UHASSELT
KNOWLEDGE IN ACTION

Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Creation of a lab-on-card based on electrical impedance spectroscopy

Kyoshi Daniels

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Bioelectronics and Nanotechnology

SUPERVISOR :

Prof. dr. ir. Ronald THOELEN

MENTOR :

De heer Frederik VREYS

2018
2019

Transnational University Limburg is a unique collaboration of two universities in two countries: the University of Hasselt and Maastricht University.

UHASSELT
KNOWLEDGE IN ACTION

www.uhasselt.be
Universiteit Hasselt
Campus Hasselt:
Martelarenlaan 42 | 3500 Hasselt
Campus Diepenbeek:
Agoralaan Gebouw D | 3590 Diepenbeek

UHASSELT
KNOWLEDGE IN ACTION

Maastricht University

Faculty of Medicine and Life Sciences
School for Life Sciences
Master of Biomedical Sciences

Master's thesis

Creation of a lab-on-card based on electrical impedance spectroscopy

Kyoshi Daniels

Thesis presented in fulfillment of the requirements for the degree of Master of Biomedical Sciences, specialization Bioelectronics and Nanotechnology

SUPERVISOR :

Prof. dr. ir. Ronald THOELEN

MENTOR :

De heer Frederik VREYS

Acknowledgements

This paper means the end of a five-year journey in biomedical sciences. It was a journey with ups and downs, but I am glad I persisted. What started as the choice for an education that was the combination of my favourite courses in high school ended in an interesting learning adventure. An adventure that increased my interest in science, resulted in new friends and makes me look forward to what my future will bring.

But first I would like to thank some people that supported me throughout my education and made this thesis possible.

First, I would like to thank the promotor of my masterthesis, professor dr. ir. Ronald Thoelen for giving me the opportunity to conduct my project in his research group. I appreciate the support he gave me and the new insights he provided when I presented him the problems I ran into. His expertise was invaluable during my internship.

I would also like to thank my daily supervisor, Frederik Vreys, for his support during my internship. He was always willing to answers my questions and really helped with understanding the sometimes difficult subject. He proposed new ideas every time I got stuck and made sure I could develop and grow as a beginning scientist. A special thank you to my second examiner, professor Marcel Ameloot, for making time to listen to my progress and proposing new ideas.

I would also like to thank my fellow students and especially Karen, Birte and Hanne for keeping me company during the breaks and helping me when experiments did not go how I wanted to. A special thanks to my family and parents for supporting me throughout my entire educational career although they did not always know what I was doing. They kept believing in me and helping me when I had a hard time. Last but not least I would like to thank my girlfriend, Eline, who was always there for me throughout my education.

Table of Contents

List of figures

List of abbreviations

CB	Coating buffer
ELISA	Enzyme-linked immune sorbent assay
LOC	Lab-on-card
MIP	Molecularly imprinted polymer
MPBS	Marvel phosphate-buffered saline
NIP	Non-imprinted polymer
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline-Tween
PCB	Printed circuit board
PET	Polyethylene terephthalate
PVC	Polyvinyl chloride
SPAG16	Sperm-associated antigen 16
SPCE	Screen-printed carbon electrodes
THIO	thioredoxin
TMB	3,3’,5,5’-Tetramethylbenzidine

Abstract

The purpose of this paper was to create an electrical impedance platform that can harbour different receptors such as molecularly imprinted polymers (MIPs), DNA and proteins. The current detection techniques are expensive and require laboratory equipment and skilled personnel. These requirements are difficult to meet in developing countries, where agriculture and livestock are the biggest source of income.

Microfluidics can solve these problems by their low-cost manufacturing, ease of use and effectiveness. They already have numerous applications in point-of-care use such as pathogen detection and critical care. Innovations in this research field led to the creation of a lab-on-chip, where multiple laboratory functions are fitted onto a small chip. In this paper, a lab-on-card (LOC) is opted for because a larger working space is necessary. These cards were made from alternating layers of polyethylene terephthalate (PET) and double-sided tape and heat-treated to increase their adhesion. Different types of contacts were tested for optimal impedance measurements. MIPs and NIPs were used as receptors. LOCs are a multi-purpose tool due to their layering technique. This property sparked the idea to incorporate an immunoassay into this type of sensor. An immunoassay was implemented to combine the two detection techniques. Antigens were used as detection tool for enzyme functionalised antibodies.

