MASTERARBEIT / MASTER’S THESIS

Titel der Masterarbeit / Title of the Master’s Thesis

„Studies for the Synthesis of MIP-based Sorbents for Bacteria“

verfasst von / submitted by
Julia Perko, BSc

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien, 2018 / Vienna 2018

Studienkennzahl lt. Studienblatt / degree programme code as it appears on
A 066 862
the student record sheet:

Studienrichtung lt. Studienblatt / Masterstudium Chemie
degree programme as it appears on
the student record sheet:

Betreut von / Supervisor: Univ.-Prof. Mag. Dr. Peter Lieberzeit
Figure 1: 1000x magnifications of E. coli ................................................................. 9
Figure 2: Set-up of a chemical sensor as used in this work........................................ 11
Figure 3: (o/w)-Pickering emulsion and (w/o)-Pickering emulsion .......................... 15
Figure 4: Interactions of target with polymer when generating MIPs .................... 17
Figure 5: Imprinting techniques ............................................................................. 18
Figure 6: Removing and rebinding step of MIP particles ........................................ 18
Figure 7: Side view of counting chamber ............................................................... 21
Figure 8: Double net (symbolized by dark squares) Neubauer-improved counting chamber ................................................................. 22
Figure 9: Example of countable (full spots) / non-countable bacteria (empty spots) within one counting net ................................................................. 22
Figure 10: Crystal violet – EtOH/water – Lugol’s iodine – Safranin solution – stained bacteria (circled) .............................................................................. 24
Figure 11: pure E. coli.............................................................................................. 24
Figure 12: E. coli contaminated with fungus ........................................................... 24
Figure 13: Atomic Force Microscope setup and cantilever tip when scanning the sample ......................................................................................... 25
Figure 14: Scanning electron microscope set-up .................................................... 28
Figure 15: Inelastic scattering (left) and elastic scattering (right) processes .......... 29
Figure 16: Images taken with the different detectors at the same sample position; the part on the left shows the Inlense detector image; in the middle a progressive transition from Inlense detector to SE2 detector; the part on the right shows the entire image of a SE2 detector ........................................................................... 31
Figure 17: Gold sputter coater Cressington 208HR ............................................. 32
Figure 18: AIBN radical starter .............................................................................. 32
Figure 19: Chain-growth polymerization of poly-(styrene-co-divinylbenzene) ....... 33
Figure 20: Sieve electrode schemes and printed gold electrodes on quartz wafer ... 34
Figure 21: Measuring cell and network analyzer Agilent 8712ET .......................... 35
Figure 22: Beads from S. epidermidis #69 ............................................................. 45
Figure 22a: Zoomed to the S. epi surface #69 ...................................................... 45
Figure 23: Surface of L. lactis bead #66 ............................................................... 45
Figure 24: Surface of B. cereus bead #66 ............................................................. 45
Figure 25: In-lens picture of UV light treated beads, bright contours with dark fillings circled in black, show removed bacteria imprints, and bright contours with bright content indicate unremoved *E. coli* cells........................................................................................................47
Figure 26: SE2 detector mode image of autoclaved imprinted polymer beads; Cavities of removed *E. coli* cells are circled in black.................................................................50
Figure 27: In-lens detector image from beads treated with a 3% glutaraldehyde solution, cavities of removed *E. coli* bacteria cells are circled in black.................................53
Figure 28: In-lens detector image of NH$_3$ (6%) treated beads, some cavities are circled in black.........................................................................................................................55
Figure 29: In-lens image of H$_2$O$_2$ (3%) treated beads, cavities of *E. coli* cells are circled in black.....................................................................................................................58
Figure 30: SE2 detector image of beads treated with a mixture of 100 mM NaCl and 1% SDS solution, some cavities are circled in white.........................................................60
Figure 31: Stamp surface of 3 % APTES in toluene .........................................................64
Figure 32: Stamp surface of 10 % APTES in ethanol..........................................................64
Figure 33: *E. coli* imprints in styrene and DVB on glass slide........................................65
Figure 34: QCM measurement of various *E. coli* concentrations....................................69
Figure 35: Bacteria-chitosan network.................................................................................70
Figure 36: Bacteria-imprinted polymer (BIP) .....................................................................71
Index of contents

1. Introduction ........................................................................................................................................... 7

2. Aims of the thesis ................................................................................................................................... 8

3. Theoretical background ........................................................................................................................... 9
   3.1 *Escherichia coli* (*E. coli*) ................................................................................................................. 9
   3.2 Gram staining ..................................................................................................................................... 10
   3.3 Chemical Sensors .............................................................................................................................. 11

4. Methods .................................................................................................................................................. 14
   4.1 Pickering Emulsions ......................................................................................................................... 14
   4.2 Molecularly Imprinted Polymers ...................................................................................................... 16

5. Techniques ............................................................................................................................................. 20
   5.1 Bacteria Culturing ............................................................................................................................. 20
   5.2 Bacteria counting ............................................................................................................................... 21
   5.3 Gram staining protocol ..................................................................................................................... 23
   5.4 Atomic Force Microscopy .................................................................................................................. 25
   5.5 SEM – Scanning Electron Microscopy .............................................................................................. 26
      5.5.1 Microscope set-up ..................................................................................................................... 28
      5.5.2 Scattering and operation modes ............................................................................................... 29
   5.6 SEM sample preparation .................................................................................................................... 31
   5.7 Radical Polymerization ..................................................................................................................... 32
   5.8 QCM manufacturing .......................................................................................................................... 34
   5.9 Chemicals, Materials and Instruments ............................................................................................. 35
6. Experiments ................................................................................................................................. 37
   6.1 Synthesis of molecularly imprinted beads ................................................................. 37
   6.2 Synthesis of a NIP polymer bead .................................................................................. 43
   6.3 Experiments with different types of bacteria .............................................................. 44
   6.4 Washing procedure - Removal of template bacteria ...................................................... 46
   6.5 Stamp imprinting ............................................................................................................ 62
   6.6 Modifications of the quartz surface and the polymer .................................................. 66
   6.7 QCM Measurements ....................................................................................................... 68
   6.8 Bacterial imprinting with Pickering emulsion based on N-acrylchitosan (NAC) .......... 70

7. Results and Discussion .............................................................................................................. 73

8. Bibliography ............................................................................................................................. 75

9. Appendix .................................................................................................................................... 83
1. Introduction

Big concerns about the quality of drinking water are present everyday not only in developing countries, but also in industrialized countries due to contaminations with viruses, bacteria and other microorganisms. In the late 19th century Georg Kaffky, coworker of Robert Koch, showed that drinking water contaminated with feces is the main reason for epidemical diseases like cholera, through the bacterium *Vibrio cholerae*. (Drews 2015) It became very important for public health protection to constantly test drinking water and the final effluents of purification plants in the environment. In the 1890s the bacterium *E. coli*, a coliform bacterium, was determined indicator in tests for contaminations because of simplicity and the costs of its detection. *E. coli* can survive up to 12 weeks in water, depending on temperature and nutrients. The first test systems were time consuming and often not specific for the target bacterium, so further progresses needed to be obtained in this field. (Edberg S. C. 2000) Today sensitive devices with quick and precise results are needed. This can be done with systems such as chemical sensors, which achieve sensitive results for water samples. Average mammalian feces contain more than one million *E. coli* cells per gram; they retain the ability to reproduce as long as the environmental surroundings provide a bacteria friendly system. (Pleydell E.J. 2007) Nevertheless *E. coli* is still a big target bacterium which causes severe diseases worldwide, exposure of humans to contaminated water is to be prevented. Therefore, it is necessary to develop analytical systems to detect bacteria in samples with as few false negatives as possible, i.e. providing high sensitivity. Furthermore, those should be stable at changing measuring conditions, fast, cost-effective to produce and use, and simple to handle.

This work shows the efforts of developing selective pre-concentration for *E. coli*, which may make the final analysis more robust and may reduce the required sample amount. For this purpose, the present thesis assesses Pickering emulsions forming molecularly imprinted poly(styrene-*co-*divinylbenzene)-beads.
2. **Aims of the thesis**

Previous works in the topic of bacteria stabilized Pickering emulsions are well established in their production, characterization and use. (Lei Y. 2014), (Firoozmand H. 2016) First goal in this thesis is the removal of the target bacterium *E. coli* from the surface of the imprinted beads. This step has to be completed without attacking the polymer. Selective cavities on the polymer beads are able to rebind to the exact same bacteria strain as used for bead’s creation. Ideally other bacteria will not bind or occupy the holes. In theory removing and rebinding should be indefinitely repeatable. This set-up can be used for separating different bacteria from each other, or separating one bacteria species from a mixture of microorganisms. The second aim is the connection of the system with QCM measurements. Due to changes in frequency an assumption of bacteria concentration can be made.

The use of stamp imprinting in the group and working systems for *E. coli* sensitive measurements with QCMs are established. In this work further stamp imprinting based researches are carried out. Improvements with the working polymer mixture of styrene and divinylbenzene are necessary for adherent MIP layers on quartz microbalances. Another aim in this work is the optimization of already working stamp imprinted QCM measurements. Therefore experiments under various conditions and new chemicals are tested.

Shen et al. use chitosan to produce *E. coli* imprinted polymer beads. (Shen X. 2014) The goal is to reproduce this recipe for use in further experiments with coupled QCM measurements.

All aims are targeted on using bacteria imprinted polymers for selective and sensitive analysis of *E. coli*. 
3. Theoretical background

3.1 *Escherichia coli* (E. coli)

*Escherichia coli*, short form *E. coli*, is one of the most widely investigated bacteria and often used as a model organism due to three non-pathogenic strains, which allow to simulate real conditions in the laboratory without harming researchers. The bacterium is rod shaped, with an average length of 2-6 µm and width of 1-1.5 µm, as can be seen in the light microscope (Figure 1); The thickness to length ratio depends on the strain. (Gangan M.S. 2017)

![Figure 1: 1000x magnifications of *E. coli*](image)

*E. coli* is a facultative anaerobic, fecal coliform, Gram-negative bacterium, which is found in the lower intestines of humans and other mammals, where it produces the fat-soluble vitamin K2. (Marley G. M. 1986) A facultative anaerobic bacterium like *E. coli* can survive and reproduce itself under aerobic and anaerobic conditions. Only Gram-negative, rod shaped, facultative anaerobic bacteria, which break down lactose and produce gases at 44°C are called fecal coliform-like bacteria. (Martin N.H. 2016)

The cell wall of Gram-negative bacteria consists of an inner phospholipid bilayer, followed by the periplasmic space with its thin peptidoglycan layer, lipoproteins that
connect the outer phospholipid membrane with the murein layer, and the outer membrane, which is absent in Gram-positive bacteria. Lipopolysaccharides are forming the outer layer of the outer membrane. Lipopolysaccharides are composed of lipid A, an endotoxin, core-polysaccharides and O-antigens which determine immunogenicity and other characteristics of the bacterium. Lipid A is secreted in vesicles during proliferation or death of the bacterium and plays a role in the immune system, when binding with receptors on macrophages. This can derange coagulation, lysis and inflammatory responses and may cause an acute circularly collapse. (Kalokerinos A. 2005) The pathogenic strains of *Escherichia coli* may cause severe diseases not only through their endotoxins secreted from the outer membrane, but also through exotoxins. Diarrhea is the main sickness caused by *E. coli*. It can lead to infections of the blood circulation, dysfunction of the kidneys till death. (Eaton K.A. 2008)

When working with microorganisms such as bacteria in this work, it’s obligatory to work at certain pH and temperature to ensure ideal conditions for living bacteria.

