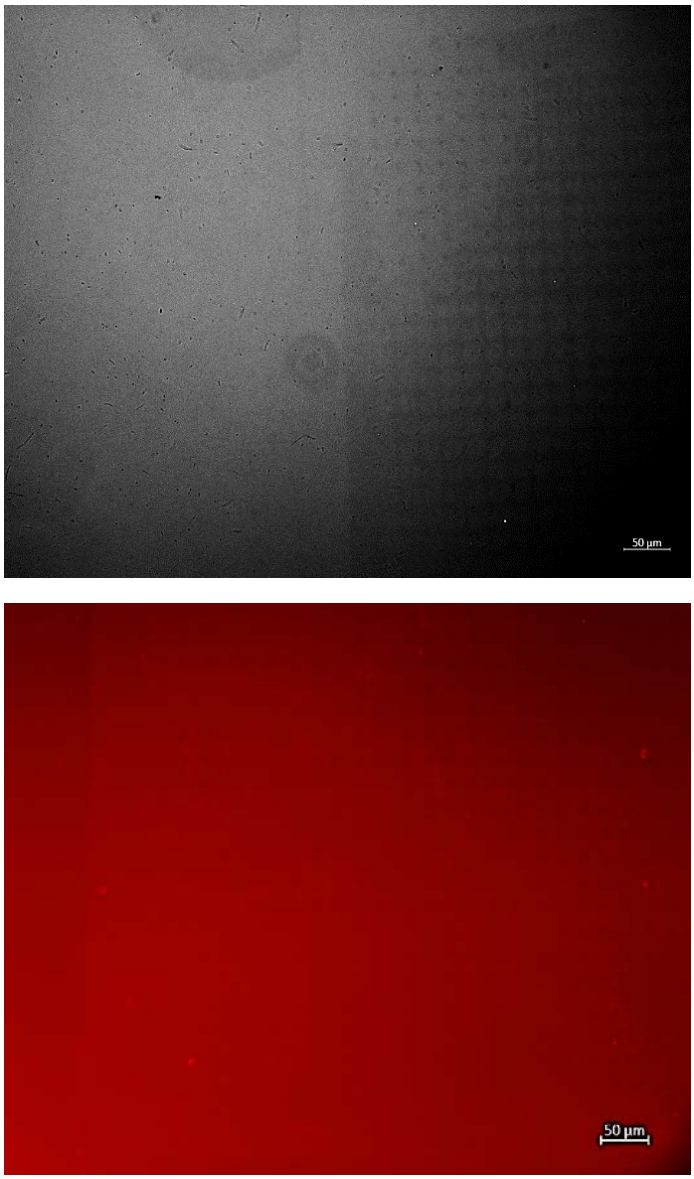

Figure 37. Pseudomonas putida on top of PDA patterned on glass (top) and PEGylated glass (bottom). Even when the pattern is visible, no specific adhesion to the spots was observed. Images were obtained using 20x lens on a Zeiss Axiobserver Z.1 microscope. mCherry filter was used for image in the bottom.

It is hard to track what could be the reason for this, because the same bacteria-adhering chemical, the same strain of bacteria and generally the same procedure for deposition of PDA and incubation of bacteria was used. Some differences were observed in the protocol, e.g. different design of the stamp. In our case, the lateral dimensions of the pillars were wider and distances between features were smaller. Also, different fluorescent protein and plasmid were used to express fluorescence. As the sizes of the bacteria-adhering chemical patches are larger in our experiments, more bacteria should be able to adhere to surface. Therefore, it is not certain that this is the reason why the array fabrication was unsuccessful. It is also unlikely that the plasmid is affecting the surface properties of the bacteria so much that there is almost no adhesion at all to functionalized patches. Some detail in the protocol might be crucial to succeed in bacterial microarray fabrication, however, it is hard to track which one, as not all elements of the method are usually covered in the articles.

As patterning using microcontact printing proved to be difficult, experiments where bacteria adhering chemicals were deposited on glass and PEGylated surfaces without using PDMS stamp were conducted (Figure 38). Comparing cleaned glass to cleaned PEGylated glass showed that PEGylation is successful in passivating the surface. During previous experiments using microcontact printing the anti-adhesive effect of PLL-g-PEG compared to glass was clearly visible as well (Figure 37). With mucin, very few bacteria were observed both on glass and PEGylated surfaces, which made the imaging difficult, thus there are no pictures shown. In the future, experiments with specific

components of mucins may help to discover a more suitable alternative for bacterial adhesion.

