SIP-HTM based noro-virus sensor using virus-like particles

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Abstract

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Surface imprinted polymers (SIP) are biomimetic receptors that have active binding cavities on their surface, and that selectively bind particles similar with template particles used in the production process. SIPs can be synthesized in a few ways, and since 2001 SIPs have been used in many applications. When a SIP is coupled with a detection platform, it can act as a biosensor revealing the presence of small particles such as viruses. SIPs have been coupled with a quartz crystal microbalance (QCM), impedance spectroscopy, surface plasmon resonance and thermal methods. In the group of Prof. Patrick Wagner at KU Leuven, a heat transfer method (HTM) is being developed that observes the change in thermal conduction due to binding of the particles on the surface of the polymer.

Virus-like particles (VLP) are particles that resemble viruses but are not infectious since they do not contain the genetic material inside. Vesa Hytönens group at Tampere University has been synthesizing Noro-VLPs in insect cells using the baculovirus expression system. The ultimate goal of the collaboration project is to produce SIP-HTMs using -VLPs as template particles, to create a biosensor that could be used to detect human viruses from liquid samples. In this Thesis work, some of the necessary fabrication steps were explored. Viruses have not been detected before using SIP-HTM and such detectors could be invaluable tools in controlling epidemics and monitoring the environment, for example with food safety issues.

In this thesis I introduce general methods used for virus detection and also the SIP-HTM sensor that is tried to be fabricated in this project. The adsorption of the VLP-particles on a PDMS surface is a vital part of the fabrication of the SIP. I have experimentally studied the adsorption of VLPs on different surfaces with varying properties. A few protocols were found following which the VLP adsorption could be optimized. Using AFM and HIM, extensive imaging of VLPs and SIPs has

been performed during this fabrication step. Images showed surface structure and topology of SIPs made using VLPs and yeasts as template particles.

Keywords: Surface imprinted polymer, biosensor, norovirus, adsorption, HIM

Tiivistelmä

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Pintaprintatut polymeerit (Surface Imprinted Polymers, SIPs) ovat biomimeettisiä reseptoreja, joiden pinnalla on aktiivisia sitomiskuoppia. Nämä kuopat sitovat selektiivisesti samanlaisia partikkeleita polymeerin pinnalle kuin ne, joita on käytetty kyseisen polymeerin valmistuksessa. Pintaprintattuja polymeerejä voidaan valmistaa muutamalla eri tavalla ja niitä on käytetty monissa sovelluksissa. Kun pintaprintatun polymeerin yhdistää sensorialustaan, toimii yhdistelmä biosensorina, joka voi havaita pienten partikkelien, kuten virusten, läsnäolon nestemäisistä näytteistä. Sensori muuttaa tiedon pinnalle adsorboituneesta partikkelista sähköiseksi signaaliksi esimerkiksi mittaamalla polymeerin massaa (QCM). Prof. Patrick Wagner KU Leuvenin yliopistosta on kehittänyt ryhmänsä kanssa metodin (Heat Transfer Method, HTM), jossa partikkeleiden läsnäolo pinnalla havaitaan pinnan muuttuneiden lämmönjohto-ominaisuuksien perusteella.

Viruksen kaltaiset partikkelit (Virus Like Particles, VLP) ovat virusta muistuttavia partikkeleita, jotka erottaa viruksista vain geneettisen materian puute. Vesa Hytönen ryhmänsä kanssa pystyy tuottamaan synteettisesti noroviruksen kaltaisia partikkeleita, Noro-VLP:tä. Tämän tutkielma liittyy yhteistyöprojektiin, jonka tarkoituksena on valmistaa SIP-HTM sensoreita käyttäen Noro-VLP partikkeleita valmistuksessa. Sensoria voitaisiin käyttää noroviruksen havaitsemiseen nestemäisistä näytteistä. SIP-HTM pohjaisia sensoreita ei ole vielä käytetty virusten tutkimiseen mutta ne voisivat olla arvokkaita työkaluja ympäristön tarkkailuun ja epidemioiden kontrollointiin.

Tässä tutkielmassa esittelen virusten havainnointiin käytettyjä yleisiä tekniikoita ja kerron myös SIP-HTM sensorien valmistuksesta. VLP partikkeleiden adsorptio PDMS:n pinnalle nesteestä on tärkeä askel sensorin valmistuksessa. Tässä tutkielmassa olen tutkinut Noro-VLP adsorptiota ja siihen vaikuttavia parametrejä. Muutama

protokolla löydettiin, joiden avulla VLP adsorbtiota PDMS pinnalle pystyttiin parantamaan. Esittelen myös atomivoimamikroskoopilla ja heliumionimikroskoopilla ottamiani kuvia pintaprintatuista polymeereistä ja Noro-VLPstä. Kuvista näkyy hyvin pintaprintattujen polymeerien pintarakenne. Kuvatuissa näytteissä mallipartikkeleina oli käytetty Noro-VLPtä ja hiivasoluja.

Avainsanat: virus-sensori, SIP, norovirus, adsorbtio, mikroskopia

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1 Introduction

This Master's Thesis is written concerning a project where a virus sensor for human noroviruses is tried to be fabricated. The project is a collaboration between three research groups located at the Catholic University of Leuven in Belgium (KU Leuven), at Tampere University and at the University of Jyväskylä. My part of this project was originally intended to be working as a member of the sensor development research group at the KU Leuven in the spring of 2020, but just after six weeks in Leuven I had to return to Finland due to the Covid-19 pandemic. The collaboration continued so that I worked here in Jyväskylä with samples that were sent from Leuven and Tampere.

The Thesis is split into two parts. In Part I, I introduce general virus detection methods and the basics of virology needed for understanding them. I also introduce SIP-HTM sensors, the type of sensors attempted to develop in the collaboration project. In Part II, I describe the fabrication process of the sensors in more detail, and introduce the work done by the collaborating research groups. I present my work done for optimizing a particular sensor fabrication step and imaging of samples related to that process, using atomic force microscopy and the Helium ion microscope.

Part I

Overview of virus detection techniques

2 Brief introduction to virology

2.1 Viruses

Microorganisms, or microbes, are microscopic unicellular organisms consisting of only a single cell. The group of microorganisms is extremely diverse. It consists of all bacteria and archaea but also some eukaryotes. Viruses are also considered to be microbes even tough they are not cellular. Viruses are defined to be genetic elements that are not able to replicate independently outside a host cell. Viruses rely on the host cell also what comes to protein synthesis, metabolism and energy generation and therefore viruses can be considered as parasites. The inability for independent function is what separates viruses the most from other microorganisms and the reason why viruses are classified as non living particles.

All viruses consist of nucleic acid and a protein coat, capsid, surrounding it. Sometimes the capsid is surrounded with a bilipid layer, envelope. Viruses can also carry some macromolecules such as enzymes. There is great variation in virus particle shape, size and chemical composition. Most viruses have a size between 0.3 to 0.02 µm. All living cells contain double-stranded DNA molecules but viral genome can be either DNA or RNA, double or single strained and either linear or circular. Viruses are often classified regarding the type of nucleic acid they contain. [1]

2.2 Reproduction, transmission and infection

Viruses replicate by forcing a host cell to synthesise all components that are needed for new viral particles. The reproduction mechanisms of viruses have great variation but regardless of the virus type, the replication process can be described via few main steps. First of all a virus has to attach to the host cell surface, which is followed by the virus penetrating into the cell. Either the whole particle enters the cell or only the viral nucleic acid can be injected in from outside. The next phase is that viruses

have to use host cell metabolism to synthesise the components for new viruses. The synthesis is followed by assembly of fabricated particles into capsids that have the viral nucleic acid inside. After this, the new virus particles have to escape from the host cell. [1]

In order for viruses to survive they have to spread from one suitable host to another, and this transmission can happen via many means. Viruses can spread through airways or direct contact between two individuals but the transmission can also be food, water or surface mediated. Some viruses can spread through different species. For example, rabies virus is maintained in infection cycles among animals but it can be transmitted also from animal to human. [2]

Infection is a name for the process when a virus enters into a cell. Viral activity in the cell can be destructive and lead to the development of a disease in the host organism. In these cases the viruses are called pathogens, organisms that can cause a disease. Antigens are specific pathogen molecules or molecular structures that interact with the immune system and usually triggers an immune response. Antigens are targeted by antibodies, soluble proteins that the human immune system starts to produce after antigen exposure. Antibodies circulate in blood and in case of target antigens are encountered they interact with them which can lead to different results. Antibodies can interact with antigens by binding to them and marking them to be destroyed by immune cells. Some antibodies can neutralize target particles. [1] In case of antigens of viruses the neutralization means the antibody binding on the virus and inactivating the virus infectivity. This can happen via many ways, for example, antibody binding can inhibit the virus adsorption to cell surfaces, lead to aggregation of virus particles or disturb the process in which the viruses of viral genome penetrates into a cell. Usually same antigen can be neutralized in multiple ways. [3]

2.3 The fight against viruses

Since viruses can cause diseases there is an obvious need for controlling the spreading of viruses in the population. The best way to prevent the spreading of the virus is artificial immunization of the population and it is a major tool of public health disease control programs. [1]. This means generating artificially a state similar to immunity one gains after a natural infection that initiates an adaptive immune response. In this state the body contains antibodies that can prevent a new infection

initiated by the same virus type. There are two types of immunity that can be artificially generated, artificial active immunity and artificial passive immunity.

When an artificial active immunity is generated a controlled amount of harmless antigen is exposed to the body. This process is known as vaccination, and it activates the human immune response that eventually leads to antibody production. Often active immunity remains throughout life.

One can also prevent the virus infection with an injection of antiserum or purified antibodies into the body, which generates artificial passive immunity. This does not initiate a response of the immune system, thus providing only temporal, rapidly decaying immunity to the injected individual. The body is not capable of fabricating antibodies by itself so the immunity is dependent on the injected antibodies.

Even though many dangerous viral diseases have been practically overcame due to vaccination there are still many viral infections against which there are no vaccines available. There are multiple reasons for the inability to generate artificial immunity against some viruses. In many cases diseases, that do not generate long lasting immunity are induced by viruses that mutate so fast the immunity developed against the virus one day does not have an effect on the virus type it is mutated into some time later. Few examples of viruses like this are influenza virus and malaria. Some viruses have multiple strains, which all have to be taken into account when developing a vaccine. This makes vaccine development difficult. Also, non-scientific reasons count for the lack of some vaccinations. For example, vaccine development can be very expensive and time consuming. [1]

In case of viruses that can not be controlled via vaccination other measures needs to be taken to prevent the virus spreading in population. One of these means is effective virus detection. The importance of availability of suitable virus detection tools can be observed from the world during the COVID19 pandemic. The virus detection has enabled tracking of the virus spreading in population indicating when more extreme spreading prevention, like restricting movement and interactions of the people, have to be considered. The detection has hindered the virus spreading between countries due to proper testing of travellers. In chapter two I introduce the main tools used for virus detection.