### 3.2 Gram staining

The bacterium *E. coli* leads to light red color in Gram staining procedures. The red color is an effect obtained from the coloring process, named after the Danish bacteriologist Hans Christian Gram. Gram-negative bacteria appear red and Gram-positive blue after coloring steps with crystal violet, Lugol’s iodine and safranin solution. The varying coloring of Gram-positive and Gram-negative bacteria occurs through the different bacteria cell wall structure. Gram-positive bacteria have a 40 times thicker murein (polysaccharide) layer, which keeps the color of the crystal violet solution. While Gram-negative bacteria have an outer membrane and a less thick polysaccharide layer, which is decolored by the washing steps in the protocol. In a counterstaining step the bacteria sample is stained with a safranin solution, which is only up taken by the colorless Gram-negative bacteria cell wall.
3.3 Chemical Sensors

The setup for chemical sensors, as shown in Figure 2, consists of a sensitive layer, also called receptor, which ideally interacts only with the target analyte, resulting in a change of measurable physical properties. The attached transducer converts this change into an electronical signal, which may be amplified and converted into a processable signal. According to literature, selective recognitions in the receptor layer can lead to a change in either electrochemical or electromagnetic parameters in the transducer. Electrochemical changes are from potentiometric, aperiometric or conductometric nature, while electromagnetic changes result in an optical change. Generally all signals besides mass changing signals are amplified before further processing steps. (Council 1995)

![Figure 2: Set-up of a chemical sensor as used in this work](image)

Chemical sensing is a method to obtain real time information about the chemical composition of a sample. They are usually robust, inexpensive and easy to handle. Their biggest advantage might be portability, which means that the detecting system is brought to the sample. This is for example shown with blood glucose meters to measure the blood glucose level and with environmental measuring stations for air pollution or water contamination. (Baraton M.I. 2004), (Bruen D. 2017) Chemical sensors are able to detect concentrations in the range of $10^{-9}$ molar or less. The sensitivity of sensors is high and is determined by the transducer, while selectivity is based on the properties of the sensitive layer and is a system parameter, which needs to be determined. (Council 1995)
3.3.1 Quartz crystal microbalance - an example for mass sensitive sensors

In this work quartz crystal microbalance (QCM) is used as mass sensitive sensor, to detect an analyte according to its mass. It consists out of a piezoelectric crystal, which generates a shift in frequency when mass is applied. For quartz microbalances a quartz crystal is used to cut out thin microbalance disks in a certain angle depending to the main axis. Most common is the AT cut of quartz (35°15’ inclination in the y-z plane) and for this work only disks with an operating frequency of 10 MHz and a thickness of 167 µm are used. (Huang X. 2017) Piezoelectric materials either work based on direct or inverse piezoelectric effect. The direct mode describes the application of pressure, pull, or other forces to form electrical charges on the crystal surface and to create measurable voltage. In the inverse piezoelectric mode, a voltage is applied to the quartz disk, which results in mechanical deformation. The relation between resonance frequency and mass loading of a QCM in the gas phase is best described by the Sauerbrey equation shown in Equation 1. Here added mass is considered added thickness of the harmonic oscillating quartz microbalance.

$$\Delta f = - \frac{2 f_0^2}{\sqrt{\rho Q \mu Q}} \frac{\Delta m}{A}$$

Equation 1: Sauerbrey equation

The parameter $f_0$ is the resonant frequency. The constants $\rho_Q$ (density of the quartz) and $\mu_Q$ (shear modulus) describe the shear stress deformation of the quartz according to an applied shear strain. $A$, the area of the electrode surface is $A = 0.1257$ cm$^2$ when using a two electrodes system with a radius of around 2 mm of each electrode, as in the system used in this work. (Bard A. J. 2008) Adsorbed mass ($\Delta m$) on a sensitive layer results in a negative frequency shift. The Sauerbrey equation may only be used for gas phase measurements, because changes in density and viscosity of liquid media are not respected in this formula. (Buttry D. A. 1992)
Advantages of this system are its high sensitivity, wide range of measuring different compounds, stability, and inexpensive production. A sensitive layer with interactions to the analyte of interest is spin-coated onto the piezoelectric microbalance surface. Too high mass loading of the coated layer may damp the oscillation of the crystal, which directs to coat thin sensitive layers on the crystal in the usual average range of up to 200 nm according to literature. (Dickert F. 2001) Besides the limit of layer thickness, another limitation for environmental and biological species is given. In Newtonian liquids like water the sensor-liquid interface causes energy loss and affects the output signal. (Janata J. 1989) A frequency decrease depending on the density and viscosity of the liquid solution is observed. For liquid surroundings, the Kanazawa-Gordon equation describes the influence of the solution’s properties when getting in contact with the quartz crystal.

The change in the resonance frequency is shown in Equation 2.

$$\Delta f = - f_0^3 \sqrt{\frac{\rho_L \eta_L}{\pi \cdot \rho_Q \mu_Q}}$$

Equation 2: Kanazawa-Gordon equation

Viscosity and the density of the liquids are named $\eta_L$ and $\rho_L$, respectively. Working in liquid media makes it necessary to control temperature during the measurement, because changes in frequency of around 8 Hz / °C are reported for water. This is caused due to the addition of shear mode oscillations and temperature depending changes of the density and viscosity of the liquid. This fact is mostly negligible when measuring in gas phases. (Stanford Research System)
4. Methods

4.1 Pickering Emulsions

A dispersed liquid in another liquid medium, which are normally not miscible, is called emulsion. This effect is shown in Figure 3 by a mixture of water and oil, either when small oil droplets are formed and surrounded by the continuous phase water – a so called oil-in-water emulsion (o/w) - or when little water drops are encircled by the oil phase- then it is called water-in-oil (w/o) emulsion. Pickering emulsions are stabilized by solid particles, aggregating on the interface between two immiscible liquids. They are highly stable and do not coalesce during extended periods of time. The stability is determined through hydrophobicity and hydrophilicity, the contact angle, shape and size of the particle. To form stable emulsions the particle needs to have a high contact angle to the interface with the dispersed liquid, so that it does not escape the spherical interface in the emulsion. This includes also the hydrophobicity of the particle itself. When the contact angle between the hydrophilic particle and the dispersed oil surface is less than 90°, an oil-in-water emulsion is formed. If the contact angle exceeds 90° at the interface, the system forms a water-in-oil emulsion. The hydrophobic characteristic of the particle leads to a contact angle of higher 90° and strengthens the interface of the liquids to form stable Pickering emulsions. When working with hydrophilic bacteria as a Pickering emulsion template particle, only oil-in-water emulsions are formed. (Kimmins S. 2011)

Best stabilizing conditions are mostly found at a contact angle of 90°. The smaller the solid particle, the smaller are the emulsion droplets; this is one way to control the final drop size.
Experiments showed that higher amounts of stabilizing solid particles lead to smaller emulsion drops, since low amounts can only stabilize small areas of the interface and form larger droplets. Excess particles in the continuous water phase lead to thickening of (o/w)-emulsions, which stabilizes them through higher viscosity and decelerated degradation steps, which may be advantageous. Not only the amount and size of solid particles influence the droplet size, but also the energy input during the emulsification process. Insufficient energy results in small interfacial areas for the particles to cover, only a few form stable emulsions while the rest stays in the dispersing phase. High energy forms too small droplets, which decompose and coalesce when the energy input is turned off. (Chevalier Y. (2013))

Best results are achieved with a mild energy emulsification process like shaking the Pickering emulsion mixture by hand.

Binding between the E. coli cells and the polymer used in this work is assumed to rely on two-step attachment. Initial adhesion is fast, reversible and based on hydrodynamic, electrostatic and physicochemical effects. Reorientation of the cells on the surface to maximize contact, loss of interfacial water, and charge the phobic areas on bacterium’s outer cell membrane and the polymer surface strengthen the
bacterial binding. In addition pili, thin appendages on the outer cell membrane of Gram-negative bacteria, and various proteins increase attachment. (Tuson H.H. 2013)

4.2 Molecularly Imprinted Polymers

Molecular imprinting is a technique, which allows the binding between polymer receptors and target molecules with high affinity and selectivity. The respective monomers and the analyte form interaction networks. After polymerization and removal of the template, cavities remain in the polymeric network. They are not only complementary in size and shape to the target analyte, but also provide binding sites and interaction points for the template molecule. (Uzunab L. 2016) The different colors and shapes of the binding sites in Figure 4 shall symbolize the various interactions between the target molecule and the polymer. The functional monomers interact via covalent, non-covalent and semi-covalent binding with the template in the mixture. Covalent linkage is stable during polymerization, but at the same time it is necessary to cleave that bond under mild conditions without harming the polymer. Useful chemical functionalities for this purpose include acetals, ketals, disulfide bonds and others. Mosbach et al. showed that covalent bonds are not essential for molecular imprinting. (Sellergren B. 1997) Non-covalent interactions such as hydrogen-bonding, hydrophobic or electrostatic interactions and others have the advantage that template removal is more straightforward than for covalently bound molecules. Semi-covalent binding combines the advantages of both methods. While strong and stable covalent bonds are used during polymerization step, the recognition of the rebound molecule takes mostly place via hydrogen-bond formation. (Lee S. 2016)
Literature distinguishes between surface and bulk imprinting, mostly depending on the size of the template. (Hussain M. 2015), (Schnettelker A. 2016) Smaller targets are easily removed after polymerization, while bigger analytes are stuck irreversibly in the polymer after polymerization, leading to unsuccessful removal and hence blocking of cavity formation. Depending on which imprinting technique cavities on or inside the polymer are formed (Figure 5). In bulk imprinting the template is mixed with the monomers, cross linker, initiator and solvent to form the pre-polymer before polymerization and removed with washing steps. Surface imprinting is the favored procedure with big size analytes. This is done either by sedimentation imprinting, where the target is placed on the surface of the polymer and diffuses into the layer during polymerization, or by stamp imprinting. Sedimentation imprinting includes a washing step after polymerization to remove the target without destroying the surface of the polymer. When washing is not complete, some cavities are still occupied with the target analyte. During stamp imprinting a stamp with covalently or non-covalently bound target analytes is pressed into the polymer matrix. After polymerization the stamp is removed and reveals the cavities in the polymer surface. The advantage of covalently bound targets is the complete removal of all analytes from the polymer, while non-covalent stamps cannot guarantee this. (Lieberzeit P. 2014)
As shown in Figure 6, selective rebinding of the target analyte into the polymeric memory is possible and the desired effect of these systems.

MIPs have the advantage of selectively binding the template species used during imprinting. This can be used for pre-concentration, distinguishing concentrations in different sample mixtures, and detecting harmful analytes in biological systems. This leads to the use of MIPs as high selective, artificial sensor layers, which satisfy with a wide resistance against temperature changes, pH and type of used solvent, partly humidity and expanded variation of target analytes. Due to the fact that any kind of
analyte can be used for molecular imprinting turns it into an all-purpose working system.

Biological systems working on a similar principle are antibodies, forming a highly selective complex with their antigen. Disadvantages of high costs, barriers in extraction and purification, heat, salt concentration, pH and more limit their working field.

In this work covalent stamp imprinting was used to generate imprinted, sensitive polymer layers on QCM devices and synthesis of Pickering emulsions with the target analyte *E. coli* as solid particle leads to molecularly imprinted polymer beads. This may also be named bacteria imprinted polymer (BIP).
5. Techniques

5.1 Bacteria Culturing

The non-pathogenic *Escherichia coli* strain ATCC® 9637™ was used as model for all experiments done within this work and commercially obtained from LGC Standards. The Luria-Bertani liquid cultivating medium was a self-prepared mixture of 1% (w/v) proteose peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) sodium chloride and 0.1% (w/v) D-(+)-Glucose Monohydrate. After heating to 100°C for 30 minutes, the hot solution was filtered through 0.2 µm syringe filters into laboratory bottles with screw caps prior to autoclaving. The standard program for autoclaving liquids was heating the medium to 121°C, which corresponded to a vapor pressure of 2 bar. The liquid was soaked for 20 minutes and slowly cooled down to 80°C before taking the medium out. All steps were carried out within one day to avoid long breaks and opportunities for contaminations. After autoclaving the culture medium was kept in the fridge at 4°C till use. The cultivating medium had a light brown to orange color after autoclaving, while the non-autoclaved liquid had a yellow color. This could be caused from caramelization of glucose in the medium and did not result from any contamination, because the solution was Gram stained before use and did not show any impurity.

All plastic tubes, screw caps and pipette tips were autoclaved before use to keep contaminations low. All steps requiring handling of bacteria were carried out under a laminar flow in a hood, which was cleaned with 96% ethanol. To culture bacteria, a suspension of bacteria and nutrient medium was incubated under stirring for less than 24 hours at 37°C. Under these conditions *E. coli* have a generation time close to 40 minutes. (Plank L.D. 1979) For culturing an amount of 100 µl of bacteria suspension was transferred into a falcon tube filled with 5 ml nutrient medium. This tube was put into a 37°C water bath with a magnet stirrer for constant dispersion and unfixed screw cap for leaking fermentation gases while culturing. After 20 – 24 hours the tube was centrifuged at 3500 rpm for 6 minutes and the supernatant
culturing liquid was decanted, followed by suspending the bacteria pellet obtained in autoclaved distilled water and subsequent centrifuging. After decanting the washing solution the washing step was repeated two times. Upon completion of the washing procedure, one part of the bacteria was used for re-culturing and the rest of the final resulting bacteria pellet was kept frozen and stored at minus 20°C till use.

5.2  **Bacteria counting**

For the experimental part it was necessary to determine the rough number of bacteria cells present in the pellet. This was done by using a counting chamber, which is a straightforward, quick and inexpensive way to get an average amount of cell concentration.

Today, the Neubauer-improved counting chamber is the standard tool for that purpose, which was used for this work. In the center third of the chamber, made from optical special galls, four grooves separate the double grid ruling in the middle from the two side parts. The middle rack is slightly lower (0.1 mm); in this part the counting chamber comprises bright net lines, fabricated through very thin lines in a metal plate, which ensures optimal contrast (see Figure 7 and 9).