PLL covered glass and PLL covered PEGylated glass had significantly higher number of bacteria compared to the rest of the surfaces. It is visible that bacteria adhere to PLL when it is deposited on top of PLL-g-PEG. However, as PLL can have an antimicrobial effect in some concentrations, it might be better to focus on improving the protocol using other bacteria-adhering chemicals.

Experiments with PDA showed fewer bacteria to be on top of the surfaces than expected. No obvious differences were visible between glass and PEGylated glass. Optimization in the stamp cleaning and surface washing procedures might be needed. It was tested to not to dry PDA with N_2 and wash the bacteria off after the incubation step with 1 ml of MilliQ instead of 2 ml. They both resulted in a greater number of bacteria on top of surfaces – quantitively more comparable to the ones observed in PLL experiments.

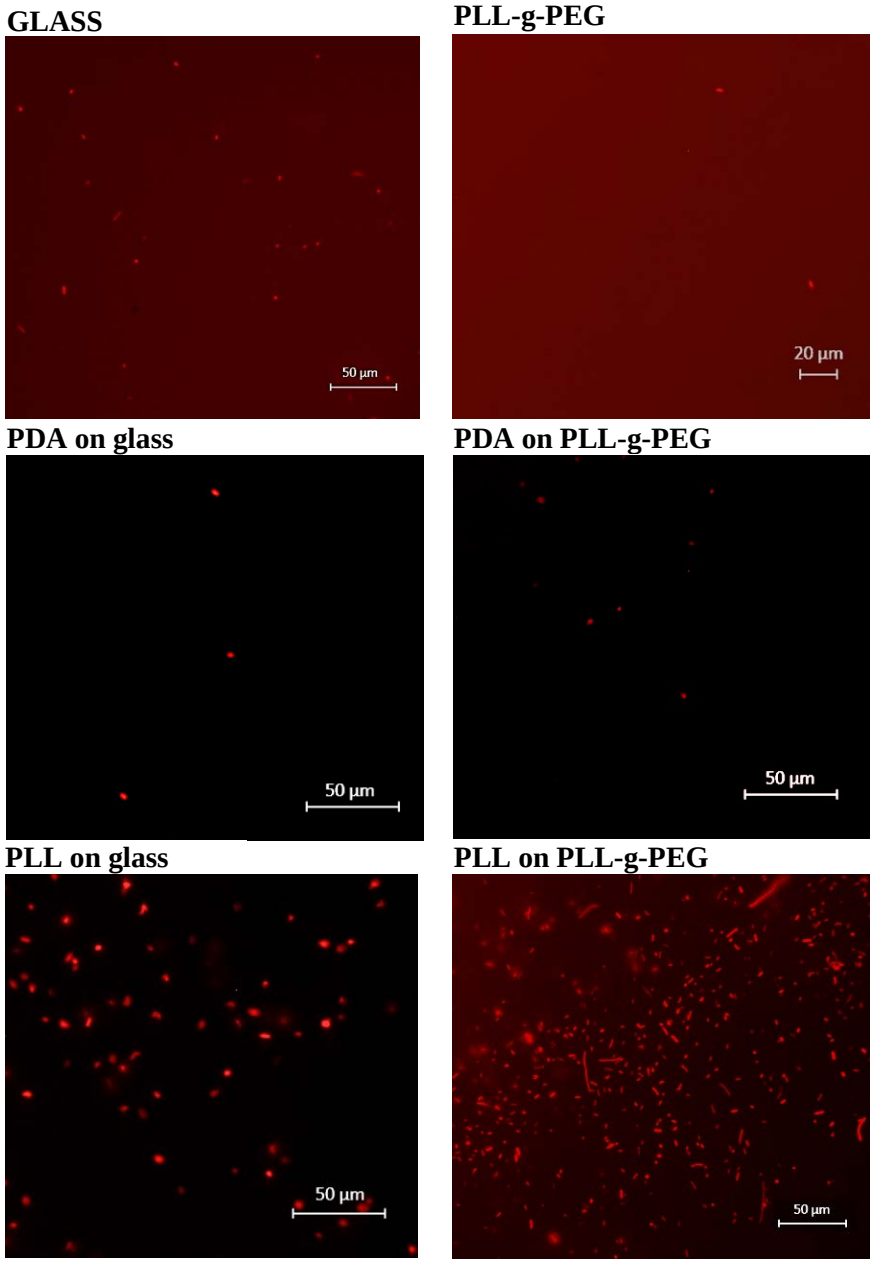

Figure 38. Comparing *Pseudomonas putida* adhesion on differently functionalized surfaces on glass and PEGylated glass. Images were obtained using 20x lens and mCherry filter on a Zeiss Axiobserver Z.1 microscope.

How do the changes in the protocol used for depositing PDA affect the bacterial array formation? This question is left to be answered in the future. Nevertheless, recommendations are made to start to test microcontact printing from fabricating stamps containing lines not patches to enlarge the area that bacteria can adhere to. This makes the optimization of the protocols easier and enables to establish conditions where bacteria adhere to desired locations.

3.3 Overall evaluation of the method

The procedure of fabricating bacterial microarrays is a multi-step process. First, a pattern needs to be designed which will be the basis for the master fabricated using photolithography techniques. The master is then used as a basis to produce PDMS stamp. The stamp is subsequently used for microcontact printing to achieve an array of patches with the material of interest. The final steps include immobilisation of bacteria on the arrayed patches and visualization of the bacterial microarray under the microscope. Each of these steps contains many sub-parts which may need optimization based on the final desired outcome. It can be argued whether the method is of high throughput, simple, applicable, cheap and reproducible.