2.4 Modified viruses in biotechnology

In biotechnology it is important to be able to modify viruses and produce its parts. Modified virus particles, that are not dangerous but can initiate the host immune system, are needed for example for vaccine development. Virus-like particles (VLP) are one example of modified virus particles that are very useful in biotechnology research. Virus-like particles are particles that resemble viruses but do not contain any nucleic acid. Basically VLPs are empty protein capsids that are not able to replicate or create infection. VLPs are useful tools in biotechnology since they can be used instead of viruses without safety measures similar to ones required when working with actual viruses. [4]

3 Microscopy in virology

Research of microorganisms and viruses require microscopes, since particles studied are far from the size that the human eye can observe. What comes to virus research even microscopes using visible light do not have a resolution high enough because the size of observed particles, below 0.2 µm, is smaller than the wavelength of visible light. First, the limitations set by the visible light wavelength were overcome in the early 20th century when an electron microscopy was developed. This led to major advancement in virology, since viruses that were earlier only assumed to exist could now be observed with a microscope. [5] Even though electron microscopes are still important tools in virology research, there are also multiple other microscopes that can be used to observe and study viruses. As microscopes are such important tools in virology and also in our studies, I will now introduce a few microscopes with high enough resolution to reveal viruses.

3.1 Electron microscopy

Electron microscopes are microscopes that use electron beams to construct an image of the sample observed. Electron microscopes reach better resolution than optical microscopes since the de Broglie wavelength of electrons is about 0.01nm. There are two types of electron microscopes: transmission electron microscopes and scanning electron microscopes.

Transmission electron microscope (TEM) can be used to observe thin specimens, with a thickness of 10-100 nm, that allows electron beams to pass through it. The common setup of transmission electron microscopy includes cathode, from where electrons are accelerated with a voltage of 10-100 kV, and "lenses" that are magnetic fields used to modify the electron trajectories. The electron beam passing specimen and couple of lenses produces magnified images of the specimen on a detector. The resolution of TEM is considered to be 0.5 nm and it is restricted by the deviation of the speeds of accelerated electrons. The speed of electrons determines the focal length of magnetic lenses and the resolution is restricted by the electron speed variation in

a beam. [6] Transmission electron microscope is rather analogical to a conventional light microscope in which the light comes trough the sample and is focused via lenses to eye. The difference is that in TEM the lenses are solenoids that create magnetic fields which guide the electron beam, and thus the beam does not interact with other matter than the sample itself. And of course, the image does not focus on the eye but on a detector surface. [7]

Scanning electron microscopes (SEM) differs from TEM in that the electron beam does not go through the specimen. In SEM the electron beam is focused as a fine line that sweeps the specimen surface. The electron beam induces releasing of secondary electrons from the sample surface that are then collected to a detector. Secondary electron production on the sample surface is dependent on the angle the electron beam hits the surface, so surface topography can be deduced from the amount of secondary electrons ending up to the detector. The resolution of SEM is considered to be approximately 10nm.

Both electron microscopes operate in a vacuum because any excess molecules would cause electron scattering resulting in image blurring. Imaging biological samples in a vacuum usually requires some sample preparation, since the vacuum destroys features of biological particles containing water. The biological particles are usually dried. When imaging with TEM the sample preparation includes cutting the sample into slices thin enough. The biological samples imaged using SEM have to be coated with a conductive layer. This is because when imaging insulating samples with SEM some charge tends to accumulate on the sample surface due to sample beam interaction which affects the image quality. The charge accumulation is prevented by coating. Transmission electron microscopy is a conventional method for imaging viruses, and it is far more used to image biological samples than SEM. [6]

3.2 Atomic Force Microscopy

Atomic force microscope (AFM) is a scanning probe microscope that uses a small tip to track the surface topology of a sample. The tip moves on the sample surface going the imaged part through completely. The movement of the tip in z-direction is traced via laser and surface topography is constructed using this data. There are multiple imaging modes that vary by the exact cantilever tip movement and feedback loop function. [8]

In contact mode, the AFM tip drags along the surface being in contact with it

the whole time. This is a mode that causes a lot of stress to the cantilever tip which is the reason why this mode is not very commonly used. In non contact mode the tip does not touch the surface, but oscillates above it. The interactions between the tip and the surface affects the frequency of the oscillation. The frequency is tried to be kept as constant via a feedback loop that lifts or lowers the tip trying to cancel the surface affection in tip frequency. This mode is not as accurate as contact mode since it does not touch the surface, but with this mode a same cantilever tip can be used for a longer time. [8]

Tapping mode is a hybrid of two former modes since the tip oscillates above sample but it touches the surface in the lowest point of the oscillation. The surface touch alters the amplitude of the oscillation, which is tried to maintain as constant via feedback loop by lifting or lowering the tip. [8] In peak force mode the tip is pushed down to the surface and the bending of the tip is then measured. With this mode also the adhesion of the surface can be measured since when the tip is lifted from the surface the tip behavior is dependent on the "softness" of the surface. [9]

The advantage of AFM is the precision in z-direction reaching sub-nanometer resolution what comes to specimen height profile. The accuracy in x-y direction is not so high and the true shape of a particle is many times different than what it seems like in the AFM image since the particle shapes in x-y dimension are convolutions of the true particle and the AFM cantilever tip. This leads to small particles appear wider in images compared to their true dimensions [10]. AFM is not a good method for mapping large areas since the imaging time is dependent on the area size, and bigger images with good resolution can take hours to take. AFM is also not good for samples with large height variation. In principle a height variation over 1 µm usually leads to poor image quality due to tip properties. [11]

AFM is not used as a method to identify virus types, but a tool for virus research to investigate virus properties on a surface, such as particle density and virus diameter in z-direction. AFM does not require sample preparation of any kind and it is not destructive for a sample.

3.3 Helium ion microscopy

Helium ion microscope (HIM) is a microscope that uses positively charged helium ion beam to construct an image of a sample. It resembles SEM in the sense that the beam excites secondary electrons from the surface of the imaged sample and since the emission of the secondary electrons is sensitive to the surface topography detecting these electrons can be used to form an image of the sample. [12]. HIM can reach resolution of a 0.5 nm. [13]

Helium ion microscope produces helium ions by generating an extremely high electric field in which the helium gas atoms are ionized. The high electric field is produced using a sharp tip and a potential difference between the tip, apex, and an electrode, extractor. The electric field is extremely high around the apex and helium gas atoms passing it in slow enough velocity ionizes. The slow velocity of helium atoms are confirmed by keeping the ionization chamber in cryogenic temperatures. After ionization the helium ions are accelerated towards an extractor that lets the ion beam trough. Before the beam hits the sample it is focused with few lenses. [12]

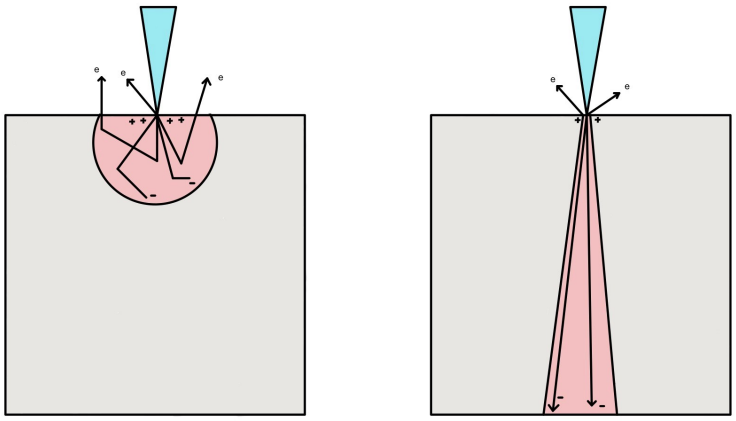

Figure 1. The left image represents secondary electron production in SEM and the right one describes the same phenomena in HIM. Helium ions are heavier particles than electrons thus generating secondary electrons from a smaller area on the sample surface. This is why HIM can reach better resolution than SEM. [12]

Like electron microscopes also HIM operates in a vacuum, so a treatment of some kind would be good for biological samples. These fixation methods aim for drying the sample while trying to maintain the structure. Compared to SEM, HIM is claimed to have better resolution, since secondary electrons are produced in narrower region

of the sample surface as is presented in figure 1. Also HIM enables imaging of non conductive samples without coating them. This is because in HIM the accumulating surface charge can be neutralized with a low energy electron beam. The surface sensitivity is increased when coating does not have to be used. [14] This feature makes HIM a valuable tool for imaging biological particles.

4 Virus detection in general

As mentioned earlier virus detection is an invaluable tool to control a virus spreading in a population. There are multiple detection tools and methods available and usually multiple different approaches can be used to detect a same microorganism. What detection method or tool would be best to analyse a virus presence in a sample depends on multiple factors.

Important features of a detection methods are their accuracy and required resources. Resources mean, for example, laboratory equipment, trained laboratory staff, time and money. Usually the accuracy and resources are proportional to each other, as high accuracy tools many times require high cost equipment and laboratory conditions while lower accuracy methods tend to be tests that one can perform at home and buy with a low price.

Besides the resources and aimed accuracy the choice of the diagnostic method is also affected by former knowledge of the infection. In the most challenging cases there are no previous strong suspicions on the microorganism that could maybe be found from a sample, so one has to use a method that reveals the microorganism regardless of what species it might be. Many times there is some suspicion of what might be the microorganism causing the infection based on information gained by analysing the symptoms of the patient. In these cases one can use a detection method that gives information about the presence of just one single type of microorganisms.

One more important aspect of virus detection is that the observation can be done by detecting the presence of the actual virus particles but also by searching other clues that can give information about the ongoing or prior virus infections. This can be done by observing antibodies that are produced in an infected individual a few weeks after the infection. Also cytopathic effects can be observed that are virus induced visual changes in infected tissue. One example of cytopathic effects is protein aggregates that emerge in cytoplasm of a specific type of nerve cell that is infected with the rabies virus. [15]

In this chapter I will introduce a few most commonly used detection methods used when a virus infection is suspected. There are some tools that can also tell

about the presence of other microorganisms than viruses. Before going into the detection methods I will say a few words about the sample collection, transport and preparation.

4.1 Sample collection, transport, storing and preparation

Sample collection, transportation and preparation have to be done correctly to get truly reliable information of possible infection and the presence of microbes in a specimen. [16] The sample has to be collected at the right time, place and quantity with right tools and sterile equipment.

The timing of the sample collection is important when trying to collect samples from the human body with high virus concentration. This usually means that collecting the sample should be done in three days after the beginning of the symptoms. When trying to detect antigens formed in the body after infection sample collection should be done weeks after symptoms have started. [1] Sample transport is done trying to maintain the samples as they were in the time of the collection. The key point in this is to keep organic material in the sample from degrading or growing. Even small changes in conditions, as in temperature, humidity, oxygen amount or ph, can initiate both unwanted changes. For example, drying the sample would cause significant harm of the pathogen, making the sample not corresponding to the conditions of the tissue it was collected from. Preserving the sample can be done by keeping it in transport media and trying to keep the time between sample collection and diagnostics as short as possible. The transport media is usually not needed for samples with liquid nature. Different types of transport media are commercially available which are typically isotonic solutions that also contain some proteins that have an ability of protecting unstable virions. Usually antibacterial and antifungal compounds are added to media to prevent unwanted growth. [15] Ideally diagnosing a sample would take place in the next 30 minutes after collection. If the sample can not be diagnosed quickly additional measures need to be taken in order to keep the sample in good condition. This usually means keeping samples in holding media and adding preservatives and anticoagulants to the sample. In cases where a sample has to be stored more that four days they are placed in -70 °C, since in higher, still negative, temperatures there can occur some formation of ice crystals that may cause damage to host cells and result in a lower viability among the viruses in the sample. [15]

Many detection methods require a sample preparation process to be performed before an actual analysis of the sample can be done. These methods variate greatly regarding the detection method that is planned to be used.