![Figure 7: Side view of counting chamber](image)

A cleaned cover glass was fixed over the middle third by water on the side racks. Then a small amount of around 20 µl of diluted bacteria suspension was pipetted next to the cover glass, which was pulled instantly into the gap between cover glass and counting chamber by capillary forces (see Figure 8).
Figure 8: Double net (symbolized by dark squares) Neubauer-improved counting chamber

Now the bacteria cells within the counting grid were counted under the light microscope. 4 x 4 small squares (each 0.025 mm²) within one big square (0.04 mm²), including two triple lines connected over one corner were counted.

Figure 9: Example of countable (full spots) / non-countable bacteria (empty spots) within one counting net

Subsequently another big square was counted and if the sums of the two squares did not differ by ten, the average is taken for final calculations of the original concentration following Equation 3. (Hallmann L. 1980)
\[
\text{Cells/ml} = \frac{\text{Number of cells} \times 1000}{\text{Counted area (mm}^2\text{)} \times \text{Chamber depth (mm)} \times \text{Dilution factor}}
\]

Equation 3: Bacteria concentration

A short example shall demonstrate the use of the calculation: \(n=25\); dilution= 1:200

\[
\text{Cells/ml} = \frac{25 \times 1000}{0.04\text{ (mm}^2\text{)} \times 0.1\text{ (mm)} \times 0.005} = 1.25 \times E9
\]

5.3 Gram staining protocol

The following protocol was used for all Gram staining cycles, which were constantly run after bacteria culturing and bacteria medium use to prove contamination free bacteria pellets and culture medium. Crystal violet and safranin solutions were purchased. Lugol’s iodine solution was prepared by mixing 0.2 g potassium iodide and 0.1 g iodine in 30 ml distilled H₂O, which formed a deep yellow solution. (Sadasivam S. 2005) To dissolve the iodine crystals completely the solution was put into the ultrasonic bath for around 15 minutes and heated to 80°C till all solid crystals were dissolved. The solution was stored in plastic tubes wrapped with aluminum foil in the fridge. (http://veterinarymicrobiology.in/grams-staining/07/14/2018)

The Gram staining protocol was as follows (Figure 10):

- Smearing a drop of bacteria solution on an alcohol cleaned glass plate with an inoculation loop or sterilized pipette tip
- Fixing the bacteria via 2-3 times pulling through an open flame
- Dropping crystal violet solution on the fixed bacteria spots for 30 seconds
- Rinsing the chemical off with distilled water first, then with 95 % ethanol for 1.5 minutes followed by distilled water again
- Dropping the Lugol’s iodine solution on the glass plate and let it soak for 30 seconds
- Pouring the iodine solution off and rinsing with distilled water, 95 % ethanol and distilled water
- Counterstain with the safranin solution for 1.5 minutes
- Pouring the chemical off and rinsing the glass plate sufficiently with distilled water
- Stained bacteria are circled in grey

Figure 10: Crystal violet – EtOH/water – Lugol’s iodine – Safranin solution – stained bacteria (circled)

Figure 11 shows pure E. coli cells in light red color, typical for Gram-negative bacteria. On Figure 12 on the side, thin hyphae are clearly visible in between the cells.

Figure 11: pure E. coli  
Figure 12: E. coli contaminated with fungus
5.4 **Atomic Force Microscopy**

Atomic Force Microscopy, abbreviated AFM, is a technique to visualize and measure surface structures of all kind of samples in three dimensions. The method is non-destructive and allows for re-using samples after measurements. Furthermore no sample preparation is necessary. In contrast to STM and SEM it is possible to characterize non-conductive samples with AFM. In 1985 Gerd Binning had the idea of imaging a surface via force instead of current, as already used in scanning tunnel microscopy. Together with Christoph Gerber and Calvin Quate he built the first prototype shortly after that. The setup is shown in Figure 13.

![Atomic Force Microscope setup and cantilever tip when scanning the sample](image)

AFM is based on a pushing force of a tip on the surface, while the tip is scanning over the sample. The tip is fixed on a cantilever made from silicon nitride, whose spring constant is smaller than the binding force between atoms to image scales of atoms and molecules, while being soft enough to not push out the atoms of the surface. The instrument usually comes with an integrated optical microscope to visualize the tip and the sample surface, while standing on a vibration isolation platform to be resilient against shakings and increase the signal to noise ratio. (Eaton P. 2010) To monitor the vertical movements of the sharp tip on the end of a soft cantilever spring, an optical detection system is sensing a reflected laser beam of the cantilever surface. Usually a photodiode detector is used to collect the reflected laser beam on four quadrants of the photodetector, which is converted into a topographic image.
Deflection is also used to monitor the force applied on the surface and serves as feedback control mechanism for the piezoelectric element to adapt the force between the tip and the sample surface. Finally, a computer converts the data into an image. (Rugar D. 1990)

Three different operating modes are established. During contact mode the tip is in physical contact with the surface of the sample. The more the cantilever is bended by topographic structures on the surface, the more the laser beam is deflected. The contact mode is generally used for solid, regular samples. The non-contact mode operates with constant distance between tip and surface. The cantilever is set to oscillation at its natural frequency by a periodic, external force. Changing surface conditions cause a change in forces and simultaneously in oscillation frequency. The non-contact mode can be used with viscous samples and liquids. The tapping mode leads to a short touching of the surface by the tip through the oscillating cantilever at its natural frequency. (Schirmeisen A. 2008) Those different operating modes make atomic force microscopy a high-capacity analysis tool for all kinds of biological and chemical samples.

5.5 SEM – Scanning Electron Microscopy

The origins of the scanning electron microscope go back to the works of Manfred von Ardenne in the year 1937. (Von Ardenne M. 1938) He intended to add scanning coils to the already existing transmission electron microscope, and a photographic plate, which is scanning in the sample simultaneously to the electron beam. First commercial microscopes are available since in the mid-1960s. Nowadays, they are standard instruments for surface characterizations. SEM yields information about surface structures, but not about the chemical constituents of the surface. In that sense it is similar to AFM. An electron microscope creates an image of the sample surface by using an electron beam scanning in a raster process over the sample. This high resolution analysis tool images a two dimensional picture of the scanned
surface with a very high depth of field which lets the user spot sample surface properties in different levels thus generating the illusion of a three-dimensional image. Advantages are the rapid scanning time, the high resolution under 1 nm, the simplicity of image creation, straightforward handling also for unexperienced users and simultaneously scanning several samples without opening the analysis chamber. Besides all the convincing benefits, some restrictions need to be considered. Not all samples can be measured without sample preparation. This includes all non-conductive samples. In this case solid samples were fixed with a double sided carbon tape onto the aluminum holder. Liquid samples were dropped and dried onto a silicon wafer and dried. To make the top surface conductive to electrons all non-conductive samples were covered with a thin gold layer. The instrument itself is highly sensitive to non-fixed, breakaway particles of the sample and dirt like dust in the air, as well to changes in temperature, which requires an air-conditioned experimental room, motions and vibrations, which ideally require vibration-free surroundings. High vacuum generating pumps are needed to avoid energy losses of the electron beam due to collisions with molecules in the air. Finally, it destroys non-conductive samples due to sample preparations, which makes it unlikely to use the same sample for further experiments or measurements. (Amelinckx S. 1997)

Scanning electron microscopy is a powerful tool for imaging and characterizing of materials. Wide range of different operation modes can be carried out within one device for answering different questions. It combines high resolutions with simplicity in use, with visualizing the surface of samples of not visible objects for human eyes.

Scanning electron microscopy represents the ideal analyzing tool for the Pickering emulsion formed polymer beads in this work. The spherical shape limits the use of AFM for surface imaging. SEM magnifications reach up to 50,000x and beyond with high resolution. Other than in atomic force microscopy and its scanning tip, the electron beam in SEM does not depend on surface rigidity. At the same time SEM creates images much faster than AFM, because the latter requires scanning of the entire surface.
5.5.1 Microscope set-up

The SEM setup is shown in Figure 14.

The electron beam is emitted by an electron gun, which generates electrons through thermionic emission of electrons from a tungsten cathode in vacuum. Electrons are accelerated through a difference in voltage between anode and cathode of up to 30 kV. Repulsing ring cathodes, also called condenser lenses, focus the beam on a small area; Modern SEM instruments use electromagnetic lenses. Scan coils are usually incorporated in the objective lens and scan the electron beam in various angles over the sample. The electron beam hits the sample in the vacuum chamber, which is fixed in the sample holder and can be tilted to all sides to vary the impact angle of the electrons for diminishing image artefacts or increasing the image information. Every microscope contains more than one detector, namely a secondary electron detector, a backscattered electron detector and a fluorescent X-rays detector for microanalysis. Only the first two mentioned detectors are
necessary to create SEM images. The signal of the detectors is amplified and converted by an analog to digital converter. Information about currents in the scan coils is processed in the computer as well to have control and knowledge over the precise beam position. A conventional scanning electron microscope with constant chamber pressure pumps the vacuum down to $10^{-4}$ Pa ($10^{-9}$ bar).

### 5.5.2 Scattering and operation modes

The principle of a scanning electron microscopy is to use a high energy electron beam, and to focus it on the surface of the sample. The beam undergoes various changes through interactions between electrons of the beam and atoms of the sample. The main interactions in SEM techniques are elastic, such as backscattered electrons, and inelastic, such as secondary electrons, scattering processes as well as X-ray emission. When the electron beam hits the surface, the energy transfer can lead to the loss of loosely bound valance electrons from the sample. These electrons emitted under irradiation of the primary electron beam, are called secondary electrons, which have energies between 0 and 50 eV. Due to this low energy they can only travel a few nanometers. Secondary electrons are generated either when an incident electron passes through the escape shell, or when backscattered electrons leave the sample or pass through the escape shell. (Figure 15)
The first mentioned scattered electrons are called SE1 electrons and contain high resolution information. The secondary electrons created through backscattering effects are called SE2 electrons and carry the lower resolution information. The signals of SE1 and SE2 electrons cannot be separated, but when operating the electron beam with lower acceleration voltage the signal of SE2 electron yield is small. This explains preferred operating acceleration voltages of less than 10 keV in SEM. Secondary electrons are pulled potentially towards the detector, hitting the scintillator, usually a YAG crystal, and leaving the detector as light beams. Secondary electron imaging is the widely used operating mode in SEM because of its simplicity of detection, electron collection, resolutions of 1 nm or less, easy handling for unspecialized users and its use obtaining topographically information of the sample surface. Topographic image information of the sample is maintained by the three dimensional effect of shadow and lighting. Bright areas are produced by a high yield of secondary electrons, which depends on the angle between the primary electron beam and the surface. High angles are seen as bright pixels in the image.

Backscattered (primary) electrons of the incident beam are scattered in the sample with an angle of less than 180° and leave the sample again. The electrons undergo energy loss and have final energies between 50 keV and the incident electron beam energy. Their yield increases with atomic number Z and energy of the incident electron beam. Backscattered electron imaging has less meaning than secondary electron imaging, also because the detector must be placed directly above the sample to collect the backscattered electrons, which is a practical problem.

In this work two different detecting systems were used for image recording. On the one hand a so called SE2 detector, which collects primarily secondary electrons from the second type, was standardly used for all surface image presentations. Besides the SE2 detector, a detector called Inlens was utilized to obtain surface information only from the outermost layers of the sample. An Inlens detector works on the principle of collecting secondary electrons, but only the SE1 type electrons, and at the same time backscattered electrons, to create images. This leads to high resolution and distortion-free imaging of the sample surface. Image 16
demonstrates the differences in the images taken with the two detectors described above.

Figure 16: Images taken with the different detectors at the same sample position; the part on the left shows the Inlense detector image; in the middle a progressive transition from Inlense detector to SE2 detector; the part on the right shows the entire image of a SE2 detector.

5.6 SEM sample preparation

SEM was used in this work for pre-treated beads for surface characterization and to characterize surfaces and determine bead diameters, as well as for assessing washing steps to remove E. coli. For that purpose the samples had to be coated with a thin gold layer to ensure electrical conductivity. The samples in this work were either coated with the instrument Leica SCD050 sputter coater in the faculty center for nanostructure research, or with the gold sputter instrument Cressington 208HR (Figure 17), following the respective instrument manuals.
5.7 Radical Polymerization

Azobisisobutyronitrile (abbreviated AIBN) shown in Figure 18, is a thermolabile white crystalline powder that forms radicals under the loss of a nitrogen molecule. It is widely used as initiator for free radical polymerizations, insoluble in water and therefore dissolved in the organic oil phase of an emulsion.

![Azobisisobutyronitrile](image)

Figure 18: AIBN radical starter
The initiated radicals start forming a polymeric chain with monomers and cross-linkers in the oil phase. The addition of unsaturated monomers to the active radical site of the propagating polymer chain is called chain-growth polymerization. The 2-cyanopropyl radical is attacking the double bond and forming a saturated single bond with another monomer while the radical position is consecutively as long as the polymerization is propagated.

![Figure 19: Chain-growth polymerization of poly-(styrene-co-divinylbenzene)](image)

It must be noticed that the chain shown in Figure 19 does not continue regularly with alternating styrene and divinylbenzene substrates. One cross-linker may be followed by several monomers before the next cross-linking molecule is attached. Polymerization stops when two radicals combine without forming a new free radical due to depletion of unreacted monomers.