PDMS stamp production step still relies on using the master mould generated with photolithography techniques, it makes the method dependent on cleanroom availability. The issue with photolithography is that it requires very specific machinery and conditions which exist mostly in cleanrooms and are not common in many labs. This might limit the applicability of the method, as the cleanrooms are not accessible in all universities around the world. However, cleanrooms have already become more and more common, and thus also more available for biologists.

Another problem to tackle in photolithography is that many methods need optimization and it may take some time to get used to the equipment and protocols, as there are many rules to follow when working in a cleanroom. In addition, special training is needed for the use of cleanrooms and machines. The protocols are greatly based on

experience and they are sometimes hard to find online. In addition to the method being time consuming, it can be rather pricey. For example, in Nanolab there is an hourly rate of 350-950 NOK (450-1300 NOK for industrial users), which can make the use of the cleanroom and the method unreachable or unattractive for many projects. However, it is possible to order a master from the cleanroom in Nanolab and possibly from other cleanroom facilities avoiding extended hours for development processes. If the master is available, the fabrication tasks could be moved outside a cleanroom, as microcontact printing and moulding can be done without using special facilities.

It is stated that the bacterial microarray can be a high throughput method with small sample volumes [6]. Usually during the experiments, 400 (20x20) or 1600 (40x40) pillars per square were fabricated depending on which design was used for the master and PDMS stamp production. Furthermore, 6-10 squares per PDMS stamp were most commonly used for microcontact printing, resulting in up to 16,000 potential bacteria adhering spots per experiment. It took approximately 10 µl of bacteria-adhering chemical solution to cover the squares if the samples were plasma cleaned, and around 100 µl if they were not. Thus, the method has the potential to investigate a high number of bacterial cells per experiment in a controlled manner. Theoretically, the number of features in an array can be increased, which can result in a higher number of potential bacteria-adhering patches for immobilization. However, as it was sometimes difficult to fabricate the array of chemicals and even more challenging to immobilize bacteria on the bacteria-adhering chemical spots, the high-throughput single cell array remains an ambitious goal for the future. Even when the challenge of bacterial

adhesion to specific spots is tackled, multiple cells adhering to one spot might still occur [6].