4.2 Viral isolation

Viral isolation is a gold standard method in viral diagnosis. Viral isolation is a detection method where viruses are tried to get replicated and grow in a specific platform. [15] A sample containing viruses is added to a culture of cells where the observed virus particles can replicate. Viruses can initiate cytopathic effects on cell culture, and the diagnosis is made based on visual changes of the cells. Many viruses do not initiate visual changes and the diagnosis require further examination. Viruses can be then tried to be identified with for example hemagglutination assay, that is introduced closer later in this chapter.

Virus isolation from cell cultures is also convenient when one wants to separate viable viruses from nonviable ones. Culture methods used to have up to 7 days turnaround time, but nowadays the process can be speeded up by using for example centrifugation enhanced culture methods which leads to a turnaround time of 24-48 hours. [17] Alongside with cell culture there is also a chance to grow the viruses in embryonated eggs and animal inoculation. These are only marginal methods when compared to cell culture. [15]

4.3 Microscopy

With microscopes one can observe samples with high resolution enough to actually see the microorganisms or visual changes they have induced in infected cells. Microscopes are important tools especially when one is observing an unknown microorganism. [17] With a microscope one does not have to have a previous knowledge of what might be the organism in hand and multiple suspects can be observed at the same time. Microscopic examinations are relatively quick to perform. Downsides of microscopic methods are that it requires an expensive microscope and trained staff to use it. Also, the organism concentration in the sample has to be relatively high so that infection can be observed with a microscope. There are methods to increase the viral concentration in samples that can be used to improve the detection limit. For example, ultracentrifugation is a method for that. Also staining of some parts

detected can be done to help [17].

4.4 Assays

Assays are analytic procedures that are used to detect the presence, the amount or functional activity of target particles. [18] Assays somehow discriminate the target particles from others according to its specific attributes, like receptors that bind selectively the target particles. After this, the presence of the target particles are somehow converted into a signal that can be measured. Assays are organism specific because of the specific reactants it uses.

4.4.1 Enzyme immunoassays

Immunoassays are biochemical tests that can reveal the presence of a target particle by using antibodies or antigens. Enzyme immunoassays (EIAs) are immunoassays that also utilize enzymes. The enzymes cause reactions in the presence of the target particles, that typically convert some substrate into a form that gives a specific color for the sample.

There are many types of enzyme immunoassays available. Three most commonly used enzyme immunoassays that are Direct EIA, indirect EIA and competitive EIA. Direct EIA observes antigenic pathogen components from specimens with function described in figure 2. Indirect EIA can be used for observing antibodies that are produced after infection of some viral disease. The operation principle differs a little from direct EIA operation principle and is introduced in figure 3. Competitive EIA is used for detection of pathogen components with generally higher sensitivity than direct EIA. The operation principle of a competitive EIA is shown in figure 4. [1]

For many viruses, for example influenza A, there are EIA tests available. [17]. The turn around time of the tests is relatively short and performing is simple. The downside of these tests is that their accuracy is not very high and it is dependent from multiple factors such as viral genotypes, viral load present and sample collection timing. [19] Assays are using antibodies that can make the assays expensive. [17].

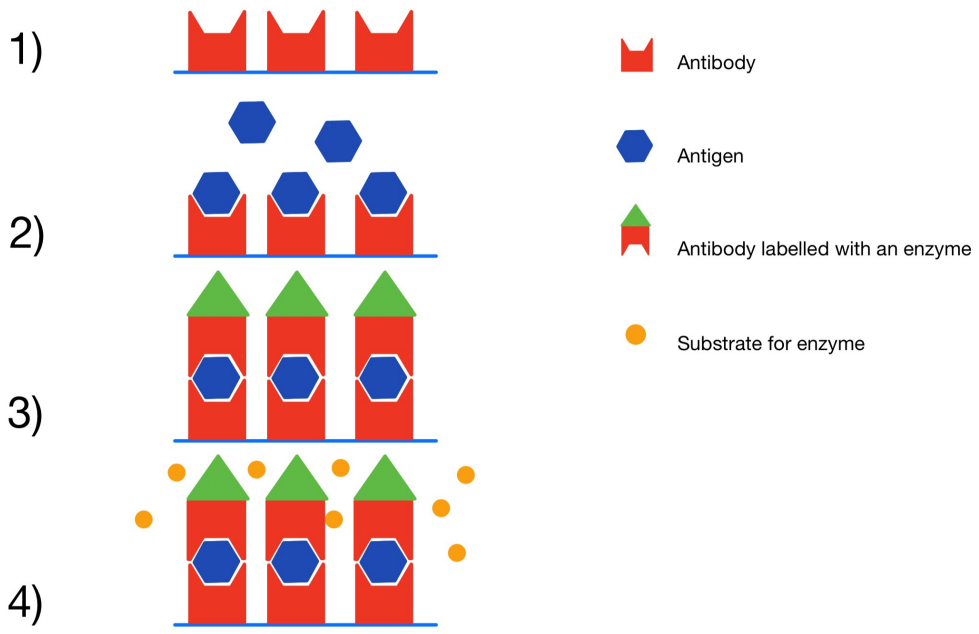

Figure 2. Direct EIA operation principle. 1) First, the substrate is coated with antibodies that can bind target antigens. 2) The specimen is introduced to this substrate and the target particles bind to the antibodies on the surface. Unbound particles are rinsed away. 3) Another liquid is introduced to the substrate, this time liquid contains antibodies that can bind to antigens. These antibodies are labelled with enzymes. 4) After unbound material is washed away and a new liquid including a substrate molecules for enzymes is placed on the surface, the enzymes induce these substrates to change in such manner that they change the color of the liquid. This color then tells about the presence of the antigens in the sample. If there are none no color change is observed. [1]

4.4.2 Hemagglutination Assay

Hemagglutination assays are the most used methods to observe viruses from cell cultures. Many viruses contain proteins that react with red blood cells. For example cells that are infected with influenza do not initiate cytopathic effect but it is known that these infected cells extract hemagglutinin, a protein that is attached to the cell surface and can bind to red blood cells. The cell culture is introduced to a suspension of red blood cells that will stick or absorb into the infected cells. From this behavior the presence of a virus can be deduced. [17]

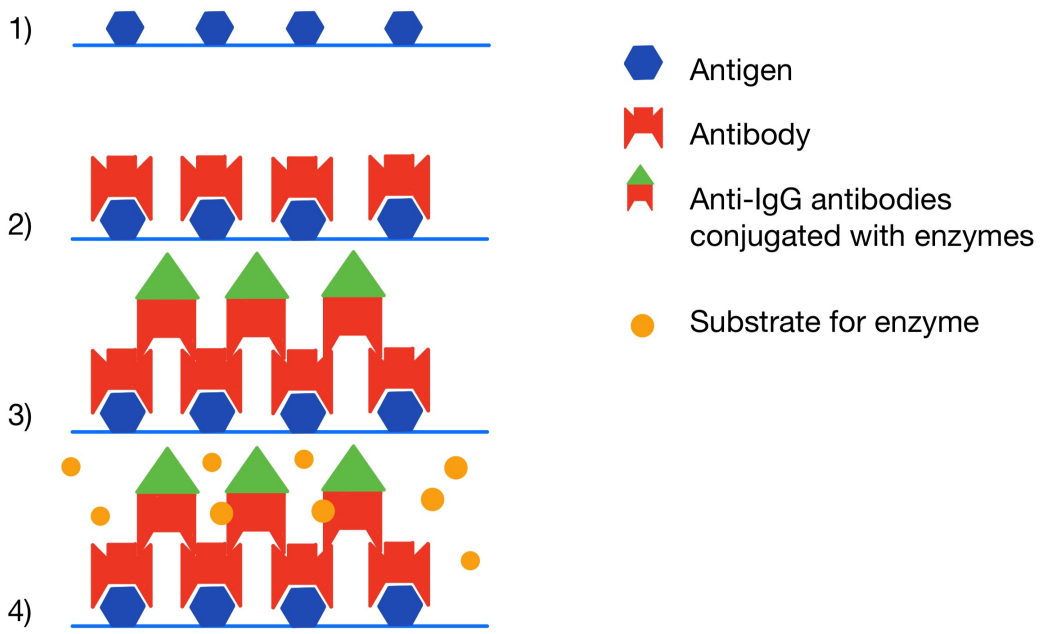

Figure 3. Operation principle of indirect EIA. 1) Modified antigens are fixed to a substrate surface. 2) When a serum sample is added the target antibodies bind to the antigens fixed to the surface. 3) After washing any excess particles away a second liquid is added, now one that includes anti-IgG enzyme complexes. Anti-IgGs are compounds that naturally bind to observed antibodies. 4) In the final part again a substrate molecule is added so that the enzyme reactions can induce a change of color from which the presence of observed antibodies can be seen. [1]

4.5 Immunofluorescent methods

Also with immunofluorescence methods one can reveal the presence of virus particles with help of antibodies, that binds selectively to the wanted virus type. While an enzyme immunoassays gives information of target particles present via enzymatic reactions that induces the sample to change color, in immunofluorescent methods the amount of target particles are observed from the intensity of fluorescent light coming from a sample.

Also fluorescent methods can be divided into direct and indirect methods. [1] Direct fluorescent antibody test starts with fixing the specimen possibly containing viruses onto glass slide after which it is immersed to liquid that contains antibodies. The antibodies are designed to bind to the observed target particles, for viruses in

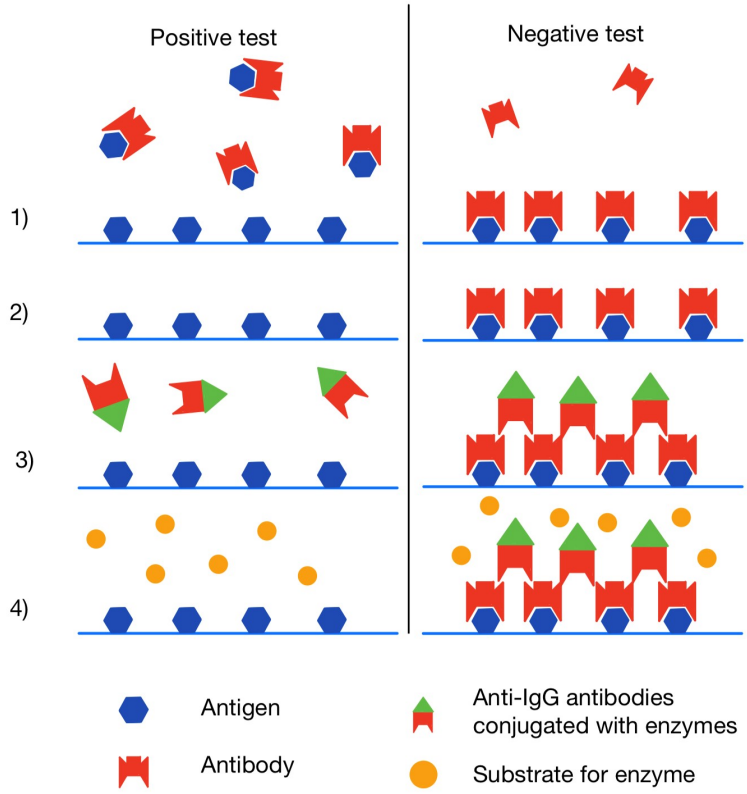

Figure 4. The operation principle of competitive EIA. 1) Substrate is covered with target antigens. Now before the specimen is added to the substrate it is mixed with a known amount of antibodies that can bind to antigens. 2) When this mix is added to substrate the free antigens bind to the antigens on the surface. If there were target antigens in the sample, there are only a little amount of free antibodies that binds to the surface. 3) The unbound particles are rinsed away and a liquid is added that contains anti-IgG conjugated with enzymes that bind to the antibodies. 4) The enzyme presence in the sample is observed as in former methods, only this time the high enzyme activity and color changes indicates the lack of antigens in the original sample. [1]

this case. The antibodies are fluorescence labelled and after unbound antibodies are rinsed away, one can observe the presence of viruses by fluorescent light coming from the sample. The fluorescent light is detected via a microscope. [15] As mentioned in direct methods antibodies linked to a pathogen surface are fluorescent unlike in indirect methods where other antibodies are linked to antibodies bound to pathogen surface. These antibodies that bind to the antibodies on a surface are fluorescent and thus the indirect methods can give information about the presence of antibodies on a sample. [1]