The amount of initiator was calculated with Equation 4, according the equivalents of double bonds present in the system from monomer and cross linker. Each double bond contributes to the calculation with 1 mol%, shown in the following example of 1 ml styrene (one double bond) and 1 ml DVB (two double bonds):
\[
\left[1\text{ ml styrene } \left( M = \frac{104.15 \text{ g}}{\text{mol}}, \delta = \frac{0.906 \text{ g}}{\text{ml}} \right) \rightarrow 8.70 \text{ mmol} \right] + \\
\left[1\text{ ml DVB } \left( M = \frac{130.19 \text{ g}}{\text{mol}}, \delta = \frac{0.914 \text{ g}}{\text{ml}} \right) \rightarrow 7.02 \text{ mmol} \rightarrow \times 2 \rightarrow 14.04 \text{ mmol} \right] \\
= 0.23 \text{ mmol} \times M_{\text{AIBN}} \left( \frac{164.21 \text{ g}}{\text{mol}} \right) = 34.34 \text{ mg AIBN}
\]

Equation 4: Amounts of initiator

5.8 QCM manufacturing

For producing QCM, two-electrode structures were screen-printed onto commercially available AT-cut quartz blanks, 167 µm thick, \( f_0 = 10 \text{ MHz} \). The sieves used for applying the gold electrodes are made in the laboratory. For this purpose the synthetic material is covered with the photo-sensitive dye Azocol Poly-Plus S, a solvent-resistant UV diazo-polymer emulsion. Models of the electrodes are laid onto the sieve. After exposure of the dye to UV, the unhardened part is washed off with distilled water. Liquid phase measurements require two different electrode diameters on the front and back side of the QCM, respectively. (Figure 20)

![Figure 20: Sieve electrode schemes and printed gold electrodes on quartz wafer](image)

The quartz chip was placed on a vacuum holder and fixed by turning the pump. On top of the quartz the electrode model for the one side was fixed and gold paste was spread with a hard rubber application tool. After removing the model and turning off
the vacuum, the gold covered quartz was fired at 400°C for 4 hours. Then the second side of the electrode was imprinted with the opposite electrode model under the same steps.

Finished QCMs were placed into the measuring cell. The small electrodes on the back were in contact with the electrode contacts on the inside of the measuring cell. Fixing the quartz with a polydimethylsiloxane cap and a screw fixed lid the network analyzer showed the damping and frequency of the quartz. (Figure 21)

Figure 21: Measuring cell and network analyzer Agilent 8712ET

Low damping and frequencies close to 10 MHz minimize the signal-to-noise ratio during sensor measurements. (Rodriguez-Pardo L. 2005)

5.9 Chemicals, Materials and Instruments

All chemicals, glassware and solvents used for this work were purchased either from Sigma-Aldrich, Merck Millipore, VWR International GmbH, or Thermo Fisher Scientific. Ammonia was bought from Thermo Fisher Scientific. Hydrogen peroxide 35%, benzoyl peroxide, styrene, divinylbenzene, Gram’s crystal violet solution, hydrochloric acid fuming, formic acid, nitric acid 65%, sulfuric acid 95-97%, orthophosphoric acid 85%, sodium hydroxide, EDTA II and EDTA III were purchased from Merck. Bacteriological peptone, glutaraldehyde 50% and TRIS hydrochloride were received from VWR International GmbH. Sodium dodecyl sulfate,
hexadecyltrimethylammonium bromide, Gram’s safranin solution were purchased from Fluka. Acid-washed glass beads of the size of 75 µm, low molecular weight chitosan, AIBN and yeast extract were purchased from Sigma-Aldrich. The gold paste for quartz electrodes was obtained from the company Heraeus Group. The bacteria strain *Escherichia coli* (*E. coli*) strain ATCC® 9637™, *Bacillus cereus* (*B. cereus*) ATCC® 11778™ and *Staphylococcus epidermidis* (*S. epidermidis*) ATCC® 12228™ were obtained from LGC Standards. For bacteria counting a Neubauer-improved counting chamber was used, purchased at Paul Marienfeld GmbH & Co. KG. The centrifuge 5702 was used for washing steps and bacteria culturing. For bacteria counting and Gram staining process analyzations the Nikon light microscope Eclipse LV 100 and its software NIS Elements D 2.30 were used. The quartz wafers, with 10 MHz resonance frequency and a diameter of 13.8 ± 0.02 mm were obtained from The Roditi International Corporation Ltd. The centrifuge 5702 from Eppendorf was used for all centrifugation steps described in this work. For the screen progress of the gold electrodes on the quartz wafers gold paste GGP A/2093-10% H from Heraeus Precious Metals GmbH & Co. KG, obtained from Keramikbedarf Ing. Skokan GmbH was used. Quartz resonance frequencies were determined with the network analyzer Agilent E5062A. Sensor measurements were carried out with the frequency counter Agilent 53131A and the power supply unit EA-PS 2032-025 from Elektro-Automatik. The measuring cell was provided by a former group member. Spin coating of thin polymer layers was carried out with the spin coater G3P-8 from Specialty Coating Systems. Sputtering the surface for SEM imaging with gold was carried out with the Cressington Sputter Coater 208HR. For surface characterization the atomic force microscope from Veeco Metrology Group and the secondary electron microscope, a Zeiss Supra 55 VP device from the Carl Zeiss AG, were used. For AFM image creation SNL-10 from Bruker cantilevers made out of silicon nitride were utilized. The LabView Routine program, used in the working group, was the computer program used for data analyzation. Finished measurements were analyzed with Microsoft Office Excel 2010.
6. Experiments

6.1 Synthesis of molecularly imprinted beads

A previous Master thesis of the group demonstrated successful synthesis of *E. coli* imprinted polymer beads. (Prinz 2017) Therefore this work focused on removing the imprinted bacteria from the polymer beads surface after polymerization. Bacteria pellets, used during early stages of this work, were contaminated with undefined bacteria strains, most likely *Bacillus Cereus*, various types of *Coccus* bacteria and also fungi. Nevertheless Pickering emulsions were synthesized with contaminated *E. coli* suspensions and compared with experiments after successful purification of the *E. coli* strain.

In a first step the recipe for obtaining Pickering emulsions needed optimizing. Among others this included choosing a suitable surfactant to form stable emulsions. In this study only cationic surfactants were used. They contain a positively charged head and surrounded by non-polar alkyl substituents. The polar group is usually a quaternary ammonium cation with a halogen atom as counterion. Within this work hexadecyltrimethylammonium bromide (HTAB), also often referred to as CTAB – cetyltrimethylammonium bromide - was used. It works similar to the surfactant dimethyldioctadecylammonium bromide. (Bucak S. 2002)

According to abovementioned previously developed protocol a ratio of 4:2 mL *E. coli* aqueous -suspension to styrene/DVB containing 1 mM of the surfactant DODAB (dimethyldioctadecylammonium bromide) in the oil phase led to the best results and had the advantage to control bead size depending on *E. coli* concentration. (Prinz 2017) Based on this experience many experiments with different ratios of oil and aqueous phase were performed as shown in Table 1, using hexadecyltrimethylammonium bromide as surfactant, because it was available in the chemical stock of the laboratory. When the respective emulsion formed stable beads
and did not separate into two phases, defined amounts of radical starter AIBN were added.

To produce non-imprinted beads 0.8 mg of HTAB was suspended in a 1:1 mixture of 2 ml styrene and 2 ml DVB and shaken by hand with 6 ml of distilled water. After formation of stable beads, 20 mg of AIBN was added and carefully mixed. Polymerization took place at 70°C in the oven over night and the next day, the beads were washed with distilled water and air-dried. Optical microscopy revealed beads with around 50µm diameter.

<table>
<thead>
<tr>
<th>#</th>
<th>Ratio (o:w)</th>
<th>Styrene/DVB (1:1) [ml]</th>
<th>HTAB [mg]</th>
<th>E. coli (~E8 – dissolved in 10 ml H2O) [ml]</th>
<th>Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1:1</td>
<td>2</td>
<td>0.72</td>
<td>2</td>
<td>✗</td>
</tr>
<tr>
<td>2</td>
<td>1:2</td>
<td>2</td>
<td>0.72</td>
<td>4</td>
<td>✗</td>
</tr>
<tr>
<td>3</td>
<td>1:3</td>
<td>2</td>
<td>0.72</td>
<td>6</td>
<td>✗</td>
</tr>
<tr>
<td>4</td>
<td>1:4</td>
<td>2</td>
<td>0.72</td>
<td>8</td>
<td>✓</td>
</tr>
<tr>
<td>5</td>
<td>1:5</td>
<td>2</td>
<td>0.72</td>
<td>10</td>
<td>✓</td>
</tr>
<tr>
<td>6</td>
<td>1:1</td>
<td>2</td>
<td>0.36</td>
<td>2</td>
<td>✗</td>
</tr>
<tr>
<td>7</td>
<td>1:2</td>
<td>2</td>
<td>0.36</td>
<td>4</td>
<td>✗</td>
</tr>
<tr>
<td>8</td>
<td>1:3</td>
<td>2</td>
<td>0.36</td>
<td>6</td>
<td>✓</td>
</tr>
<tr>
<td>9</td>
<td>1:4</td>
<td>2</td>
<td>0.36</td>
<td>8</td>
<td>✓</td>
</tr>
<tr>
<td>10</td>
<td>1:5</td>
<td>2</td>
<td>0.36</td>
<td>10</td>
<td>✓</td>
</tr>
<tr>
<td>11</td>
<td>1:2</td>
<td>1</td>
<td>-</td>
<td>2</td>
<td>✓</td>
</tr>
<tr>
<td>12</td>
<td>1:3</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>✓</td>
</tr>
<tr>
<td>13</td>
<td>1:4</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>14</td>
<td>1:5</td>
<td>2</td>
<td>-</td>
<td>10</td>
<td>✓</td>
</tr>
</tbody>
</table>

Table 1: Ratio experiments with contaminated E. coli
As it can be seen from the results of experiment 11 to 14 in Table 1, adding HTAB is not essential. Hence it turned out that no surfactant is needed to form stable Pickering emulsions with *E. coli*. This is of course favorable, because fewer chemicals are needed, which makes the reaction less expensive and more environmentally friendly. Interestingly, large amounts of surfactant with respect to the ratio of oil and aqueous phase increase the probability of destabilization or separation into two immiscible phases. This can be an effect of secondary particle formation of the surfactant without forming desired polymer beads. It results in destabilization of the Pickering emulsion and either final decomposition of the phases, or formation of white particle coagulations. (Wang Z. 1995) The next step comprised experiments to assess, how the monomer composition in the oil phase influences the amount, size, and quality of particles. The results are summarized in Table 2.
<table>
<thead>
<tr>
<th>#</th>
<th>Styrene [ml]</th>
<th>DVB [ml]</th>
<th>Ratio (Sty:DVB)</th>
<th>HTAB [mg]</th>
<th>E. coli (~E8 – dissolved in 10 ml H2O) [ml]</th>
<th>Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>1</td>
<td>0.5</td>
<td>2:1</td>
<td>-</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>16</td>
<td>0.5</td>
<td>1</td>
<td>1:2</td>
<td>-</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>0.3</td>
<td>3:1</td>
<td>-</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>18</td>
<td>0.3</td>
<td>1</td>
<td>1:3</td>
<td>-</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>19</td>
<td>1</td>
<td>0.25</td>
<td>4:1</td>
<td>-</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>20</td>
<td>0.25</td>
<td>1</td>
<td>1:4</td>
<td>-</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>0.5</td>
<td>2:1</td>
<td>0.27</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>22</td>
<td>0.5</td>
<td>1</td>
<td>1:2</td>
<td>0.27</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>23</td>
<td>1</td>
<td>0.3</td>
<td>3:1</td>
<td>0.27</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>24</td>
<td>0.3</td>
<td>1</td>
<td>1:3</td>
<td>0.27</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>0.25</td>
<td>4:1</td>
<td>0.27</td>
<td>5</td>
<td>×</td>
</tr>
<tr>
<td>26</td>
<td>0.25</td>
<td>1</td>
<td>1:4</td>
<td>0.27</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>27</td>
<td>1</td>
<td>0.5</td>
<td>2:1</td>
<td>-</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>28</td>
<td>0.5</td>
<td>1</td>
<td>1:2</td>
<td>-</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>29</td>
<td>1</td>
<td>0.3</td>
<td>3:1</td>
<td>-</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>30</td>
<td>0.3</td>
<td>1</td>
<td>1:3</td>
<td>-</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>31</td>
<td>1</td>
<td>0.25</td>
<td>4:1</td>
<td>-</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>32</td>
<td>0.25</td>
<td>1</td>
<td>1:4</td>
<td>-</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>33</td>
<td>1</td>
<td>0.5</td>
<td>2:1</td>
<td>0.27</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>34</td>
<td>0.5</td>
<td>1</td>
<td>1:2</td>
<td>0.27</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>35</td>
<td>1</td>
<td>0.3</td>
<td>3:1</td>
<td>0.27</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>36</td>
<td>0.3</td>
<td>1</td>
<td>1:3</td>
<td>0.27</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>37</td>
<td>1</td>
<td>0.25</td>
<td>4:1</td>
<td>0.27</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>38</td>
<td>0.25</td>
<td>1</td>
<td>1:4</td>
<td>0.27</td>
<td>4</td>
<td>✓</td>
</tr>
<tr>
<td>39</td>
<td>1</td>
<td>0.5</td>
<td>2:1</td>
<td>-</td>
<td>3</td>
<td>✓</td>
</tr>
<tr>
<td>40</td>
<td>0.5</td>
<td>1</td>
<td>1:2</td>
<td>-</td>
<td>3</td>
<td>✓</td>
</tr>
<tr>
<td>41</td>
<td>1</td>
<td>0.3</td>
<td>3:1</td>
<td>-</td>
<td>3</td>
<td>×</td>
</tr>
<tr>
<td>42</td>
<td>0.3</td>
<td>1</td>
<td>1:3</td>
<td>-</td>
<td>3</td>
<td>×</td>
</tr>
<tr>
<td>43</td>
<td>1</td>
<td>0.25</td>
<td>4:1</td>
<td>-</td>
<td>3</td>
<td>×</td>
</tr>
<tr>
<td>44</td>
<td>0.25</td>
<td>1</td>
<td>1:4</td>
<td>-</td>
<td>3</td>
<td>✓</td>
</tr>
<tr>
<td>45</td>
<td>1</td>
<td>0.5</td>
<td>2:1</td>
<td>0.27</td>
<td>3</td>
<td>✓</td>
</tr>
</tbody>
</table>
Table 2: Influence of styrene/DVB ratio on polymerization