The overall robustness, repeatability and reproducibility of the method is at the present rather poor and further optimization is thus needed. As the microarray production is a multistep process, doing one mishap can spoil the whole experiment. It is important to acknowledge that the issues faced in primary steps (like comets on the resist-covered wafer or exposure problems with MLA) carry on to PDMS and can therefore affect microcontact printing. Furthermore, it is essential to carefully handle the stamp as it is quite sensitive to small changes. Small details, e.g. the shape of weights, i.e. if weights are flat or hollow on the bottom, can ruin the stamping. Hollow weights are harder to firmly place on top of the glass, as they have the tendency to fall off, resulting in contact problems between the stamp and the surface. If the contact between the stamp and the glass is not stable, then it might ruin the stamping procedure, causing multiple patterns to be arrayed near each other. Sensitivity of the stamp also affects repeatability of the method, as the results may vary from experiment to experiment or even when two of the same experiments are run parallel with each other. An experiment with a single stamp can have variable results in different regions around the patterned area, e.g. droplets in one region and spots in another. Every now and then the PDMS gets stuck on the surface. Occasionally there are micro droplet aggregates or circular liquid patterns in stamped areas, in other times there are single droplets, sometimes there are no droplets visible at all. It can be quite challenging to detect the cause for these conditions, as they can occur in the areas close to each other where similar conditions have been applied during the stamping process.

It is also hard to track if the use of plasma treatment on PDMS stamp is necessary. A regular array of droplets with PDA or PLL was obtained when using a stamp that had been treated with 50% O_2 and 50% or 100% generator power. The regular array was not obtainable for PLL without plasma treatment. However, it is not clear if an array of droplets is necessary to obtain bacterial microarrays or spots with smaller deposition of bacteria-adhering chemical are enough. Furthermore, plasma treatment equipment is not usually part of common lab equipment, thus it might be reasonable to develop the method without using the plasma cleaning step. As not many successful bacterial arrays were fabricated, finding out if plasma treatment is needed and, if the droplets are essential for bacterial array production will be the aim of future experiments.

The method might need optimization when different bacteria-adhering chemicals or bacterial species are used. Also, optimization is needed for the washing protocol, as it can wash away the bacteria-adhering chemicals and bacteria. In addition, the materials used for adhesion can have antimicrobial effects, so it is important to find new chemicals or test different concentrations and conditions, which promote adhesion without potentially harming bacteria. It is also interesting to continue experiments with both *Escherichia coli* and *Pseudomonas putida*, as both have been immobilised into an array – *E. coli* in our experiments and *P. putida* by Arnfinnsdottir, et al. [6]. In the future, it is recommended to start testing new chemicals using lines instead of patches to enlarge the area that bacteria can adhere to, making sure that microcontact printing works as expected.

4 Conclusion and future perspectives

The main objective of this master project was to prepare master moulds and PDMS stamps which could be used for microcontact printing. In addition, the aim was to develop and characterize chemical arrays to fabricate bacterial arrays, continuing the work initiated by Karen Dunker and Aurora Resell. The master moulds and the PDMS stamps were produced using lithographic methods present in the NTNU Nanolab. The stamp was then used to microcontact print PLL-FITC, PLL, PDA, and mucin onto PEGylated and non-PEGylated glass surfaces. Bacterial microarrays were then fabricated by incubating bacteria on PLL, PDA, or mucin functionalized patches. PDMS stamps, functionalized surfaces, and bacterial microarrays were characterised using different microscopy methods, including scanning electron microscopy, light microscopy, and atomic force microscopy.

Testing and optimization of the protocol for using new photoresist in the Nanolab, mr-DWL 5, was part of this master thesis. mr-DWL 5 proved to be a suitable resist to work with, as the master mould production was sufficient to generate functional PDMS stamps. The PDMS stamps were successfully used to pattern PLL-FITC. Plasma treating the stamp once or twice did not affect the obtained patterns of the fluorescent dye. Several plasma treatments, however, might have long term effect on the stamp surface affecting the patterns.

PEGylation of the glass surfaces to generate an anti-adhesive layer was achieved, as the number of bacteria attached to the passivated surface was reduced compared to untreated glass. Depositing bacteria-adhering chemicals was somewhat accomplished with PDA and PLL, as regular

patterns were obtained, however, the stamped patterns were inconsistent and the detection of the issues occurring during the stamping proved to be challenging. Plasma treating PDMS stamps made the application of molecular inks easier, but slightly extended the drying step. In addition, the patterns of PDA and PLL contained the most regular droplets when a plasma treated stamp was used for microcontact printing. However, as the microcontact printing step was very sensitive to small movements, it is difficult to say if the patterns were uniform due to the plasma treatment or due to no movements occurring during the stamping process. Moreover, the treatment might have been the cause for the pillars to break off. It is therefore advisable to reduce the contact time, to not use such heavy weights and to continue curing the PDMS 4h at 65 degrees when plasma treating the stamp is necessary.