The turn around time of this detection method is generally between 1 and 2 hours. Downsides of this method are cross reactivity with similar virus types and sometimes high cost if used antibodies are expensive. Also, this method needs relatively high concentration of the virus in the sample. [16] This method is generally considered less sensitive than enzyme immunoassays and nucleic acid based methods introduced next. [17]

4.6 Nucleic acid based detection

Nucleic acid based detection methods are extremely sensitive tools that use species-specific nucleic acid sequences for detecting pathogens. [1] Polymerase Chain Reaction (PCR) is method for replicating specific DNA sequences exponentially. The basic operation principle of PCR is described in figure 5. PCR uses DNA polymerase enzymes to copy the DNA and it also requires artificially synthesized primers that initiate DNA synthesis. The presence of target genome in a sample can be observed from the amplification of the amount of genome in a sample. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is a similar tool than PCR but it is able to replicate RNA sequences.

Both PCR and RT-PCR are powerful tools that are extremely sensitive and specific. The operation takes just a few hours to perform analysis and the amplification can be initiated by just a small amount of target genome. [1] Besides requirements for laboratory machines and staff PCR has downsides related to viral viability observations and the variability of viruses.

PCR does reveal whether there are virus genome in a sample with high precision, but it does not tell about the infectivity of viruses, since the observed genomes can originate both from noninfectious, damaged virus particles and infectious particles. This problem can be made less severe by pretreating the samples before PCR with a process that reduces the amount of genomes from damaged particles. Even tough samples are pretreated all noninfectious virus genomes still can not be removed from samples. [20]

Genetic variability of viruses is a problem in PCR measurements. The primers used in PCR have to be an exact match to target virus genome sequence and used sequences are therefore usually form highly conservative regions. If the virus has been mutated from these regions primers are from, the viruses can not be recognized. PCR has additional disadvantages when observing food samples. In food, there are

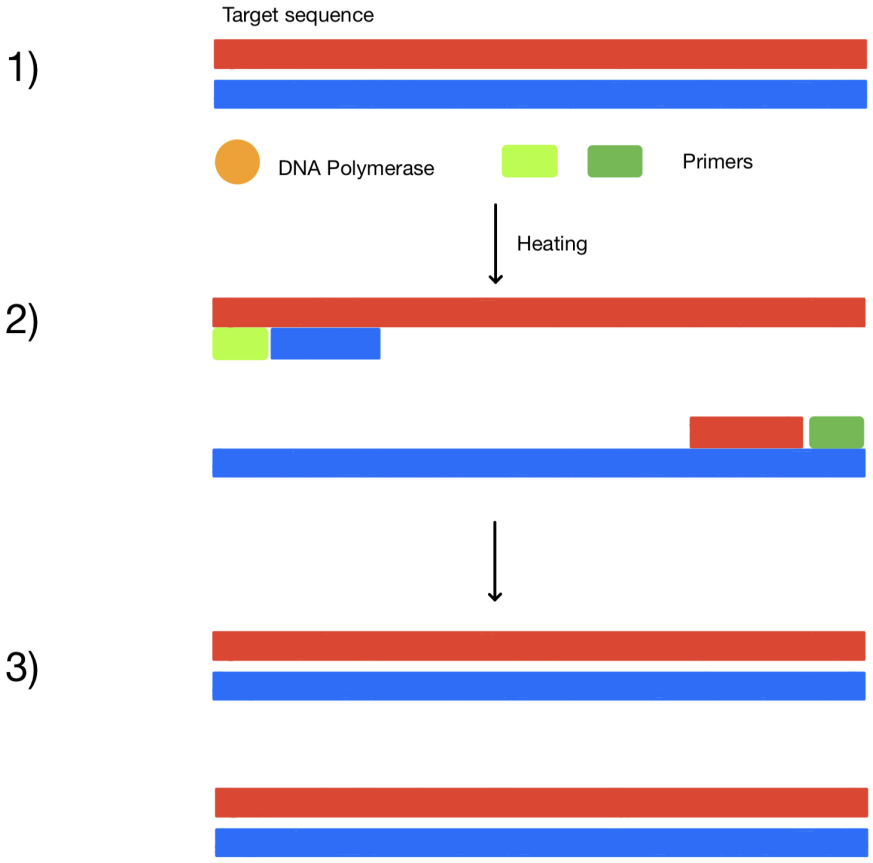

Figure 5. The basic operation principle of PCR. DNA polymerase enzyme and primers are added to a sample containing target DNA. When the sample is heated two DNA strains separates and a DNA polymerase enzyme builds complementary strains for both of them. In the end of a PRC cycle the number of target sequences is doubled. The cycle is then repeated leading in an exponential growth of the amount of DNA. [1]

compounds present that naturally inhibit the PCR reaction. Virus detection from food samples is usually hard since the amount of viruses in samples is usually very low. [20]

4.7 The future of virus detection

Virus detection methods are under constant development since low cost, easy to use diagnostic methods with short turnaround time and no requirement of laboratory conditions and trained staff are still missing for many viruses. Due to COVID-19 there has been huge pressure to develop more easy tests to observe possible infection, and a lot of funding has been directed into this field. Most probably this will give a

boost to the overall virus detection field and we will see many new innovations in the following years.

5 SIP-based detection

5.1 From receptors to biosensors

As one can see from the previous chapter there are many approaches on how one can construct a sensor that gives information the presence of a virus or antigen in a sample. One of the most simple sensor types that can be used to detect biological particles is sensors that contain two parts, a receptor that binds selectively to some bio particles and a detection platform that changes the information of the particle binding to a receptor into an electric signal. The idea of combining a natural receptor with a sensor platform was first introduced in 1962 and the development of this kind of sensors have been under a great interest ever since.

Natural receptors bind ligands with great sensitivity and selectivity making them good components for sensor development. There are also downsides with using natural receptors. They have a low stability over time and the synthesis or isolation typically requires a lot of resources. Thus scientists have been developing synthetic receptors that mimic natural receptors with good binding selectivity but being more stable in time and having an easier synthesis process. These receptors are called biomimetic. [21]

5.2 Surface imprinted polymers

Surface imprinted polymers are an example of biomimetic receptors. Surface imprinted polymers (SIPs) are polymers that have specific binding cavities on its surface that binds selectively only particles similar to ones used as template particles in the fabrication process.

SIPs can be fabricated in many ways but in principle the fabrication contains introducing template particles on a semicure oligomer layer. When a semicure oligomer layer is cured the polymer chains cross links with each other resulting in toughening or hardening of the polymer. Polymers interact also with functional groups on the template particle surfaces. After the polymer is cured the template particles are removed from the surface leaving behind cavities that geometrically and

chemically matches to particles similar to template particles. Different fabrication techniques introduce template particles to the matrix via different means, in stamping technique the template particles are stamped on the polymer surface while in molding technique the template particles are first immobilized to a surface and the polymer is poured on top of them. In self assembly technique the template particles are mixed in the polymer. [21]

SIPs were first introduced two decades ago after which they have been used in many applications. SIPs can be used on their own, for example, to separate bacteria or sort cells. [22] When SIPs are coupled with a detection platform it can be used as a sensor. SIPs have been succesfully created using many different bioparticles, including viruses, as templates in fabrication [23].

5.3 Detection platforms

The function of a detection platform coupled with a SIP is to change the adsorption of the particles on the polymer surface into an electrical signal. In the first SIP based biosensors the polymer was deposited on a quartz crystal microbalance chip (QCM) that allowed the detection of the adsorbed particles via observing the increase of mass on the polymer surface with high precision. [21] In QCM this is done by observing changes in the resonance frequency of a piezoelectric crystal. Also dissipation of the frequency is dependent on the viscoelastic properties in the solid-liquid interface. QCM is still the most commonly used sensor platform coupled with SIP. [24] Also surface plasmon resonance (SPR) has been used to detect the amount of attached particles on the surface. [21] In SPR measurements the adsorption of particles on the surface can be observed from shifting of the resonance frequency of surface plasmon polarisation, when the surface is illuminated with light. [25]

Electrochemical impedance spectroscopy has also been used to sense adsorption on SIP surface [21]. Electrochemical impedance spectroscopy measures the impedance of the system by generating a voltage difference across the observed sample trough which a current is then measured. The impedance is the ratio between the voltage and the current and depends on the properties of the observed system. [26]. Thermal wave transport analysis (TWTA) can also give information from particle adsorption on the surface in electrical form. The thermal transport analysis gives information about the phase shift of the thermal waves travelling across a functional interface. [27]

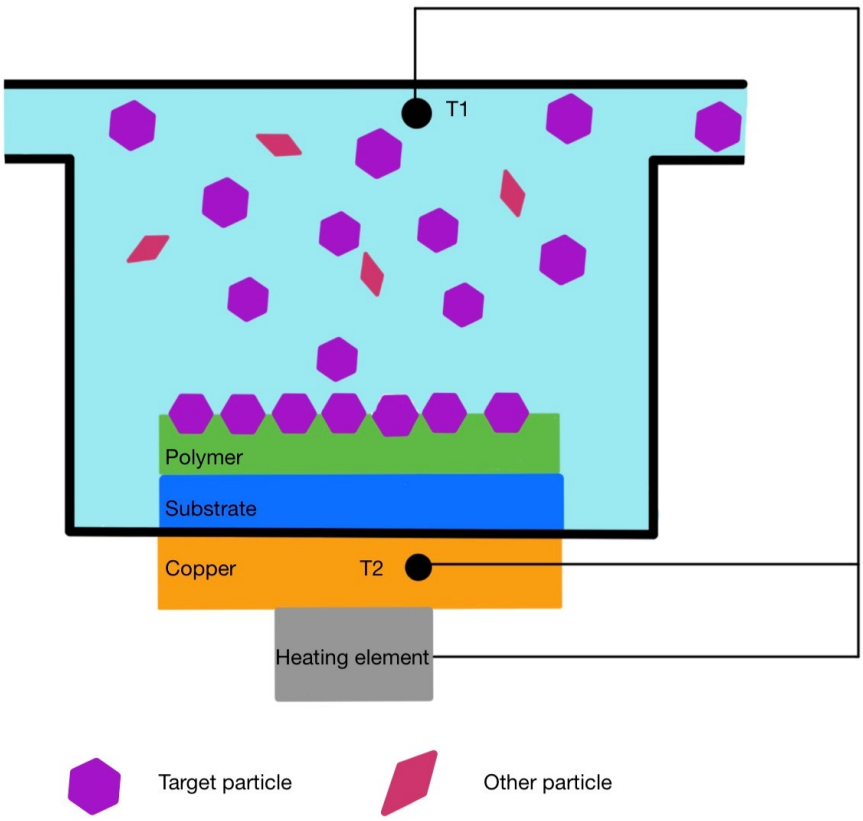

Figure 6. Heat transfer method used for observing the adsorption of particles on the polymer surface. Layer of adsorbed particles increases the thermal resistance across the surface-liquid interface. The thermal resistance can be calculated when two temperatures and the heating power is measured. [21]