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>46</td>
<td>0.5</td>
<td>1</td>
<td>1:2</td>
<td>0.27</td>
<td>3</td>
</tr>
<tr>
<td>47</td>
<td>1</td>
<td>0.3</td>
<td>3:1</td>
<td>0.27</td>
<td>3</td>
</tr>
<tr>
<td>48</td>
<td>0.3</td>
<td>1</td>
<td>1:3</td>
<td>0.27</td>
<td>3</td>
</tr>
<tr>
<td>49</td>
<td>1</td>
<td>0.25</td>
<td>4:1</td>
<td>0.27</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>0.25</td>
<td>1</td>
<td>1:4</td>
<td>0.27</td>
<td>3</td>
</tr>
</tbody>
</table>

Recipes leading to stable emulsion and not phase-separating within 24 hours were polymerized, due to simplicity, with 20 mg AIBN even if the optimal calculated amount of initiator (according to Equation 1) was less.

As it can be seen in Table 2, the ratio of monomer and cross linker per se does not have an influence on the formation of the bead. It can be argued that less cross linked polymers interact less strongly with the template and led to easier removal. Like already mentioned before, Table 2 shows the success of forming imprinted polymer beads without presence of additional surfactant. This further underpins that no additional surfactant is necessary when producing stable polymer beads via Pickering emulsion.

Table 1 and Table 2 show that the overall ratio of oil and aqueous phase has a big influence on the stability. The data in the tables above show that oil phase to aqueous phase ratios of 1:4 to 1:5 resulted in the most stable Pickering emulsions (experiments number #4/5; #9/10; #13/14 and #15-38). The less water phase, in this case equitable with less bacteria cells, is present, the more likely the emulsion separate into two phases due to insufficient stabilization. To proof this assumption the following experiments were carried out.
Based on the expectation of finding improved protocols to create Pickering emulsion polymer beads, it was surprising that experiment #51 was successful, while the following two #52 and #53 were not. Table 3 summarizes what was mentioned before, namely that increasing the concentration of additional surfactant HTAB too much lead to decomposition in combination with high oil to aqueous phase ratio.

The most promising experiments were repeated with exact knowledge of the amount of *E. coli* bacteria and with characterizing the size of the beads under the light microscope. Table 4 summarizes those results.

<table>
<thead>
<tr>
<th>#</th>
<th>Ratio (o:w)</th>
<th>Styrene/DVB (1:1) [ml]</th>
<th>HTAB [mg]</th>
<th><em>E. coli</em> (~E8 - dissolved in 6 ml H₂O) [ml]</th>
<th>Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>2:3</td>
<td>4</td>
<td>0.18</td>
<td>6</td>
<td>✓</td>
</tr>
<tr>
<td>52</td>
<td>2:3</td>
<td>4</td>
<td>0.36</td>
<td>6</td>
<td>×</td>
</tr>
<tr>
<td>53</td>
<td>2:3</td>
<td>4</td>
<td>0.72</td>
<td>6</td>
<td>×</td>
</tr>
</tbody>
</table>

**Table 3: Experiments with different ratio and higher concentration in the bacteria suspension**

The most promising experiments were repeated with exact knowledge of the amount of *E. coli* bacteria and with characterizing the size of the beads under the light microscope. Table 4 summarizes those results.

<table>
<thead>
<tr>
<th>#</th>
<th>Ratio</th>
<th>Styrene/DVB (1:1) [ml]</th>
<th>HTAB [mg]</th>
<th><em>E. coli</em> suspension [ml] (concentration [cells/ml])</th>
<th>Ø Beads size [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>1:2</td>
<td>0.5</td>
<td>-</td>
<td>1 (7.1 E8)</td>
<td>300</td>
</tr>
<tr>
<td>55</td>
<td>1:3</td>
<td>0.5</td>
<td>0.09</td>
<td>1.5 (7.8 E8)</td>
<td>50</td>
</tr>
<tr>
<td>56</td>
<td>1:3</td>
<td>0.5</td>
<td>-</td>
<td>1.5 (1.3 E9)</td>
<td>80</td>
</tr>
<tr>
<td>57</td>
<td>1:4</td>
<td>0.5</td>
<td>0.09</td>
<td>2 (7.8 E8)</td>
<td>90</td>
</tr>
<tr>
<td>58</td>
<td>1:4</td>
<td>0.5</td>
<td>-</td>
<td>2 (7.1 E8)</td>
<td>120</td>
</tr>
<tr>
<td>59</td>
<td>1:5</td>
<td>0.5</td>
<td>0.09</td>
<td>2.5 (7.8 E8)</td>
<td>70</td>
</tr>
<tr>
<td>60</td>
<td>1:5</td>
<td>0.5</td>
<td>-</td>
<td>2.5 (7.1 E8)</td>
<td>200</td>
</tr>
<tr>
<td>61</td>
<td>1:5</td>
<td>0.5</td>
<td>0.18</td>
<td>2.5 (7.1 E8)</td>
<td>100</td>
</tr>
<tr>
<td>62</td>
<td>2:3</td>
<td>4</td>
<td>0.18</td>
<td>6 (1.4 E9)</td>
<td>90</td>
</tr>
</tbody>
</table>

**Table 4: Promising protocols**
After successful pretesting with contaminated \textit{E. coli} strains the experiments from Table 4 were examined with an in the meantime purified \textit{E. coli} strain ATCC\textsuperscript{®} 9637\textsuperscript{TM}. The results were comparable to the ones in Table 4.

6.2 Synthesis of a NIP polymer bead

Uptake tests require non-imprinted Pickering emulsion polymer beads out of the same monomer and cross linker system to compare selective binding of bacteria cells into cavities of MIP beads to smooth surfaces of NIP beads.

Experiments were carried out with different amounts of surfactant added to the oil phase: To 4 ml oil phase of a 1:1 mixture of styrene and DVB 1.0 mg; 0.8 mg; 0.6 mg; 0.4 mg and 0.2 mg of HTAB as surfactant, respectively, were added. All mixtures were shaken by hand and formed stable Pickering emulsions; After adding 20 mg AIBN to each falcon tube and polymerization overnight at 70°C, it turned out that only surfactant amounts over 0.6 mg HTAB formed spherical polymer beads. At amounts of 0.6 mg HTAB and less only splinters were seen below the microscope, which proves that a minimum concentration of surfactant is needed to form stable bacteria free/non-imprinted polymer beads.
6.3 Experiments with different types of bacteria

As it can be seen in Table 5, experiments carried out with Gram-positive, spherical *Staphylococcus epidermidis* (*S. epi*) always resulted in unstable emulsions. Therefore no beads imprinted with *S. epidermidis* were obtained. *Lactococcus lactis* is also Gram-positive and globular. It is used for the caseation of cheese and partly formed stable Pickering emulsions. Compared to emulsions with *Bacillus cereus* as solid particle, no clear explanations for the few stable emulsions were found. The fact that the Gram-positive, but rod-shaped *B. cereus* formed mostly stable polymer beads, may lead to the conclusion that the shape of the microorganism has an influence on the emulsion’s stability, because *E. coli* and *B. cereus* - both rod shaped - strongly favor Pickering emulsion.

<table>
<thead>
<tr>
<th>#</th>
<th>Ratio</th>
<th>Styrene/DVB (1:1) [ml]</th>
<th>HTAB [mg]</th>
<th>Bacteria (~E8 - dissolved in 10 ml H2O) [ml]</th>
<th>Beads</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>S. epi</td>
</tr>
<tr>
<td>63</td>
<td>1:5</td>
<td>1</td>
<td>0.18</td>
<td>5</td>
<td>×</td>
</tr>
<tr>
<td>64</td>
<td>1:4</td>
<td>1</td>
<td>0.18</td>
<td>4</td>
<td>×</td>
</tr>
<tr>
<td>65</td>
<td>1:3</td>
<td>1</td>
<td>0.18</td>
<td>3</td>
<td>×</td>
</tr>
<tr>
<td>66</td>
<td>1:5</td>
<td>1</td>
<td>-</td>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>67</td>
<td>1:4</td>
<td>1</td>
<td>-</td>
<td>4</td>
<td>×</td>
</tr>
<tr>
<td>68</td>
<td>1:3</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>×</td>
</tr>
<tr>
<td>69</td>
<td>1:5</td>
<td>1</td>
<td>0.36</td>
<td>5</td>
<td>×</td>
</tr>
<tr>
<td>70</td>
<td>1:4</td>
<td>1</td>
<td>0.36</td>
<td>4</td>
<td>×</td>
</tr>
<tr>
<td>71</td>
<td>1:3</td>
<td>1</td>
<td>0.36</td>
<td>3</td>
<td>×</td>
</tr>
</tbody>
</table>

Table 5: Pickering emulsions with different types of bacteria
Figure 22a demonstrates that *S. epidermidis* did not stick to the surface of the formed beads, but somehow it was stabilizing the oil-water-interface, so that stable beads were formed, which is not possible without surfactant. This led to speculations if some contaminations were present in the bacteria pellets, which led to stabilization or destabilization of the emulsion. Generally it is difficult to say what effect contaminations have on emulsions. However, they cannot be controlled or influenced when working under impure conditions. Some bacteria partly cover the surface, as shown in Figure 23 and Figure 24, circled in black.
6.4 Washing procedure - Removal of template bacteria

After studying the various conditions of Pickering emulsions and finding the best ratios for the experiments, the further scope of the work was establishing a working procedure for removing the imprinted bacteria from bead surfaces. For this part the properties of *E. coli* were examined more in detail to get an idea which conditions and chemicals are harmful to bacteria and may cause their destruction. The difficulty was not to find ways to kill and disable bacteria, because most likely all bacteria were killed during polymerization at 70°C, but to remove dead cells from the system without destroying the polymer surface.

Literature almost only talks about inactivating and killing bacteria to stop further contaminations, bacterial growth, infections and many more, but effectively destroying and eliminating bacteria from any kind of surface or bulk medium is not well established and investigated yet. This leads to the consideration about how bacteria are removed in microbiology, chemistry and medicine. The approaches developed so far can roughly be separated into two main groups, namely chemical ways on the one hand, and mechanical/physical strategies on the other.

6.4.1 Physical trials for *E. coli* removal

*Ultraviolet light*

The use of ultraviolet light for disinfection and cleaning of surfaces and instruments is well established. UV light damages the cell wall, DNA, ribosome and proteins through direct change of DNA and protein characteristics or through generation of radical oxygen species, which interfere with cell growth, reproduction, and the cell
The idea behind it is that damages in the cell wall weaken binding of the bacterium to the polymeric surface and facilitate removal of cell fractions. For this reason polymeric beads were left under UV light at 312 nm for 1 hour and 18 hours. The idea behind it is that damages in the cell wall weaken binding of the bacterium to the polymeric surface and facilitate removal of cell fractions. Afterwards bead were washed with distilled water with and without the support of vibrations for 1 minute in the ultrasonic bath. After characterizing the surface of the treated beads with SEM (Figure 25), some imprints can be seen, but most of the surface is still covered with the bacteria cells. The SEM images also show that no cell wall damages are achieved with UV light, because no partly fractioned cells are seen on the pictures.