Experiments with mucin were rather unsuccessful, as very few patterns were obtained. No pattern specific attachment was observed. In the future, alternative mucins or specific compounds of the mucin known to adhere bacteria could be tested.

It is advisable to continue using mr-DWL 5 photoresist following the recommendations proposed in this thesis and to silanize the wafer to ease the peeling of PDMS from the master mould. Degassing the pre-polymer mixture for 5 minutes is enough to eliminate most of the bubbles – the rest can be removed by using manual air pump. Curing PDMS pre-polymer for 4 h at 65 degrees is recommended to avoid the breaking of the pillars. Using flat-bottom weights makes it easier to obtain a stable contact between the substrate and the stamp and is therefore recommended for the stamping step.

Although multiple masters as well as functional stamps from them were obtained, it was not possible to repeatedly fabricate bacterial microarrays even though various conditions were tested. Pattern specific attachment occurred only once, using *Escherichia coli* on polydopamine. However, in most of the experiments no adhesion with any of the used bacteria-adhering chemicals was observed, even when the pattern of the chemical was visible. Optimization for microcontact printing and bacterial incubation steps is still needed in order to obtain functional bacterial microarrays.

References

1. Microresist. *Negative Tone Photoresists mr-DWL*. 2013 [cited 18.02.2018; Available from: https://cmi.epfl.ch/photo/photo_process/files/mr-DWL_en_102013.pdf.
2. B. Alberts, et al., *Molecular Biology of the Cell, Sixth Edition*. 6th ed. 2014, New York and Abingdon, UK: Garland Science. 1464.
3. B. F. Brehm-Stecher and E. A. Johnson, *Single-cell microbiology: tools, technologies, and applications.* Microbiol Mol Biol Rev, 2004. **68**(3): p. 538-59, table of contents.
4. E. Y. Bridson and G. W. Gould, *Quantal microbiology.* Letters in Applied Microbiology, 2000. **30**(2): p. 95-98.
5. D. S. Chen and M. M. Davis, *Molecular and functional analysis using live cell microarrays.* Curr Opin Chem Biol, 2006. **10**(1): p. 28-34.
6. N. B. Arnfinnsdottir, et al., *The Design of Simple Bacterial Microarrays: Development towards Immobilizing Single Living Bacteria on Predefined Micro-Sized Spots on Patterned Surfaces.* PLOS ONE, 2015. **10**(6): p. e0128162.
7. R. Iino, et al., *Design of a large-scale femtoliter droplet array for single-cell analysis of drug-tolerant and drug-resistant bacteria.* Frontiers in Microbiology, 2013. **4**: p. 300.
8. G. M. Wallraff and W. D. Hinsberg, *Lithographic Imaging Techniques for the Formation of Nanoscopic Features.* Chem Rev, 1999. **99**(7): p. 1801-1822.
9. Microchemicals. *Exposure of Photoresists*. 2013 [cited 18.02.2018; Available from: https://www.microchemicals.com/technical_information/exposure_photoresist.pdf.

10. Z. Cui, *Nanofabrication: Principles, Capabilities and Limits, Second edition*. 2017, Switzerland: Springer Nature. 432.
11. S. Diez. *The next generation of maskless lithography*. in *SPIE OPTO*. 2016. SPIE.
12. R. A. Cone, *Barrier properties of mucus.* Adv Drug Deliv Rev, 2009. **61**(2): p. 75-85.
13. K. Bell. *Photoresist edge bead removal processes*. 2017 [cited 15.02.2018; Available from: https://www.doeingalls.com/photoresist-edge-bead-removal-processes/.
14. Microchemicals. *Lithography Trouble Shooter*. 2012 [cited 09.05.2018; Available from: https://www.microchemicals.com/technical_information/TroubleShooter_EN.pdf.
15. H. J. Levinson, *Principles of Lithography, Third Edition*. 2011, Bellingham, Washington, USA: SPIE. 526.
16. T. Besson. *Maskless photolithography with the MLA150*. [cited 18.02.2018; Available from: https://www.himt.de/files/Application%20Notes/Maskless%20photolithography%20with%20the%20MLA150.pdf.
17. Heidelberg. *Maskless aligner*. [cited 18.02.2018; Available from: https://www.himt.de/index.php/MLA150.html.
18. D. Qin, Y. Xia, and G. M. Whitesides, *Soft lithography for micro- and nanoscale patterning.* Nature Protocols, 2010. **5**: p. 491.
19. Younan Xia and George M. Whitesides, *SOFT LITHOGRAPHY.* Annual Review of Materials Science, 1998. **28**(1): p. 153-184.
20. E. Mele and D. Pisignano, *Nanobiotechnology: Soft Lithography*, in *Biosilica in Evolution, Morphogenesis, and Nanobiotechnology: Case Study*