Heat transfer method (HTM) is an approach for observing the attached particles on the surface by detecting thermal resistance of polymer-liquid interface. Thermal resistance represents the thermal conductance through the interface. Thermal resistance increases via particle adsorption since biological particles have typically poor thermal transport properties, thus particles at the surface are acting as an insulating layer between chip surface and liquid. [28]

SIP-HTM biosensor function is described in figure 6. A SIP-HTM sensor contains substrate on top of which the SIP is placed. Substrate is attached to a copper chip that is placed on a heating element. This sensor is then placed in a flow cell, a container like chamber, through which the observed liquid sample flows. Now the target particle adsorption is observed via monitoring the thermal resistance through the solid-liquid interface. This is done by heating the system with a specific heating

power and then measuring two temperatures, one from the sensor copper and other from the liquid, some distance away from the polymer surface. The thermal resistance of the solid liquid interface can be calculated by dividing this temperature difference with the heating power used [21]

$$R_H = \frac{T2 - T1}{P}. \tag{1}$$

The thermal resistance measurement is done in following way. The liquid sample is injected in flow cell, after which the flow is stopped for a sedimentation time. During the sedimentation time particles adsorb to polymer surface cavities. Then the flow cell is flushed with PBS in order to remove any unbound particles. After a while the PBS flow is stopped for equilibration time. The thermal resistance of the interface is measured in the end of both cell sedimentation time and equilibration time. At both these times the thermal resistance have reached a constant value with no variation over time. [22]

The thermal resistance of a solid liquid interface increases as particle concentration in the sample gets higher. The absolute thermal resistance is highly dependent on device properties and environmental conditions thus the analyses of the particle concentration in a sample needs to be made by comparing a baseline resistance to resistance measured for the sample. Baseline resistance is measured from a liquid sample in which there are no particles that could adsorb on the polymer surface. [22]

5.4 What has been detected using SIP-HTMs

The SIP HTM biosensors have been used to detect human cancer cells in 2013. The separation of the cancer cells from healthy blood cells were witnessed. [28] Studies of Chinese hamster ovary cells (CHOs) showed how SIP could separate the CHOs with specific membrane proteins from ones without them [29]. SIP-HTM sensors have also been used to observe E coli bacteria presence from a solution. This study showed that the detection limit SIP-HTM sensor achieved was among best when compared to other available E.coli sensors. [22]

Part II

SIP-HTM project

6 Introduction: The KU Leuven SIP-HTM project

This Thesis work aims to contribute to the goal of designing a SIP-HTM sensor for the detection of Noro-VLP and ultimately the norovirus. It is a collaborative project with the KU Leuven, that is actively working on the SIP-HTM sensor concept, and the University of Tampere, that synthesizes the Noro-VLPs. The research work on the SIP-HTM sensor design at the KU Leuven is therefore the foundation of this Thesis. Professor Patrick Wagner is the head of the research group and has good experience in this field. Our contribution here at the University of Jyväskylä is described in this Thesis work.

Norovirus is a virus that can cause infection and gastroenteritis when it infects the human body. Norovirus is the most prevalent foodborne pathogen [30] and it can cause an infection with as low concentrations as 10^2 copies/mL. Virus is quite resistant to heat and acid as also for chlorine, so the prevention of the spreading is difficult. Human noroviruses have high diversity and rapid evolution which creates trouble for both development of norovirus vaccines and detection methods. [20] There is no available vaccine for noroviruses.

There is no commonly used method for cultivating human noroviruses in cell culture [20]. Traditionally noroviruses have been observed from samples mainly by transmission electron microscopy and by detecting antigens from serum samples. From 1990 on human noroviruses have been possible to be detected via techniques based on sequence of the viral genome, which provides very accurate identification. [16] The standard method for detecting noroviruses nowadays is RT-PCR. [20]

There is an obvious need for high sensitivity, easy to use sensors that could be used for NoV detection. SIP-HTM based biosensors could therefore provide low cost and effective detection tools.

The Part II of this Thesis describes the contribution done here at the University of Jyväskylä, and is structured as follows: In subchapters 6.1-6.2 actions taken by our collaborators are described. In subchapter 6.3, an overall view of my work is

given. A motivation for the work is given by explaining what requirements and problems certain fabrication steps of the SIP-HTM sensor may involve. In Chapter 7 I describe the experiments undertaken, and their results are presented in Chapter 8. Finally some concluding remarks are given in Chapter 9.

6.1 KU Leuven contribution: SIP-HTM sensor fabrication

6.1.1 SIP fabrication via stamping method

As introduced earlier surface imprinted polymer fabrication aims for producing a polymer with specific binding cavities in its surface. The binding cavities are such that they match geometrically and chemically the target particles they are trying to bind in the sensor. [31] There are multiple ways a SIP can be fabricated. The different techniques are self assembly, molding technique, miniemulsion polymerization and stamping technique. A major approach for SIP synthesizing is the stamping technique which is the technique used in this sensor fabrication project. [21] The fabrication process has four main steps described below and in figure 7.

The SIP fabrication via stamping methods starts with producing a stamp. The stamp is a polydimethylsiloxane (PDMS) chip that is covered with the template particles. The template particles spreading is done by pipetting liquid that contains the template particles on the chip after which it is let to incubate for some time. The fabrication process is done in glovebox under high humidity atmosphere, which keeps the liquid on the chip from drying. After sedimentation time has passed the excess liquid is removed from the chip surface by spinning.

The second step in the fabrication process is to prepare a substrate that is covered with a semicure oligomer layer which is applied onto a substrate using a spinner. The third step is to press the stamp on the polymer surface. When a stamp is pressed onto the polymer the template particles on the stamp surface are partly buried into polymer. Now when oligomer is heated cross linking happens during which polymers interact with each other and with functional groups on the surface of the template particles. Stamp is kept on the surface for hours while also keeping it heated in order to cure the polymer. Curing the sample makes the previously viscous polymer harder.

After the polymer is cured the stamp is removed from the surface and brought under a normal atmosphere. Extraction process is followed in order to remove

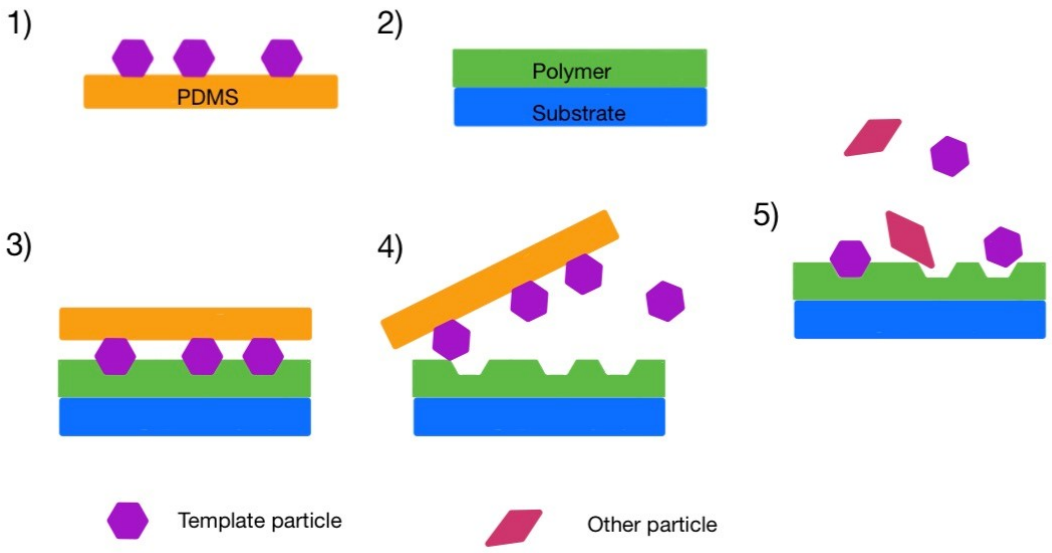

Figure 7. SIP fabrication using stamping technique. 1) Stamp production, fabricating a PDMS chip that has template particles on its surface. 2) Preparing a polymer on substrate that in this point is a semicured oligomer layer. 3) Stamping, the fabricated stamp is pressed on the prepared polymer. 4) After some time stamp is removed and particles are rinsed away. 5) As a result one has a surface imprinted polymer that has specific binding cavities on the surface that are able to rebind particles similar to ones used as a template.

template particles from polymer surface. Now a surface imprinted polymer is ready and when placed in a liquid it can rebind particles similar to ones used as template particles in the fabrication process. [21]

6.1.2 Testing of function: NIPs and cross testing

No imprinted particles (NIPs) are polymers produced in a similar manner as SIPs, but in the stamping phase the NIPs are treated with PDMS chips that do not have template particles on its surface. Thus NIPs do not have template induced binding cavities on its surface. NIPs are used as a standard with which the functionality of SIPs can be compared. The increase of thermal resistance of the surface happen to both for SIPs and NIPs when immersed to a sample containing particles, but the change is greater for SIPs since the NIPs do not have as good affinity to the target particles as the SIPs do. [32]

6.2 Tampere contribution: Virus like particle production

The production of SIPs requires using target particles as template particles in the fabrication process. In this project the actual noroviruses are not used but their role is taken by norovirus like particles (Noro-VLPs) that resembles noroviruses but does not have nucleic acid inside the particles. Noro-VLPs are fabricated in Tampere University. Professor Vesa Hytönen is the principal investigator of the project at Tampere.

Noro-VLPs in solution produced in Tampere University have spherical capsid of about 38-40nm in diameter. The capsid is formed from 180 proteins that are assembled in T=3 icosahedral symmetry. Noro-VLPs are produced in insect cells using baculoviruses as vectors. Baculovirus genome is modified to contain genes that codes norovirus capsides. Baculoviruses are relased into insect cell culture where the cells eventually start to produce material according baculovirus genome leading into VLP production. VLPs are separated from other material using ultrasentrifugation and anion exchange.

Ultracentrifugation is a method where particles in a liquid solution can be separated according to their mass. [33] The solution is placed in a tube that is spinned fast enough for particles in solution to experience forces with magnitudes of 70 000 g. After several hours of centrifugation, the solution have spread into distinct layers from which one can collect a layer containing desired particles. Spinning and collection is done multiple times in order to separate the VLPs as well as possible from other particles.

Further purification is done via ion exchange cromatography. Ion exchange cromatography is commonly used method for protein separation [34]. The solution containing VLPs is poured trough a column containing charged polymer beads to which VLPs attach during the first wash. The binding of VLPs to the column can be modified by altering the ph of the washing solution. In the second wash the VLPs are separated from the column resulting in VLP solution extraction.