![Figure 25: In-lens picture of UV light treated beads, bright contours with dark fillings circled in black, show removed bacteria imprints, and bright contours with bright content indicate unremoved E. coli cells](image)

**Ultra-sonication**

Trials to remove bacteria from the bead surface from a former co-worker in the group lead to the conclusion that sonication of the polymer beads may have the effect of leaving some imprints, but not homogeneously. (Prinz 2017) Based on this
previous knowledge further experiments with ultra-sonication were carried out, also in combination with other chemical and physical trials. The physical action leading to cell destruction and bacteria death is formation of small cavities in the liquid medium through ultrasonic waves. These cavities are quickly collapsing bubbles in the irradiated medium and provoke tensile stress and thus rupture of the cell wall. (Turner A. 1975)

Sonication for one minute or less has a positive effect on bacteria removal; longer sonication times cause disruption of the polymeric beads, by smoothing the surface. In combination with other pre-treatments no enhanced amount of imprints are seen on the SEM images, which lead to the conclusion that additional ultra-sonication is not essential for complete bacteria removal.

**Abrasion**

Basic assumption is that applying direct shear force to the surface leads to mechanical removal through abrasion of the cells off. For this purpose smooth glass beads of 70 µm were mixed with imprinted polymeric beads under the following conditions:

- One mixture was stirred in an argon gas flow for one hour
- One was purged with argon for one hour in distilled water
- Another set-up was a mix of glass and polymer beads with distilled water, stirred with a magnetic stirrer for one hour
- Stirring of glass beads and bacteria imprinted beads for one hour

Afterwards addition of chloroform (CHCl₃; ρ= 1.48 g/ml) separated the glass from the polymer beads due to the density of the glass beads of ρ= 2.42-2.50 g/ml, which were sinking in chloroform and the polymeric beads with a density of around ρ= 1.3 g/ml, floating at the surface. Afterwards all polymeric beads were washed with water and dried at room temperature.
Another abrasion experiment was carried out with a standard kitchen sponge with fine pores and a vortex mixer. Polymer imprinted beads and sponge were put into an Eppendorf tube and constantly mixed on the vortex mixer for one hour. Through the shear motions of the sponge on the bead’s surface, removal of bacteria was expected. Some beads made their way through the sponge within one hour and afterwards a SEM image was taken, but removal did not take place.

**High temperature**

Cooking the imprinted beads in water for 1 hour at 100°C did not show any effect on bacteria cells on the polymer surface. Maybe the cooking process deactivates any E. coli still alive, but cells were not fractioned or destroyed, they just lost their activity.

**Autoclaving**

The principle of steam autoclaving is to reduce possible contaminations, when exposing bacteria to hot steam at 121°C (2 bar) for 20 minutes. Temperature and duration can vary. Autoclaving not only moderates the number of augmentable bacteria to a minimum, but eventually also kills their spores, which are more resistant against dry heat. (Alderton G. 1969) Literature does not contain much information on how autoclaving renders bacteria innocuous, so one idea is that it simply leads to cell rupture and removal of the cells from the surface: another chain of thoughts is that high pressure and high temperatures may soften the surface of the imprinted beads so that bacteria can diffuse from the surface, because binding is weaker. SEM images revealed that this was not the case here: the surfaces of the beads are still widely covered with bacteria after autoclaving (Figure 26).
Neither technique thus led to removing major part of *E. coli* bacteria from particle surfaces thus leading only to a few random imprints. Bacteria cells were not influenced by mechanical stress and cell fractioning like expected with ultrasonication; UV irradiation also did not lead to the desired effect. The most satisfying results came from utilizing the ultrasonic bath for bacterial removal, though no improvements of chemically treated samples were observable. It has to be considered that ultrasonic waves were quite harsh and did not only damage the bacteria cell walls, but also caused bond breaking interactions with the polymer.

### 6.4.2 Chemical trials for *E. coli* removal

Various chemicals have devitalizing effects on bacteria, such as antibiotics, saline solutions, organic solvents (Sardessai Y. 2002), as well as acids and bases. Antibiotics and other drugs have not been tried in this work due to the reason that almost all of them only intervene at some point in the transcription of proteins or
the correct composition of the ribosome subunit and thus cause inactivation of the damaging characteristics of the bacterium or lead to cell death. Standard antibiotics do not disrupt the cell wall of the microorganism or somehow else degrade and remove the individual cells, so that lethargic bacteria cells are excreted of the individual’s body. A few antibiotics attack the cell wall itself, but never disrupt it: They only prevent further cell wall growth. (Aktories K. 2005)

*Organic solvents*

Organic solvents are classified as volatile, containing carbon atoms, and able to dissolve compounds. Examples include alcohols, aldehydes, ketones, aromatic/cyclic or aliphatic hydrocarbons and others. (Firestone J. 2009) Organic solvents are cytotoxic compounds that cause disruption of the bacterial cell membrane and thus deforming integrity and functionality of the cell. It is known that already small concentrations of solvents are highly toxic to microorganisms, though more and more resistant bacteria strains develop, if concentrations are low. It’s presumed that Gram-negative bacteria might be more resistant against organic solvents due to their outer cell wall, and Gram-positive cells are disrupted more easily. (Sardessai Y. 2002)

Based on this assumption the following organic solvents, listed in Table 6, were tested for bacteria removal. Experiments were carried out with the synthesized bead load according to experiment #13 (0.5 ml styrene + 0.5 ml DVB; no surfactant; 4 ml pure *E. coli* solution).
Glutaraldehyde is a disinfectant used for instruments in medicine and pharmacy deployed against bacteria and fungi. (Schneemann H. 1995) The second reason for using glutaraldehyde as washing solution is its bactericidal and sporicidal effect: It is used in diluted concentrations as disinfectant in the public health sector. (Gorman S. P. 1980), (R. A. Munton T. J. 1973) Based on its bacteria inactivating application, various concentrations of glutaraldehyde were mixed with the imprinted beads prevenient to remove the bacteria from the surface. Unfortunately SEM images showed partial, but incomplete removal of the imprinted E. coli cells. Most likely glutaraldehyde kills living microorganisms, but does not disrupt their cell compartments. This is not the desired result when one can assume that all bacteria cells are already dead after polymerization.

Experiments with organic solutions showed either no or only few imprints next to cells on the surface. Best results were obtained with a 3% glutaraldehyde solution as can be seen in Figure 27.

<table>
<thead>
<tr>
<th>#13/x</th>
<th>Washing procedure</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>96 % ethanol 72 h</td>
<td>Some imprints</td>
</tr>
<tr>
<td>B</td>
<td>96 % ethanol 24 h + ultra sonicatio</td>
<td>AFFECTED SURFACE</td>
</tr>
<tr>
<td>C</td>
<td>3 % glutaraldehyde</td>
<td>Some imprints</td>
</tr>
<tr>
<td>D</td>
<td>12 % glutaraldehyde</td>
<td>No imprints</td>
</tr>
<tr>
<td>E</td>
<td>50 % glutaraldehyde for 48 hours</td>
<td>AFFECTED SURFACE</td>
</tr>
<tr>
<td>F</td>
<td>3 % glutaraldehyde + ultra sonicatio</td>
<td>Some imprints</td>
</tr>
</tbody>
</table>

Table 6: Washing procedures with organic solutions
Figure 27: In-lens detector image from beads treated with a 3% glutaraldehyde solution, cavities of removed *E. coli* bacteria cells are circled in black

**Saline solutions**

Bacteria cell walls are permeable for water and small molecules to ensure correct osmolaric ratio and pressure inside the cell. Considerations of the known osmolaric effect on blood cells in hyper- or hypoosmolaric surroundings lead to the idea of using saline solutions to remove bacteria. Hyperosmotic relations cause shrinking of the cell, because water is pumped out to dilute the high salt concentrations of the osmolaric solution. In contrast, hypoosmotic (hypotone) environments cause water being taken up by the cell to reduce the concentration gradient between inside and outside. At one point, when too much water is absorbed, pressure in the cell exceeds the possible maximum and the cell disrupts. This leads to cell lysis and should be useful to destroy *E. coli* cells on the surface of the polymeric beads. Various concentrations of different salt solutions were tested plus a sugar solution relying on the same principle. Images showed that removal of the bacteria did not take place.
Acids and bases

For every cell there is a defined pH range for optimal reproduction rates, chance of survival, and living optimum. In the case of Escherichia coli, this pH range is around pH 7, so it is a so-called neutrophil bacterium. The pH range can be extended by approximately ±2 pH units without strongly harming the bacteria. When exposing E. coli cells to more acidic or alkaline media, cell functions will degrade: Especially proteins change their conformation. This causes cell death in most cases. (Simon E. W. 1951), (Krulwich T. A. 2011) Highly acidic conditions are used for food preservation with organic acids to prevent food spoilage. (Breidt F. 2004) While hydrochloric acid and enzymes are an important part of digestion, the acid plays a major in gastric juice to kill bacteria. Below certain pH levels bacteria such as E. coli die without the presence of pepsin. (Zhu H. 2006) Hence, acids and bases seem a suitable way to remove bacteria from bead surfaces.

All following results were obtained with beads prepared according to protocol #13:

A solution of 6 % NH₃ was prepared followed by incubating beads 24 hours. A second batch was kept in the ultrasonic bath for 30 seconds after incubation. The SEM images taken show that this procedure worked partially and left a couple of imprints on the surface (Figure 28). Nonetheless, most of the bacteria cells are still visible. Longer incubation time of five days did not show any positive effect at bacteria removal. Even fewer imprints are visible, than with shorter incubation time. Due to the first positive results the conditions of the ammonia solution were changed. In the next step fresh beads were incubated for 48 hours in concentrated ammonia (25 %) and washed with distilled water before drying. Another trial included six washing steps with 12 % ammonia solution for three minutes each. Both, concentrated and 12 % ammonia solutions, did not reveal any imprints in SEM images.
The stronger base sodium hydroxide (NaOH) was used for experiments to destroy *E. coli* cells on the beads without damaging their surface. For this a 10% NaOH was prepared and beads were incubated for 24 hours. Parts of the beads were ultra-sonicated for 30 seconds after the incubation and before drying. The 10% solution was further diluted 1:1 and 1:10 and as well beads were incubated 24 hours. SEM reveals some imprints, but the surface of the beads got battered when treating the beads with the 1:10 dilution of the 10% sodium hydroxide solution. (Bimboim H.C. 1979)

Besides bases also various acids were used for bacteria removal experiments. The following concentrated acids were diluted 1:10 for parallel incubation experiments. SEM images were taken of all trials. The chemicals used were concentrated formic acid (HCOOH 90%), concentrated nitric acid (HNO₃ 70%), concentrated phosphoric acid (H₃PO₄ 85%), concentrated sulfuric acid (H₂SO₄ 96%) and fuming hydrochloric acid (HCl 37%). The experiment with the diluted hydrochloric acid was carried out twice to hold the second batch into the ultra-sonic bath for 30 seconds after incubation time.

Figure 28: In-lens detector image of NH₃ (6%) treated beads; some cavities are circled in black
SEM images show that diluted hydrochloric acid left behind a few single imprints, but most of the bacteria cells still covered the surface. Treating the beads in the ultra-sonic bath did not remove any cells. Also formic acid and nitric acid caused some random, single imprints, but the use of phosphoric acid and sulfuric acid attacked the surface and removed bacteria with deforming the surface entirely. Sulfuric acid treated beads turned black during the incubation step, because the acid is an extremely strong oxidizing agent and decomposes organic compounds to carbon.

**Further chemical removal experiments**

There are reports in literature that mixtures based on a combination of TRIS-HCl (tris(hydroxymethyl)aminomethane), EDTA (ethylenediaminetetraacetic acid) and lysozyme secrete cell proteins through osmotic shock. (Vázquez-Laslop N. 2001), (Wooley R. E. 1974) EDTA is a chelating agent and binds cations that are necessary in Gram-negative bacteria outer cell membrane with the effect of dissociation of proteins and polysaccharides from the cell wall. EDTA induced disruption of the outer cell membrane lets lysozyme attack the cell wall with partial release of its peptidoglycan layers. TRIS-HCl buffer has an enhancing effect of EDTA on Gram-negative bacteria cells. (Wooley R. E. 1974)

The experiment based on this principle was carried out to lyse and fraction *E. coli* cells on the beads surface. 0.05 M TRIS-HCl buffer solution (pH 8) containing 45 µg/ml lysozyme and 3 µmol of EDTA per milliliter was prepared. As literature did not specify which EDTA derivative was used, both its sodium salt and the pure acid were tested: For Na₂EDTA 1.12 mg per milliliter buffer was used and for EDTA 0.88 mg/ml was needed. After mixing all components the buffer was adjusted to pH 8 with sodium hydroxide solution. Beads were kept in the respective EDTA-TRIS buffer solutions for one day and were afterwards centrifuged and dried. SEM images were taken and show for both experiments that beads are completely destroyed. This might be due to the long incubation time to ensure complete fractioning and removal of *E. coli* cells.
The paper of Vàzquez-Laslop Nora et al. indicates cell fractioning through salt solutions. The experiment was carried out with mixing a buffer containing 20% sucrose, 30 mM TRIS-HCl, 50 µg/ml lysozyme and 10 mM Na-EDTA. As above, both EDTA species were used, namely 1.12 µg/l for Na₂EDTA and 0.88 µg/l for EDTA. After mixing all components the buffer was adjusted to pH 8 with sodium hydroxide solution. The surface imprinted beads were kept in the different solutions for one hour at room temperature, centrifuged at 3000 rpm for 15 minutes and dried afterwards. Literature says that within this hour up to 99% of all bacteria cells become spheroplasts, which means that the outer cell wall is completely removed. (Vàzquez-Laslop N. 2001), (Liu I. 2006) SEM images show for both EDTA salts some imprints, but in total most bacteria cells still stick in the polymer and aren’t removed with this procedure.