Lake Baikal, W.E.G. Müller and M.A. Grachev, Editors. 2009, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 341-358.
21. Dow Corning Corporation. *Sylgard® 184 Silicone Elastomer*. 2014 [cited 25.04.2018; Available from: http://igem.org/wiki/images/2/29/T--Technion_Israel-HardwarespecsPDMS.pdf.
22. M. H. Madsen, et al., *Accounting for PDMS shrinkage when replicating structures.* Journal of Micromechanics and Microengineering, 2014. **24**(12): p. 127002.
23. G. Csucs, et al., *Microcontact printing of novel co-polymers in combination with proteins for cell-biological applications.* Biomaterials, 2003. **24**(10): p. 1713-20.
24. C. D. James, et al., *Patterned Protein Layers on Solid Substrates by Thin Stamp Microcontact Printing.* Langmuir, 1998. **14**(4): p. 741-744.
25. A. Perl, D. N. Reinhoudt, and J. Huskens, *Microcontact Printing: Limitations and Achievements.* Advanced Materials, 2009. **21**(22): p. 2257-2268.
26. S. Rozhok, et al., *Methods for fabricating microarrays of motile bacteria.* Small, 2005. **1**(4): p. 445-51.
27. J-H. Choi, et al., *Influence of pH and Surface Chemistry on Poly(l-lysine) Adsorption onto Solid Supports Investigated by Quartz Crystal Microbalance with Dissipation Monitoring.* The Journal of Physical Chemistry B, 2015. **119**(33): p. 10554-10565.
28. G. L. Kenausis, et al., *Poly(l-lysine)-g-Poly(ethylene glycol) Layers on Metal Oxide Surfaces: Attachment Mechanism and Effects of Polymer Architecture on Resistance to Protein Adsorption.* The Journal of Physical Chemistry B, 2000. **104**(14): p. 3298-3309.

29. D. Mazia, G. Schatten, and W. Sale, *Adhesion of cells to surfaces coated with polylysine. Applications to electron microscopy.* The Journal of Cell Biology, 1975. **66**(1): p. 198.
30. M. Conte, et al., *Antimicrobial activity of various cationic molecules on foodborne pathogens.* World J Microbiol Biotechnol, 2007. **23**(12): p. 1679-83.
31. K. Colville, et al., *Effects of poly(L-lysine) substrates on attached Escherichia coli bacteria.* Langmuir, 2010. **26**(4): p. 2639-44.
32. ThermoFisher. *Fluorescein (FITC).* [cited 24.01.2018; Available from: https://www.thermofisher.com/no/en/home/life-science/cell-analysis/fluorophores/fluorescein.html.
33. SuSoS. *SuSoS PLL-g-PEG Polymers.* [cited 23.01.2018; Available from: https://susos.com/en/beschichtungstechnologien/pll-g-peg-polymere/.
34. B. Yu, et al., *Pdop layer exhibiting zwitterionicity: a simple electrochemical interface for governing ion permeability.* Chem Commun (Camb), 2010. **46**(32): p. 5900-2.
35. Zafar Iqbal, Edward P. C. Lai, and Tyler J. Avis, *Antimicrobial effect of polydopamine coating on Escherichia coli.* Journal of Materials Chemistry, 2012. **22**(40): p. 21608-21612.
36. Y. H. Ding, M. Floren, and W. Tan, *Mussel-inspired polydopamine for bio-surface functionalization.* Biosurface and Biotribology, 2016. **2**(4): p. 121-136.
37. K. Patel, et al., *Polydopamine films change their physicochemical and antimicrobial properties with a change in reaction conditions.* Physical Chemistry Chemical Physics, 2018. **20**(8): p. 5744-5755.
38. S. K. Linden, et al., *Mucins in the mucosal barrier to infection.* Mucosal Immunol, 2008. **1**(3): p. 183-97.