In Tampere the quality of the final VLP solutions is observed using dynamic light scattering (DLS) that gives information about the size distribution of the particles in the solution [35]. VLPs are also imaged using TEM.

6.3 Jyväskylä contribution: imaging and adsorption studies

As was mentioned in Chapter 1, the Introduction, the very original plans of this project were interrupted by the Covid-19 pandemy. This necessitated a sudden switch into a different work plan where this author worked with the facilities at Jyväskylä and different samples were sent there from KU Leuven and/or Univ. of Tampere. Of course this change was very regrettable from the point of view of this Thesis, since the author lost the close interaction with the expertise at KU Leuven of the topic of the Thesis. For the new situation, it was attempted to work in such a way where the facilities at Jyväskylä can be put to good use in the development of the norovirus SIP-HTM sensor. The SIP fabrication was deemed to be such subtopic within the entire development work, which had problems of the kind where the microscopy instrumentation at Jyväskylä was very useful. The SIP fabrication scheme was illustrated in Figure 8. An optimal fabrication requires that a monolayer of the template particles are deposited on the PDMS stamp, and that these create a mirror image of cavities of this deposition on the SIP. This is of course very demanding, and in practice much goes wrong until a working recipe is found. The different steps of the SIP fabrication need to be investigated with modern microscopy, which thus became the basic idea of this Thesis work.

At first our task in this project was to image samples sent from KU Leuven using AFM and HIM. This was done for a few samples received in spring 2021. We also received some samples in the fall of 2020. The samples were stamps used for SIP fabrication, PDMS chips that were supposed to have Noro-VLPs on the surface. We did image the stamps with AFM but we did not find any virus-like particles from the surface. From that point on my main task in this project has been to optimize the stamp fabrication process by trying to find fabrication parameters that conclude into the VLPs adsorption on the PDMS surface as a monolayer with suitable particle density.

7 Experiments

My experimental work in this sensor project consists in general of imaging tasks of different samples from the SIP fabrication process. Moreover, it consists of the work on the stamp fabrication optimization, where VLPs adsorb on a surface. Much of the imaging work was occupied with samples sent from KU Leuven. All imaging was made using AFM (Bruker Dimension Icon AFM, mode used was Peakforce Quantitive nanomechanical mapping in air) and HIM (Zeiss Orion Nanofab Helium Ion Microscope).

The investigation of the SIP fabrication process requires microscopy images from different stages of the process. These stages are at least as follows. After the stamp has been deposited with the template particles, the quality of the deposition is of interest. Right after the stamping step, the SIP surface might still include the semi-immersed template particles, so this stage should be imaged. The template particles are removed with some treatment, so naturally the post-treatment SIP surface, that now should be filled with cavities, is of interest for imaging. All of these imaging tasks could not be undertaken, but some examples are described in subchapter 7.1.

When making the stamp the template particles needs to sediment on the PDMS surface. When dealing with larger and heavier template particles, for example E.coli, the particles sediment on the PDMS from the solution in about 30min due to gravity. VLP-particles, on the other hand, are so light that they do not sediment, which inhibits VLP adsorption on the surface.

The adsorption process can be modified by altering the surface properties of viruses by varying parameters of the virus-solution. The pH of the solution affects the surface charge of the viruses, thus making the viruses attach better to an oppositely changed surface. [36] In neutral ph Noro-VLPs have a negatively charged outer layer [37].

The VLP adsorption to a surface can be enhanced also by modifying the properties of the surface. [38] Surface modification can be done either by altering the surface's hydrophilicity or the surface charge. One way to modify the virus adsorption

properties is to coat the surface with poly-l-lysine (pll). The treatment should make the surface positive and more hydrophilic. Surface hydrophilicity can be modified using a reactive ion etcher that can perform etching and O2 plasma treatments. I did study the effect of these surface treatments on VLP adsorption. These experiments are explained in more detail in this Chapter. The results are presented in the following Chapter 8.

7.1 Description of samples sent from KU Leuven

We received the most important samples for imaging from KU Leuven at the end of the spring 2021. These are described in Table 1. The samples were SIPs made with either VLPs or yeasts used as template particles. The samples included three different VLP SIPs which differ in polymer layer thickness and stamp wetting. There was also a NIP sent, a non imprinted polymer that could be observed as a reference to other samples. The samples were ready made SIPs that were not treated in any way in Jyväskylä.

Table 1. The samples received from KU Leuven at spring 2021. Pulyuretan (PU) is the polymer commonly used in SIPs.

Number	Sample
1	Noro-VLP SIPs with thin PU layer dry stamps
2	Noro-VLP SIPs with thick PU layer
3	Noro-VLP SIPs with thin PU layer and spin coated stamps
4	Stainless steel SIPs with Dr Oetker yeast
5	Non imprinted polymers (NIPs)

7.2 Stamp fabrication optimization: VLP adsorption studies

The stamp fabrication process, that was tried to be optimized, was done by placing a drop of solution containing the Noro-VLPs on a PDMS chip from which excess liquid was spinned or blown away using a nitrogen gun after some sedimentation time. Although the process is rather simple it has a lot of parameters that could influence on the VLP adsorption on the surface. Parameter considered were virus concentration in the liquid, surface treatment of PDMS, droplet size, sedimentation

time, drying techniques and different washing protocols. Besides PDMS studies we also observed VLP adsorption on silicon surfaces. Summary of the considered parameters and the studies are in table 2.

Table 2. Parameters considered in VLP adsorption tests.

Parameter	What tested
Chip materials	PDMS, silicon
Surface treatments	RIE O2 plasma treatment, pll coating
VLP concentration	Original solutions and dilutions made with factors 10, 50 and 100
Drying protocols	Letting the drop dry on a chip, removing the drop from a chip with a nitrogen gun
Washing protocols	Washing the wet or dry chip, not washing at all
Drop size	5 - 20 µm
Incubation time	From few minutes to days

7.2.1 VLP solutions and concentrations

Standard VLP-dispersion was obtained from Vesa Hytönen's group in Tampere. VLP-dispersion had originally a VLP density of 0,82 mg/ml corresponding to $45 \cdot 10^9$ particles/µl. Also diluted dispersions with dilution factors of 10x, 50x and 100x were prepared. The dispersions were conventional PBS-based solutions containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, and 2 mM KH2PO4 and having pH of 7.4. Some sodium azide was applied to the solutions in Jyväskylä to prevent unwanted bacterial growth. The solutions were stored in a refrigerator.

7.2.2 Substrates and surface cleaning protocols

Both silicon and PDMS were used as a substrate on which the VLP adsorption was studied. The basic washing protocol was always followed in order to clean the surfaces before experiments.

PDMS chips were first cut from larger PDMS plate into chips with a size of about 5x5 mm. Then the PDMS chips were placed in isopropanol where the chip surfaces were wiped using cotton sticks. After this the chips were dried with a nitrogen gun.

Silicon plate was cut into 8x8mm chips after which the chips were placed in acetone and treated with a sonicator. After that the chips were cleaned using cotton

sticks after which the chips were placed into isopropanol where the chips were again wiped with cotton sticks. After this the chip surface was dried using a nitrogen gun.

7.2.3 Surface treatments

Surface treatments were performed for both PDMS and silicon. RIE O2 plasma treatment was done for both substrates, poly-l-lysine coating only for PDMS.

Both silicon and PDMS surfaces were treated with Oxford Plasmalab 80 Plus RIE, Reactive Ion Etcher (RIE) using O2 Plasma treatments. The recipes used for the surfaces differ slightly. To modify silicon surface to be more hydrophilic, oxygen plasma treatment was done for a 2 min period and 200W forward power. For PDMS the treatment was done using power of 60W. Silicon surfaces were also treated with RIE to be more hydrophobic. This was done by oxide etching.

Modifying the surface was done in order to change it more suitable for virus adsorption was also tried by coating it with polymers. We used poly-l-lysine (pll), that is a positively charged amino acid polymer, as a 0.10-percent solution in water. The pll promotes the adhesion of a virus particle to a solid surface by interacting with negative charged outer parts of the VLPs. Coating surfaces with pll is a commonly used method for promoting adhesion. Below the basic operation principle of pll coating is described.

The pll was added on a cleaned PMDS surface using a pipet. The drop size was about 20-50 µl depending of the size of the used PDMS chip. The amount of pll was such that the whole surface was entirely covered with the drop. The pll was let to stay on the surface for five minutes after which the surface was washed with ultraclean water. This was done by pipetting a few ml of water on the chip while keeping it in tilted position. The washing was done twice. After that the chip was left to dry overnight before applying VLP solution on substrate.

7.2.4 Adsorption tests

The adsorption tests were done after cleaning and performing a surface treatment. The adsorption test was done by pipetting a drop of VLP solution on a substrate which was then let to incubate for variating times. The process was done both in a high-humidity enclosure and in a normal atmosphere. The droplets of the samples placed inside high-humidity enclosures did not dry on the surface as they did on the samples kept under normal atmosphere. The samples on which the droplets did not dry were dried placing them in a normal atmosphere or blowing the drop away from the surface using a nitrogen gun. For some samples washing was done by pipetting a few milliliters of ultraclean water on the substrate surface. This done either before or after the drying of the sample surface. The adsorption of particles on the sample surface was observed by imaging the samples with AFM.

8 Results

8.1 Imaging samples sent from KU Leuven

8.1.1 AFM: VLP imprints

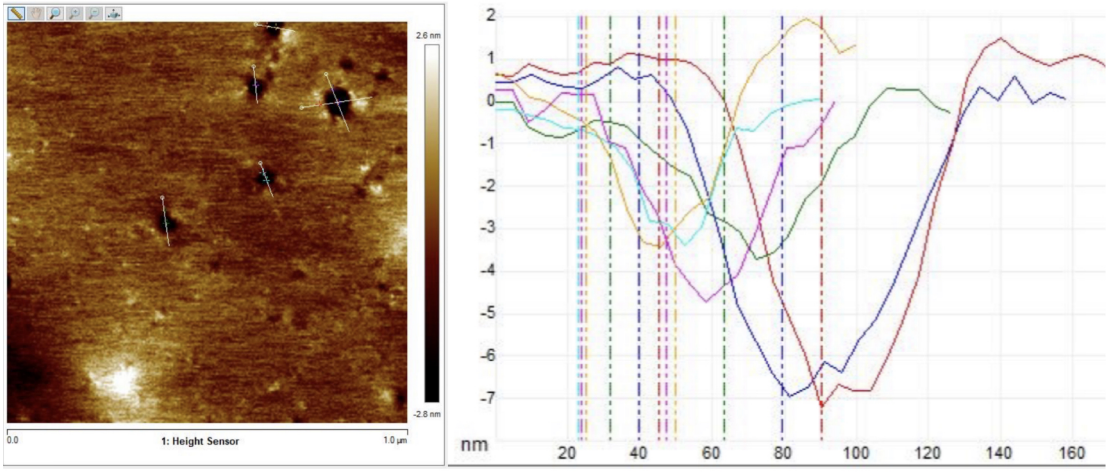

Figure 8. An AFM image of Noro-VLP SIPs with a thin PU layer. Shallow cavities could be found from the polymer surface with a maximum depth of 7 nm.