Most likely EDTA- lysozyme- TRIS-HCl buffer combinations did not work because the paper only describes disruption and fractionizing of the outer membrane in Gram-negative bacteria, but does not lead to a complete deletion of the entire cell wall.

Hydrogen peroxide (H₂O₂) at various concentrations is a widely used bactericidal disinfectant. Its DNA damaging effect in Escherichia coli cause cell death. H₂O₂, the superoxide radical anion, and the hydroxyl radical are all formed by cells during oxidative stress. These highly reactive molecules can oxidize lipids and proteins among others. (Asad N. R. 1998) Based on this fact, H₂O₂ is used as a lysing agent to cause damages not only in the nucleic acid, but in the cell membrane of the Gram-negative bacteria, and cell rupture. Like the germicidal 3% H₂O₂ solution used in microbiology, a solution of the same concentration was used to treat bacteria imprinted polymer beads. The beads were incubated in hydrogen peroxide (3% solution) for 18 hours and for five days, respectively, and afterwards kept in the ultra-sonic bath for 30 seconds. Bacteria imprinted beads were placed 48 hours into concentrated H₂O₂ solution (35%) and afterwards washed with distilled water. After decanting the liquids, the different treated beads were dried and SEM images were taken. Image 29 shows some imprints on the surface and a partly working method for bacteria removal. Complete and full removal was never possible though.
In a further attempt different chemical agents with different interaction points with *E. coli* bacteria were combined to definitely disrupt a weak cell wall in two or more steps. Therefore the bactericidal effects of lysozyme and SDS (sodium dodecyl sulfate) solution in combination with osmotically active saline solution or bases were used. Additionally, also short time exposures to mechanical cell wall destruction by ultrasound were examined. The executed washing procedures with the bacterial imprinted beads #13 are listed below.

The beads were treated with a 1:1 (v/v) mixture of 0.1 mg lysozyme (pH 8.6) and 30 mM sodium chloride solution at 37°C for 5 minutes. Afterwards they were washed with a 100 mM sodium hydroxide and 1 % SDS mixture with ration 1:1 (v/v). Followed by a five times washing with distilled water. A second attempt was carried out in the exact same way like described above, besides the fact that the beads were treated for 30 minutes at 37°C instead of five minutes.

The experiment was redone with a different concentration of lysozyme. This time a 1 µg/ml solution of lysozyme at pH 9.3 was used in a 1:1 (v/v) ratio with
30 mM NaCl solution and incubated with beads for 5 minutes at 37°C. The procedure was continued with a 1:1 (v/v) mixture of 100 mM NaOH and 1 % SDS solution, worked up with washing the beads five times with distilled water. In the second attempt the incubation time was again elongated to 30 minutes.

A reaction mixture of 0.1 mg/ml lysozyme at pH 8.6, 30 mM sodium hydroxide solution and 1 % SDS solution in a 1:1:1 (v/v) ratio was admixed with imprinted polymer beads and afterwards washed with distilled water five times. A second test was sonicated 45 seconds in the ultra-sonic bath before being washed with water.

1:1 (v/v) mixtures of 100 mM NaCl and 1 % SDS solution were intermixed with beads and one batch was sonicated for 45 seconds before washing, while the other batch was directly washed with distilled water five times. Figure 30 shows the effect after treating the beads with 100 mM NaCl plus 1 % SDS solution and several washing steps. In the same manner mixtures of 50 mM sodium chloride and 1 % SDS with ration 1:1 (v/v) were tested as well as a 1:1 (v/v) mixture of 1 M sodium hydroxide and 1 % SDS solution.
Interestingly the experiments with the 1 M sodium hydroxide and 1 % SDS mixture dissolved the beads with and without ultra-sonication treatment before performing the final washing steps. The attempt with the 1:1 (v/v) mixtures of 100 mM NaCl and 1 % SDS solution followed by 45 seconds sonication resulted in the same effect.

SEM images of the other reaction mixtures show some randomly placed imprints, but widely covering of the surface with *E. coli* cells. It needs to be assumed that the few imprints are not caused by the chemical treatment. Probably bacteria cells, which are not strongly bound during the polymerization step, might be removed already in earlier steps, before bacteria removal acts are undertaken.
**SDS as a lysing agent**

Several papers talk about the use of sodium dodecyl sulfate (SDS) as a lysing agent for bacteria cell walls. SDS is the commonly used surfactant for protein denaturation in electrophoresis. (R. A. Munton T. J. 1972), (Chen S. 2017)

A 0.05 mM SDS solution was prepared and adjusted with hydrochloric acid to pH 3 and with sodium hydroxide to pH 8 according to literature. The beads were added to the two solutions and kept at 40°C in the water bath for one hour. Afterwards the mixture was ultra-sonicated for 30 seconds, centrifuged and dried. SEM images of the sample at pH 8 show no removal of the *E. coli* cells. (Munton T.J. 1972)

The paper of Chen S. et al. working with *Escherichia coli* based biosensor uses another SDS recipe for bacteria template removal. (Chen S. 2017) 5 wt % acetic acid/ SDS solution was incubated with the bacteria imprinted beads for 18 hours. The original paper does not state the exact composition of the AAc/SDS mixture. Therefore, two different solutions were prepared: On the one hand 5 wt % SDS solution was used to dilute concentrated acetic acid 1:20 (50 mg acetic acid + 950 5 wt % SDS solution), on the other hand equal parts of concentrated acetic acid and SDS solution were mixed to a 5 wt % acetic acid/ SDS mixture. For this 25 mg conc. acetic acid were mixed with 25 mg of a 5 wt % SDS solution. Polymeric beads were incubated with the prepared solutions each for 48 hours, afterwards centrifuged and dried.

SEM images of both samples show abrasion of the polymer surface including all the bacteria cells. No imprints are visible anymore after this comparably harsh procedure.
6.5 Stamp imprinting

After it turned out that completely removing bacteria cells from the beads surfaces was a challenge, the same system was tested in a two-dimensional setting. For this case covalent stamp imprinting was used to create MIP layers for QCM settings. To obtain bacteria stamps the following two protocols were tested:

1. Glass slides were cleaned for 5 minutes each in ethyl alcohol and distilled water, respectively, and afterwards 20 minutes in Piranha solution (a mixture of sulfuric acid and hydrogen peroxide (30 %), 3:1 v/v). Then the glass slides were dipped into distilled water to wash off cleaning solutions and dried with argon gas. The glass slides were chemically functionalized with 3 % APTES (3-(aminopropyl)triethoxysilane) in toluene for 2 hours to introduce amino groups to the surface. No plastic petri dishes could be used in this step because APTES/toluene mixtures cause plastic-to-glass sealing; glass slides could not be removed from the plastic dish afterwards. After functionalization the glass slides were dipped into toluene for washing off excess APTES and dried with argon gas. By adding 3 % (v/v) glutaraldehyde in phosphate buffer (pH 7.4) for 2 hours, the amino groups induced were modified. Dipping the slides in phosphate buffer removed excess glutaraldehyde and was followed by dipping into distilled water and blow drying with argon gas. A suspension of E. coli cells (bacteria pellet plus 10 ml distilled water ~ 10⁸ CFU/ml) was added in 200 µl amounts each onto the glass slides. The suspension was subsequently drying at room temperature overnight. During this step, bacteria cells covalently bound to the glutaraldehyde tails. The next day the glass slides were dipped into distilled water and dried with argon gas. In a closed petri dish, stamps can be kept at 4°C in the fridge for several weeks. (Perçin I. 2017 (17)6, 1375)

2. In the other stamp recipe glass slides were cleaned 10 minutes each in the ultrasonic bath with 1 molar hydrochloric acid, 1 molar sodium hydroxide solution and pure 96 % ethanol, respectively. Between each steps the glass
slides were dipped into distilled water and after the final cleaning step dried with argon gas. Amino groups were introduced onto the surfaces of the clean glass slides by using 10% APTES in ethanol (v/v) mixture for one hour at room temperature. Then the slides were dipped in ethanol to remove unbounded APTES and dried with argon gas. Modified slides were dunked into a 5% glutaraldehyde in 10 mM sodium phosphate buffer (pH 7.4) solution for two hours at room temperature. Unbound glutaraldehyde was washed off by dipping the slides into phosphate buffer and afterwards distilled water before drying them with argon gas. For *E. coli* binding a suspension (~10^8 CFU/ml) was dropped onto the glass slides and dried at room temperature overnight. The next day the glass slides were dipped into distilled water and dried with argon gas. In a closed petri dish, stamps can be kept at 4°C in the fridge until use. (Idil N. 2017 (87))

Both stamp recipes show constant and complete coverage of the glass slide. Covalently bound *E. coli* cells are not removed in imprinting procedures or further washing steps with distilled water.

To find the pre-polymer gel point the respective monomer mixture was heated to 70°C in a water bath. When the organic phase starts to become viscous the gel point is reached and reaction is immediately stopped by immersing the reaction tube into an ice bath. The experiments were carried out as followed: mix of 0.5 ml styrene and 0.5 ml DVB, adding 20 mg of AIBN and purging with argon for ten minutes. Afterwards it was polymerized in the water bath. After seven to eight minutes, depending on the exact amount of initiator added, the polymer reached its gel point and the reaction was stopped in an ice bath. Then the viscous pre-polymer was spin coated in a thin layer on cleaned glass slides and a bacteria stamp was pressed onto it. After full polymerization overnight in the oven at 70°C the stamp was carefully removed without peeling the polymer layer off. Both stamp surface and imprinted polymer layer were characterized with AFM as shown in Figure 31 and 32. Both recipes work perfectly fine and are straightforward in their procedure. Benefits of the recipe of Idil N. et al. are the absence of piranha solution, which makes the work
less hazardous and the less time consuming step when introducing covalently bound amino groups with the APTES solution.

In Figure 31 and 32 the AFM images of the stamp surface of the two described protocols are shown. Bacteria cover the stamp all over without clotting.
Both AFM images show the homogeneous spreading of the cells all over the stamp. Dimension measurements on the right-hand side show that the bacteria cells have usual *E. coli* size: Furthermore, they protrude around 600µm from the surface, which allows for assuming that more than the half of the cell width is protuding.

![AFM image of cells spreading](image)

**Figure 33: E. coli imprints in styrene and DVB on glass slide**

Figure 33 demonstrates the ideal situation after pulling off the stamp from the polymer. Many clear imprints are visible and measurements show that their sizes match the average *E. coli* cell.

After these successful pretests, the system was tested on QCM to obtain the corresponding bacteria sensors. The pre-polymer consisted of 0.5 ml styrene, 0.5 ml DVB and 20 mg AIBN, followed by argon purging and polymerization in the water bath at 70°C for 10 minutes and 30 seconds. Then 10 µl of the pre-polymer were spin coated for 10 seconds at 2000 rpm onto the surface. A stamp produced after the recipe described in chapter 6.5, with only one hour of glass slide functionalization, was pressed with clamps onto the polymer and hardened in the oven over night. The next day, the clamp and stamp was removed carefully.

Generally, difficulties occurred when pulling off the stamp from the polymerized quartz surface, because the very hydrophobic polymer layer did not stick to the
quartz surface and was easily peeled off when removing the stamp. Attempts to soften binding of the stamp to the polymer by soaking the quartz in distilled water did not lead to the desired effect. Modifications of the gold electrodes on the quartz surface were carried out to make the lipophilic polymer stick to the quartz.

6.6 Modifications of the quartz surface and the polymer

Based on various ways found in literature, the gold electrodes of the quartz were modified with thiol containing chemicals, including 3-mercapto-1-propanoic acid, 11-mercapto-1-undecanol, 1-dodecanethiol and 1-octadecanethiol.

The quartzes were pretreated in different ways.

- Ultra-sonication in acetone for 10 minutes followed by quick dipping into piranha solution and water before drying with argon gas. Keeping the quartzes too long in the piranha solution (sulfuric acid and hydrogen peroxide, 7:3 v/v), led to removal of the gold electrodes.
- Ultra-sonication in acetone for 10 minutes
- Dipping the quartz into the piranha solution and water, blow-dried with argon gas and irradiated them for 5 minutes with UV-light at 365 nm.
- Dipping into piranha solution, washing with water and 96 % ethanol, and dried with argon gas.
- Quartzes were placed in an acetone bath for 10 minutes, followed by washing with water and 96 % ethanol and dried under argon gas.