39. K. Hori and S. Matsumoto, *Bacterial adhesion: From mechanism to control.* Biochemical Engineering Journal, 2010. **48**(3): p. 424-434.
40. I. Schwartz and G. P. Wormser, *Bacterial pathogenesis: a molecular approach.* Clinical Infectious Diseases, 2002. **35**(5): p. 638-639.
41. R. R. Hill, H. M. Cowley, and A. Andremont, *Influence of colonizing micro-flora on the mucin histochemistry of the neonatal mouse colon.* Histochem J, 1990. **22**(2): p. 102-5.
42. J-F. Sicard, et al., *Interactions of Intestinal Bacteria with Components of the Intestinal Mucus.* Frontiers in Cellular and Infection Microbiology, 2017. **7**(387).
43. H. Wu, et al., *Adhesion of Pseudomonas putida onto kaolinite at different growth phases.* Chemical Geology, 2014. **390**: p. 1-8.
44. UniProt. *Proteomes - Escherichia coli (strain B / BL21-DE3)*. 2018 [cited 03.05.2018; Available from: http://www.uniprot.org/proteomes/UP000001509#proteome-info.
45. K. Rogers and R. J. Kadner. *Bacteria*. 2018 [cited 03.05.2018; Available from: https://www.britannica.com/science/bacteria/Diversity-of-structure-of-bacteria.
46. John Innes Centre. *What is Light Microscopy?* [cited 14.02.2018; Available from: https://www.jic.ac.uk/microscopy/intro_LM.html.
47. J. W. Lichtman and J. A. Conchello, *Fluorescence microscopy.* Nat Methods, 2005. **2**(12): p. 910-9.
48. P. J. de Pablo and M. Carrión-Vázquez, *Imaging Biological Samples with Atomic Force Microscopy.* Cold Spring Harbor Protocols, 2014. **2014**(2): p. pdb.top080473.
49. K. Dunker, *Microarrays for High Throughput Analysis of Cellular Heterogeneity*, in *Department of*

Biotechnology and Food Science. 2017, Norwegian University of Technology: Trondheim. p. 61.
50. N. B. Arnfinnsdottir, *Microscale tools for the development of bacterial microarrays*, in *Department of Physics*. 2016, Norwegian Universtiy of Science and Technology: Trondheim. p. 76.
51. A. Beaussart, et al., *Single-Cell Force Spectroscopy of Probiotic Bacteria.* Biophysical Journal, 2013. **104**(9): p. 1886-1892.
52. L. Su, et al., *Strong Antibacterial Polydopamine Coatings Prepared by a Shaking-assisted Method.* Scientific Reports, 2016. **6**.
53. G. Sitterley. *Poly-lysine*. 2008 [cited 23.01.2018; Available from: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/articles/biofiles/volume3.8a12-poly-lysine/Poly-lysine.pdf.
54. Sigma-Aldrich. *Poly-L-lysine solution*. 2018 [cited 12.05.2018; Available from: https://www.sigmaaldrich.com/catalog/product/sigma/p4707?lang=en&region=NO.
55. R. Potter, L. Truelstrup Hansen, and T. A. Gill, *Inhibition of foodborne bacteria by native and modified protamine: Importance of electrostatic interactions.* International Journal of Food Microbiology, 2005. **103**(1): p. 23-34.
56. I. A. Bushnak, et al., *Adhesion of microorganisms to bovine submaxillary mucin coatings: effect of coating deposition conditions.* Biofouling, 2010. **26**(4): p. 387-97.
57. T. Crouzier, et al., *Cell Patterning with Mucin Biopolymers.* Biomacromolecules, 2013. **14**(9): p. 3010-3016.
58. J. Y. Co, T. Crouzier, and K. Ribbeck, *Probing the Role of Mucin-Bound Glycans in Bacterial Repulsion*

by Mucin Coatings. Advanced Materials Interfaces, 2015. **2**(17): p. 1500179.