All the samples described in table 1 were imaged using AFM. From the fourth sample, yeast SIPs, good AFM images were not obtained due to large surface height variation. Rest of the samples were successfully imaged. The first two samples, Noro-VLP SIPs, showed shallow cavities on the polymer surface with a depth of 7nm or less. The most cavities were only a few nanometers deep and a few tens of nanometers wide. The cavity density on the surface did vary greatly since in some images I did not observe any cavities while at best there were about 10 cavities in 1 µm x 1 µm sized area.

Based on the images I took it seems like there were more cavities on the surface of the second sample (SIP with a thick PU layer) compared to the first one (SIP with a thin PU layer). It looks like the cavities were deeper on the first sample. The third sample had different surface characteristics than the first two and I did not

observe any cavities on its surface. I did not find any similar cavities when imaging NIPs either.

8.1.2 HIM: yeast SIPs

Samples described in table 1 were imaged with a helium ion microscope. The yeast sample was the easiest to image since there were large particles on the surface. The samples with only cavities and no particles on the surface were more difficult to image and I did not succeed in the time I had to take any good images on the VLP samples.

Figures 9 and 10 show images taken with HIM of the sample four, the yeast SIPs. One can observe yeast particles on the SIP, a polymer surface that is filled with cavities. The cavities on the polymer surface seem to match geometrically well to the yeast particles. There are yeast particles both in and out of surface cavities. The SIPs are ready made in KU Leuven so they have gone through the extraction process that should have removed the yeast particles, that have been used in the fabrication, from the surface. The extraction has obviously failed to remove all the yeast particles.

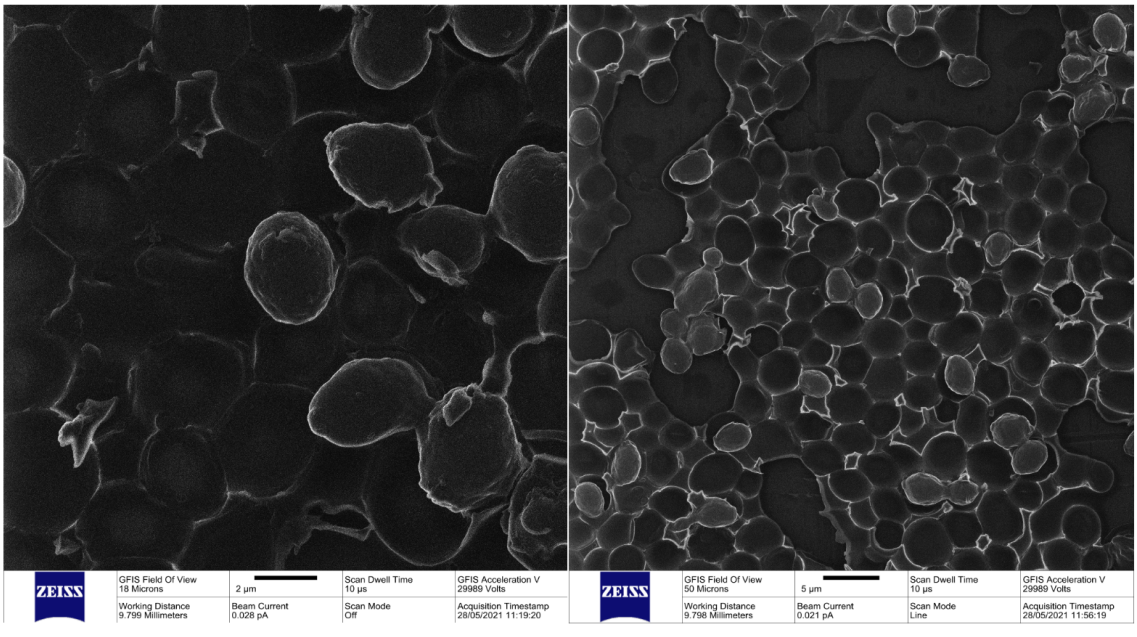

Figure 9. HIM images of yeast particles on SIP. The polymer surface is filled with cavities geometrically matching to the yeast particles. The field of view in the left image is 2 µm and in the right image 5 µm.

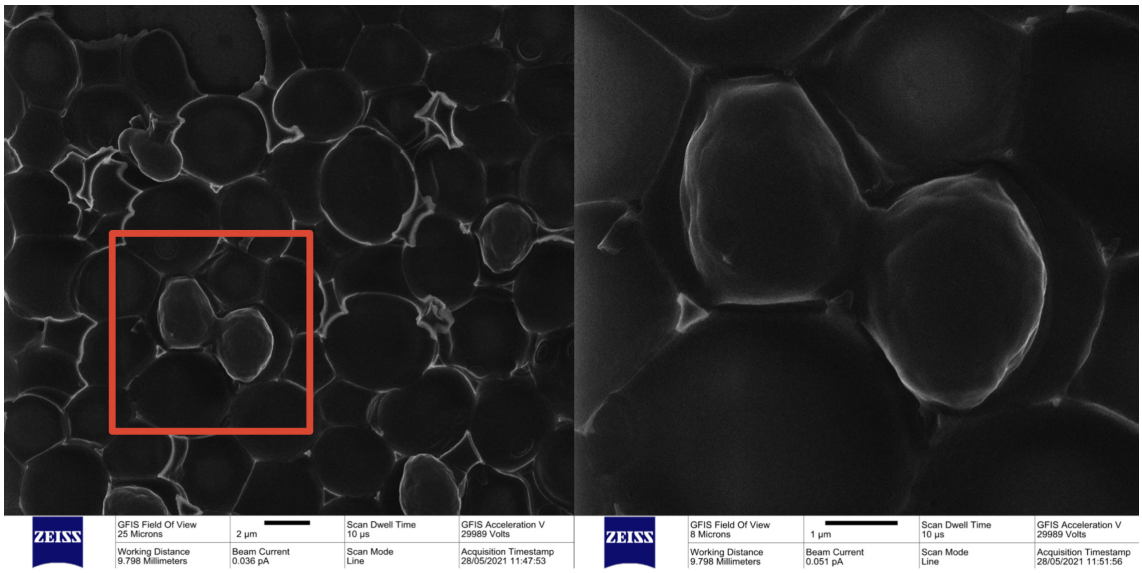

Figure 10. HIM images of yeast particles on SIP. The field of view in the left image is 2 µm and in the right image 1 µm. The right image is taken from an area marked with red in the left picture. The left picture shows two yeast particles in SIP surface cavities.

8.2 VLP adsorption studies

8.2.1 General observations about VLPs in AFM images

From AFM imaging of the adsorption test samples, round particles were discovered from the surface with uniform height and size and sometimes two or three particles were stuck together. In case of studies where the drop on the chip was blown away the VLPs were discovered from the place the drop was initially placed on the chip but also from the route the drop moved away from the surface. These observations should exclude any other possibility than VLPs. The height of the particle was around 12 nm, while in theory the particles should have 40 nm diameter. Figure 12 shows VLPs and their height profile on a silicon surface.

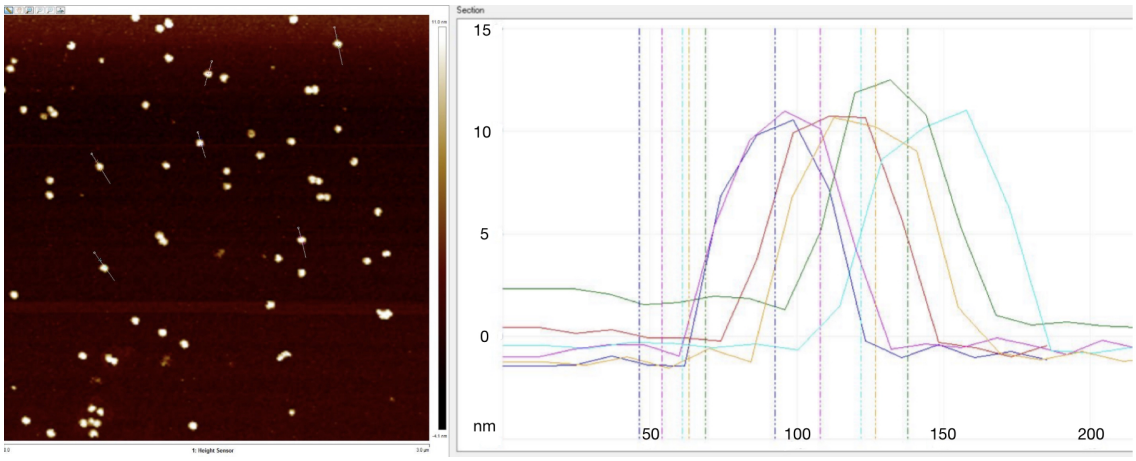

Figure 11. AFM image of VLPs on a silicon surface. The sample was prepared using 100 x diluted VLP solution and incubation time of 30 minutes. The height curves in the right shows a uniform height profile of observed VLPs. The observed VLP height was around 12 nm. The width of the imaged area is 3 µm.

8.2.2 Observations of drying and washing

When drying the solutions, we observed that if a drop of VLP solution was let to dry on the surface there was always a lot of salts on the surface after drying. In order to get salt free surface, the drop containing VLPs was never allowed to dry on the surface, but was eventually blown away with a nitrogen gun. This means that the adsorption test needed to be done in a high-humidity chamber to keep the VLP solution from drying during incubation time in order to get salt free samples where one could differentiate VLPs.

There were still some salt on the surface even though the drop was not left to dry on the surface. This salt could be tried to remove by washing the surface. The washing needed to be done also in such a manner that before it the drop had not dried on the surface.

8.2.3 VLP adsorption on silicon

VLP adsorption on the silicon surface was observed regardless if it was treated hydrophilic, hydrophobic, or that the surface was not treated at all. The VLP concentration in the solution used did make a difference to the adsorption. With the original concentration the surface was observed to be almost full on VLPs, and with 100x diluted samples with VLP with relative long distances apart from each other was obtained. Also the variation in sedimentation time had an effect on the

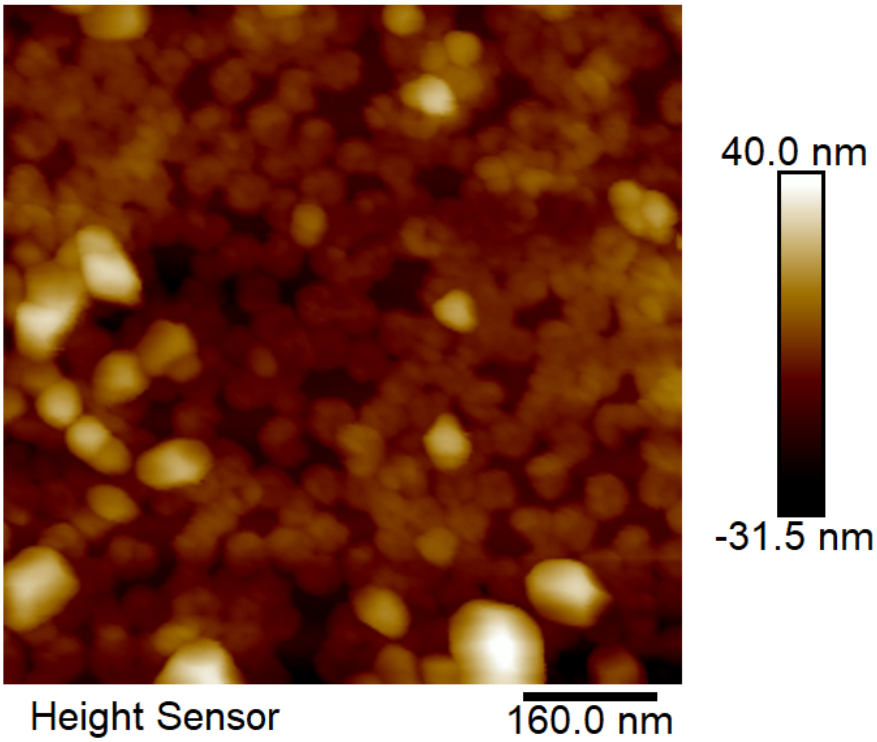

Figure 12. Virus like particles on an untreated silicon surface using a sedimentation time of 30min. VLPs are the round, uniform particles on the background while the large bright particles are contamination.

adsorption density. The longer the sedimentation time was, the more viruses were found. Figure 13 shows VLPs on a silicon surface.