The single pretreated quartzes were incubated over night with 10 mM solutions of each thiol reagent mentioned before. The next day the 20 resulting quartzes were washed with 96 % ethanol and dried under argon gas flow.
Besides these surface preparations, the treatment mentioned in the paper of Ramirez P. et al., was assessed. Therefore QCM were placed each into a 5 mM solution of 3-mercaptoproprionic acid, 11-mercapto-1-undecanol, 1-dodecanethiol and 1-octadecanethiol overnight. The solvent used in these experiments was a mixture of distilled water and pure ethanol in the ratio 1:3 (v/v). The next day, the quartzes were dipped in pure ethanol and dried with argon gas. (Ramírez P. 2008)

On each quartz 30 µl of the poly(styrene-co-DVB) pre-polymer was spin coated on the electrode surface. The NIP side was covered with a cleaned glass plate, while on the MIP side a bacteria stamp was pressed. Stamp and glass slide were covered with a glass slide. Polymerization was finalized in the oven at 70°C overnight. After that the chips were taken out and placed in a water bath to soften the stamp and glass slide over the polymeric layer. Modifications of the surface led to slightly better adhesion of the polymer to the quartz electrodes. It is notable that pretreating the quartz by dipping it into the piranha solution and water, blow-drying them with argon gas and final irradiation under UV-light at 365 nm for 5 minutes; 3-mercaptoproprionic acid, 11-mercapto-1-undecanol and 1-dodecanethiol show much better adhesion of the polymer on the quartz surface. Only QCM treated with 1-octadecanethiol showed partial removing of the gold electrode when pulling the stamp off.

Further tests included polymers containing sulfur groups to achieve strong interaction between the gold surface and the thiol groups in the polymer. The batches summarized in Table 8 were pre-polymerized at 70°C in the water bath before 50 µl of each mix was dispersed on the quartz surface and stamp and glass slide were placed on the gold electrodes. Full polymerization was carried out in the oven at 70°C overnight. The next days, quartzes were placed in a water bath to remove the glass slide and stamp easier.
For polymer 5 and 6 sensitive layers were obtained on the quartz surface, the other mixtures did not show any better adhesion effect of the polymer on the quartz and most experimental set-ups broke when removing the stamp.

### 6.7 QCM Measurements

For the QCM measurement a quartz was coated with styrene/DVB pre-polymer mixture. On each electrode 4 µl of polymer were pipetted. On the MIP channel a bacteria stamp was pressed onto the electrode, while a glass plate was pressed onto the NIP side. After polymerization in the oven at 70°C overnight, the stamps were removed the next day. The quartz was put in the measuring cell and the above volume was filled with distilled water through the small plastic pipes in the cap. The analyzer was adjusted to the quartz’s frequency and it was waited for a stable baseline signal. (Figure 34)
When adding the first bacteria concentration (~E8 CFU/ml) a frequency shift of around 6000 Hz is recorded. For washing, ten times distilled water was flushed through the tubes and free volume above the quartz. The line went back to base line, which indicated that the washing step was successful. Adding a 1:1 (v/v) dilution of the concentrated *E. coli* solution led to a smaller frequency shift of only a few Hertz. Removing the bacteria from the quartz surface was again successful with ten times distilled water as washing steps. As control a second time the concentrated *E. coli* solution (~E8 CFU/ml) was injected into the measuring cell. A frequency shift of about 3000 Hz was registered, but far less than the first time inserting the bacteria solution. The experiment was continued with a 1:5 (v/v) dilution of the highly concentrated bacteria solution and followed by a final use of the concentrated *E. coli* solution. The frequency shift of the concentrated *E. coli* solution was always different, which might indicate some kind of saturation of the quartz surface and cavities occupation. Nevertheless this experiment showed that distilled water as washing step works perfectly to remove the *E. coli* cells from polymer cavities.

**Figure 34: QCM measurement of various *E. coli* concentrations**
6.8 Bacterial imprinting with Pickering emulsion based on N-acrylchitosan (NAC)

Bacteria stabilized Pickering emulsions are also producible with various phase conditions. For the oil phase, modified polysaccharides form stable emulsions with \textit{E. coli} bacteria in an aqueous buffer solution. The article of Lei Ye et al. shows impressive \textit{E. coli} bacteria removal with a chitosan based polymer for Pickering emulsions. (Shen X. 2014) In this work the principle of bacteria-stabilized oil-in-water emulsions was used to mix an aqueous phase of bacteria treated \textit{N}-acrylchitosan as monomer with the oil phase containing the cross linker dispersed in an aqueous buffer. The bacteria cells form a network with the functionalized chitosan monomers, which are stabilizing the oil-water-interface of the afterwards produced Pickering emulsion. (Figure 35)

![Network formation](image)

\textit{E. coli} bacteria cells + \textit{N}-acrylchitosan → \textbf{Bacteria-pre-polymer complex}

Figure 35: Bacteria-chitosan network

The initiator for the polymerization is benzoyle peroxide (BPO), which is a radical starter initiating the reaction at room temperature. The molecule dissociates at room temperature into two benzoic acid radicals and one molecule oxygen. (Hamdu H. 2014) After polymerization of the oil phase at room temperature, the bacteria are removed by solvent extraction. (Figure 36) (B. J. Shen X. 2014)
The first step in the synthesis of the bacteria imprinted polymers was the functionalization of chitosan to N-acrylchitosan. In a mixture of 2 ml trimethylamine and 40 ml N,N-dimethylacetamide (DMAC) 1.61 g of low molecular weight chitosan was dissolved in an ice bath. The solution was purged with argon gas for 10 minutes, to remove present oxygen while being cooled. A solution of 5 ml DMAC with 161 µl of acryloyl chloride was added dropwise to the precooled and purged solution present in the two-neck round-bottom flask. Stirring the mixture for 4 hours at 0°C was followed by stirring for 20 hours at 25°C in the water bath. The lightly yellow powder formed was filtrated via a suction strainer and washed with DMAC, followed by dichloromethane and methanol. The obtained N-acrylchitosan powder was dried in the vacuum chamber overnight.

The next step comprised modification of the aqueous NAC monomer phase with E. coli cells to form a functionalized network. For that purpose, 9 mg of as-synthesized NAC were dissolved in 30 ml of a 0.03 % acetic acid solution. 0.3 ml of this prepared mixture was mixed with 0.9 ml of an E. coli suspension (bacteria pellet was dissolved in 10 ml of a 10mM phosphate-buffered saline at pH 7.4) and incubated for 30 minutes at 37°C. The oil phase contained a mixture of DVB and TRIM (1-(2-Trifluoromethylphenyl)imidazole) in the ratio 1:1 (v/v), 31.3 µl of dimethylamine (DMA) and 6.2 mg of benzoyl peroxide (BPO). The oil phase was incubated for 30 minutes at 37°C as well. After incubation the aqueous phase was poured into the oil phase falcon tube and shaken by hand. A stable Pickering emulsion was formed and polymerized at room temperature for 24 hours. The experiment was repeated with medium weight chitosan as starting chemical under the same procedure.
Low molecular weight chitosan, formed stable Pickering emulsions, but did not cure during the polymerization. Also after several days and heating the mixture to 50°C the Pickering emulsion did not harden to imprinted polymeric beads. With the achieved N-acrylchitosan of low molecular weight the polymerization was carried out as described in literature besides taking AIBN (azobisisobutyronitrile) as the radical starter. The amount used of AIBN is 4.2 mg equivalent to the primarily used BPO. In contrast to the prior formed Pickering emulsion when using benzoyl peroxide, the two phases decomposed at the AIBN based reaction.

The washing protocol was investigated though on the styrene/DVB bacterial imprinted beads, if the E. coli cells of the bead’s load #13 could be removed. In the paper the bacteria are removed by washing the beads six times with a 10 % acetic acid (v/v) solution containing 1 % SDS (m/V), followed by six times washing with distilled water and two times with methanol. The treated beads were dried in the vacuum chamber and SEM images were taken. SEM images show that only a few bacteria cells were removed from the polymeric surface. Complete removal was not possible with these washing steps.
7. Results and Discussion

Creating *E. coli* stabilized bacteria MIP beads is straightforward, inexpensive, does not need special equipment. On top of that, it is a safe and quick procedure when using poly(styrene-co-divinylbenzene). Nevertheless removing the bacteria from the surface without destroying the polymer itself is challenging and leaves questions unanswered. During this work efforts were undertaken by using many bactericides and cytotoxic compounds to lyse the bacteria cells. Some reactions are too aggressive for the polymer and lead to abrasion and dissolution of the artificial macromolecule such as sulfuric acid, sonication and long-time glutaraldehyde. In collaboration with another Master student of the group it was possible to use Raman spectroscopy to analyze if the *E. coli* bacteria cells were covered by the polymeric oil phase during polymerization or were situated more than half of their volume inside the polymer. This might be a reason why it was not possible to remove the cells from the surface with the experiments carried out. Due to instrumental limits it was not possible, however, to focus the laser beam on a small area like the interface of bacterium and polymer. The spectra of the system show typical bacteria bands as well as polymer bands. Light microscope and scanning electron microscope are not designed to yield three-dimensional information or revealing if the polymer covered the bacterium fully or partly. Should the bacteria be covered somehow by the polymer or embedded in the polymer with more than half of their volume, removing is physically not realizable without demolishing the surrounding polymer. Under the conditions tried in the laboratory the production of imprinted particles was not possible and adequate and satisfying result for further bacteria concentration measurements were not achieved.

It can be said that the use of benzoyl peroxide may have a positive effect on *E. coli* cells during polymerization. Bacteria cells can survive polymerization due to the reaction at room temperature, which is less harsh than hardening in the oven at 70°C when using AIBN. During this work it has not been shown how the cells withstand the highly toxic properties of styrene or if the cell organism suffer under the environment of styrene and cell death occurs when forming styrene based
emulsions. (Mingardon F. 2015) Due to this poisonous quality of styrene the use of chitosan, a poly amino sugar, as monomer base may lead to viable *E. coli* cells on the bead surface.

When preparing QCM with thin MIP films, removal of the stamp on the molecularly imprinted side and the glass slide on the non-imprinted side proved to be difficult. Hydrophobicity of the poly(styrene-*co*-divinylbenzene) layer causes difficulties trying to produce thin, agglutinated layers on the quartz surface. Experiments showed that great amounts of pre-polymer show decreasing affinity on the quartz surface and formed more likely a not affiliated layer with the surface. Volumes in the range of 5 - 50 µl are sufficient for electrode covering without forming too thick and therefore cohesion and repulsive induced spalling. Finding the best parameters for stamp imprinted QCMs for the polymer mixture of styrene and DVB could still be optimized. QCM measurements carried out with different *E. coli* concentrations show that the principle of the mass sensitive device worked. Washing steps and removing the cells of the QCM surface was successful, further experiments with QCM measurements and improving the process may lead to constantly working devices of poly(styrene-*co*-divinylbenzene) sensitive layers for *E. coli* detection.
8. Bibliography


Hamdu H., H. „An isocratic normal-phase high-performance liquid chromatographic method for the simultaneous determination of benzoyl peroxide and benzoic acid in one pharmaceutical preparation and their stability in different solvents." *IOSR Journal of Pharmacy and Biological Sciences*, 2014: Volume 9, Issue 1, 4-12.


9. Appendix

9.1 Abstract

Pickering emulsion poly(styrene-co-divinylbenzene) MIP using *E. coli* as templates led to stable particles that required further optimization. Bead size was controlled by varying the bacteria. Beads were characterized by scanning electron microscopy. Removal of the bacteria from the bead surface was carried out with various mechanical and chemical treatments. Unfortunately none of the tested strategies lead to complete removal of imprinted cells. Partial removal probably did not occur through the treatment, but most likely cells are broken off through mechanical abrasion in uncontrolled handling steps. In general this work shall help and indicate others later on the difficulties of bacteria imprinted beads and their removal.

The success of surface imprinted sensitive layers of poly(styrene-co-divinylbenzene) is shown in chapter 6.5. Two stamp generating recipes are established in this work and show straightforward and sufficient imprinting. This working system is used for surface imprinting of quartz crystal microbalance devices and measurements with *E. coli* solutions are carried out.
9.2 Zusammenfassung


Erfolgreich wurden zwei Stempel für E. coli geprägte Oberflächen etabliert, welche vielversprechende Resultate für sensitive Poly(styrol-co-divinylbenzol) Schichten ergaben. Die im Kapitel 6.5 gezeigten Techniken sind einfach und führen zu ausreichender Prägung, welche für Oberflächenprägung auf Quarz Kristall Mikrowaagen genutzt wurde. Dieses funktionierende System wurde für Messungen von E. coli Konzentrationslösungen verwendet.