8.2.4 VLP adsorption on PDMS

From the PDMS surface any VLPs were not found unless the surface was treated with some kind of surface treatment. Both RIE O2 plasma treatment and poly-l-lysine coating resulted in VLP adsorption on the surface.

After the PDMS chip was treated with O2 plasma treatment VLP adsorption on the surface was observed. The density of VLPs on the surface seemed to be directly proportional to VLP concentration of the solution. 30min sedimentation time and original concentration resulted in a surface filled with VLP, 100x diluted solution gave only few VLPs in μm^2 with equal sedimentation time. Samples treated with RIE did have salt on the surface which is problematic, since the salts typically had the same or larger sizes as VLPs in which case the salts would obviously be harmful in the SIP fabrication process. The salt was tried to be removed from the

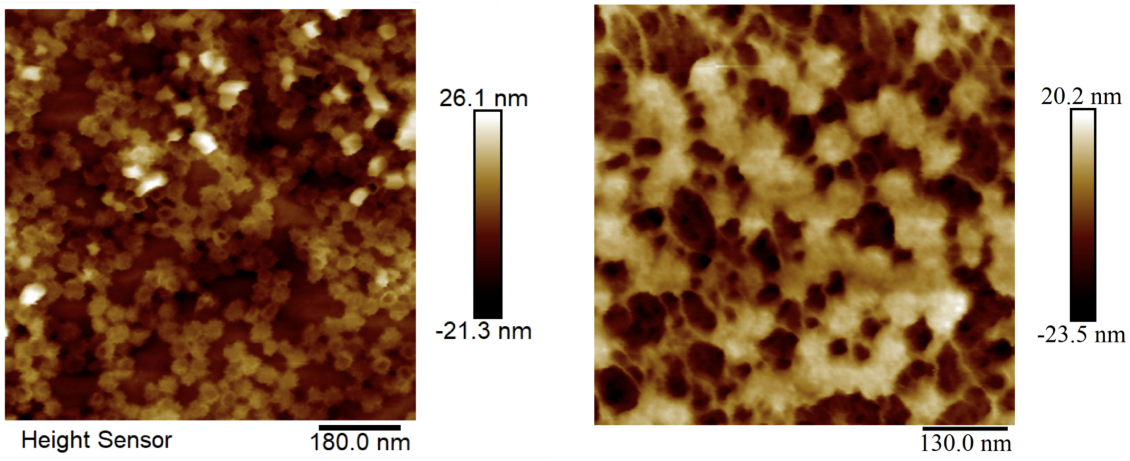

Figure 13. The left image shows VLPs on PDMS that has treated to be hydrophilic. The sedimentation time used was 30 min. The right image shows VLPs on poly-l-lysine treated PDMS surface. The poly-l-lysine coating created a spiderweb like surface. The observed VLP heights were slightly bigger than the ones observed when VLPs did adsorb to other surfaces. On poly-l-lysine VLP heights were about 20 nm.

chip surface by washing the samples but it also led to the VLP particles to leave the surface too. The VLP adhesion on the surface could be enhanced to last the washing on the surface. This could be done by adding some charged particles on the solution.

With poly-l-lysine coating we observed adsorption of VLPs on the PDMS surface. The washing of the surface could be done since the VLP adhesion was strong enough that the VLPs did survive the washing staying on the surface. Thus with pll coating a surface with VLPs but not large amounts of salt was obtained. An example of a nice adsorption pattern was gained using 30 min sedimentation time, 10 µl drop and 10x dilution. The concentration of the solution did affect the density of adsorbed particles on the surface. We did observe little to no adsorption with 100x diluted solution, and very much when using undiluted solution. The best adsorption densities for a creation of VLP monolayer were 10x and 50x diluted suspensions. The sedimentation time did not clearly correlate with particle density on the surface with a similar manner it did on a silicon surface. Similar adsorption densities were observed regardless the particles were let to sediment for overnight or just for a couple of minutes. Also the drop size did not affect on the adsorption considerably since adsorption seemed similar when done with 10 µl and 20 µl drops.

8.3 HIM imaging of VLPs adsorbed on a surface

When imaging VLPs on a silicon substrate we did observe particles with HIM as one can see from the picture 14. The VLPs were clearly suffering from conditions inside HIM, vacuum and the cold, thus losing the particle shape. Here imaged silicon sample is such that the washing has not been done for them, as one can see from the pictures the surface is filled with unwanted particles bigger than VLPs.

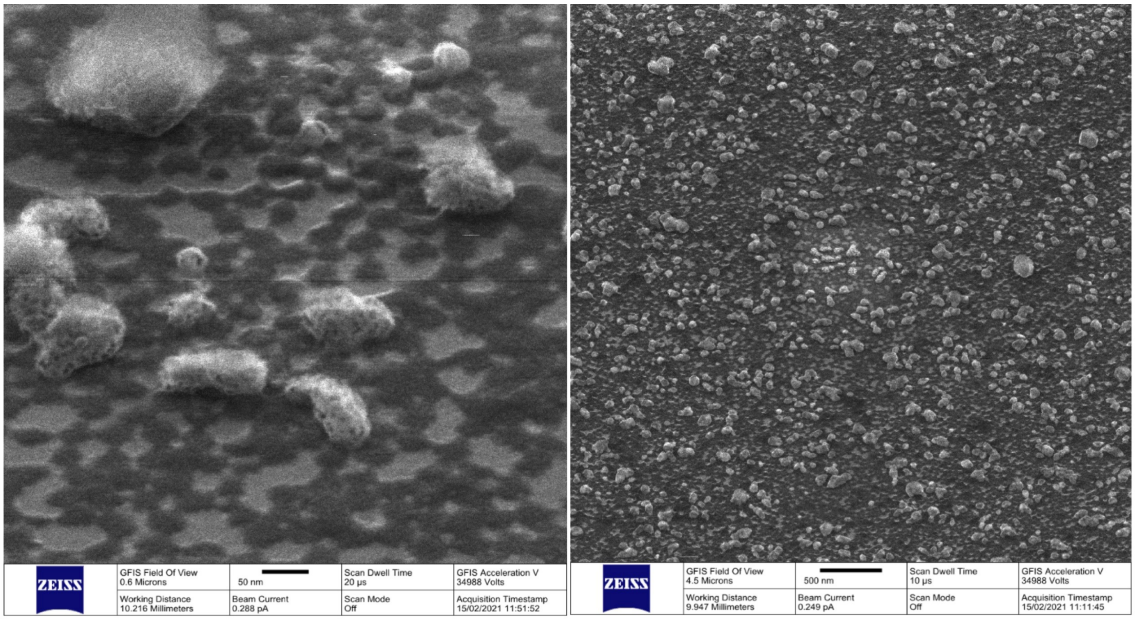

Figure 14. HIM images of VLPs on the silicon surface. VLPs are the black round spots on the surface while the bigger particles were considered to be something else. The VLPs structures were clearly suffering from conditions inside the microscope. The field of view in the left image is 0.6 µm and in the right image 4.5 µm

From the samples where VLPs were on the PDMS surface the VLPs did not stand out from the surface for some reason. We imaged multiple PDMS chips that were shown to have VLPs on a surface according to AFM images of the samples. With HIM nothing was observed from the surface of these samples.

9 Conclusions

Virus sensors are essential tools that help controlling the virus spreading in the population. There is constant need for low cost high accuracy detection tools that would be easy to use and have a short turnaround time. The SIP-HTM is an example of these kind of sensor.

The optimization of a SIP fabrication step, the stamp fabrication, was pursued in this Thesis. The optimization was in practice about developing a protocol with which a PDMS surface was covered with a nice VLP monolayer. The optimization included studying the effect of varying the parameters of VLP adsorption. VLPs were noted not to adsorb onto untreated PDMS surfaces so the main thing influencing on VLP adsorption was the surface treatment. Two separate surface treatments were found to enhance the VLP adsorption on a surface. Treating PDMS chips with RIE O2 plasma changed the surface hydrophilic properties in more VLP adsorption favourable direction. On the other hand, coating the PDMS surface with poly-l-lysine positively charged polymers did attract VLPs with a negative surface charge. Besides surface treatment, we also observed the effect of other parameters on the VLP adsorption. The parameters were VLP concentration in used solution, incubation time, drop size and different drying and washing protocols. The impact of these parameters on adsorption was different, depending on the used surface treatments. With both RIE and pll treated surfaces the higher VLP concentration in the solution concluded into denser VLP adsorption on the surface. For RIE treated surfaces, longer incubation time resulted in more adsorption, while in pll surface incubation time was not observed to have a correlation with incubation time in a timescale from few minutes to days.

Drying of the sample surfaces was noted to have to be done in such a manner that the drop never dried on the chip, but was blown away with a nitrogen gun. This was done by keeping the sample under a high humidity enclosure during incubation time after which the drop was blown away from the surface. If the drop dried on the surface a lot of salt crystals were also found there with high volume enough to bury the VLPs under. The salt was also found in all samples that were not washed. When

this was done on O2 plasma treated surfaces also the VLPs were washed away. The particles did last over washing on the surface when it was coated with poly-l-lysine. The poly-l-lysine coating was then observed to be a better way to create PDMS chips with VLPs on a surface without salt residue.

An important part of the experimental work of this thesis was observing samples with microscopes since the studied phenomena were on the nano- and microscale. AFM was the most used microscope and it suited well for observing the VLP adsorption on different surfaces showing the z-dimensions of the adsorbed VLPs with high precision. The AFM was also suitable for observing SIPs made with VLPs.

The samples were also imaged using HIM. We did try to image VLPs on both silicon and PDMS surfaces. HIM images did show VLPs on silicon surface but not on PDMS, and the VLPs observed on a silicon surface had expectedly lost their shape. Some fixation protocols should be followed in order to get nice HIM images of VLPs. The VLP-SIPs were also imaged but the cavities were not successfully recognized from the images. HIM was a successful tool for imaging yeast particles on SIP surfaces since the particles did hold their shape rather well in the microscope. Also the yeast particles were considerably larger than VLPs making HIM imaging easier. Besides for imaging large biological particles, HIM was also suitable for overall surface observation and for example statistical analysis of the amount of salt on a surface could be done using HIM.

The SIP-HTM NoV sensor development is still under progress in June 2021 when this Thesis is finished so analysis on the sensor function can not be yet made.

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