Initially, an LOC was created that used molecularly imprinted polymers and non-imprinted polymers (NIPs), sandwiched between two paper layers, to detect nicotine. Contacts in a PCB slab were fitted in between the different layers of PET and double-sided tape to measure impedance. Afterwards, a card was successfully constructed where a stable impedance signal could be recorded with aluminium contacts. Due to the layering technique, the focus was shifted to the implementation of an immunoassay into an electrical impedance platform. An altered ELISA protocol was used to determine the base impedance values for certain steps of this process. A change in impedance was observed when the antigens and blocking agents were introduced. Once this combination is realised it can be used for the detection of numerous molecules in developed and developing countries.

Abstract in het Nederlands

Het doel van dit werk was het creëren van een elektrisch impedantie platform dat verschillende receptoren kan herbergen zoals MIPs, DNA en eiwitten. De huidige detectie-technieken zijn duur en vereisen gespecialiseerde toestellen en ervaren personeel. Aan deze vereisten is echter moeilijk te voldoen in ontwikkelingslanden, waar landbouw en veeteelt de grootste bron van inkomen zijn.

Microfluidica kunnen deze problemen oplossen door hun goedkope fabricage, gemakkelijk gebruik en effectiviteit. Ze hebben al vele applicaties in point-of-care, zoals in pathogeen detectie en in de kritieke zorg. Innovaties in dit onderzoeksveld hebben geleid tot de creatie van een lab-on-chip, waar verschillende laboratoriumtechnieken op een kleine chip verzameld worden. In dit werk wordt voor een lab-on-card (LOC) gekozen omdat een grotere werkruimte nodig is. Deze kaarten worden gemaakt van afwisselende lagen van PET en dubbelzijdige plakband en ze worden verhit om hun adhesie te vergroten. Verschillende soorten contacten werden getest om optimale impedantie metingen te bekomen. MIPs en NIPs werden gebruikt als receptoren. Lab-on-cards zijn zeer breed inzetbaar door hun lagen techniek. Deze eigenschap deed het idee ontstaan om een immunoassay in dit type sensor te verwerken. Een immunoassay werd dan toegevoegd aan een LOC om de twee detectiemethoden te combineren. Antigenen werden gebruikt als receptor voor enzym-gebonden antilichamen.

Initieel werd er een lab-on-card gemaakt dat MIPs en NIPs, die vastzaten tussen 2 lagen papier, gebruikte om nicotine te detecteren. Contacten in een PCB plaat werden tussen de verschillende lagen van PET en dubbelzijdige plakband geplaats om impedantie te meten. Hierna werd een kaart met aluminium-contacten gemaakt waarbij een stabiel impedantie-signaal gemeten werd. Door de lagen techniek werd de focus verlegd naar het combineren van een immunoassay met een elektrisch impedantie platform. Een aangepast ELISA protocol werd gebruikt om stabiele impedantie-signalen te verkrijgen voor bepaalde stappen van het proces. Een verandering in impedantie werd opgemerkt bij het aanbrengen van antigenen en blokkers. Eenmaal deze combinatie gerealiseerd wordt in een LOC kan deze gebruikt worden voor de detectie van moleculen in ontwikkelde landen en ontwikkelingslanden.

1. Introduction

1.1 Background

There have been several threats to global health throughout history. For example, the Ebola virus and Zika virus are the most recent ones, surfacing in 2014 and 2015 respectively. Tuberculosis too has been a threat to mankind for thousands of years and still kills 1.7 million people each year (1). These diseases can be contained but this depends on the ability to identify the root cause and the diagnosis of the afflicted. Diagnosis is crucial in managing the spread of the disease, but shortage of laboratory equipment and scarcity of trained personnel impede this process (2). Techniques such as electroluminescence and immunoassays to detect target molecules in fluids have these shortcomings and are time-consuming and costly too (3). The equipment and price of these methods especially are a big problem for developing countries, which rely on agriculture and livestock. These countries have little to no infrastructure and medical personnel (4). These problems can be solved by using microfluidics.

Microfluidics integrate multiple liquid handling processes at the microscale, creating miniature diagnostic assays (5-7). Their biggest advantages are the decrease in fluid volume from millilitres to nanolitres or less and the decrease in time (8). The number of error-prone steps is diminished as well. Their small size makes these devices appropriate for point-of-care use (9-11). This type of devices has numerous applications in pathogen detection, critical care and haematology. Advances in microfluidic technology led to an innovation in diagnostics called a lab-on-chip (12).

A lab-on-card has the same working principle, but the laboratory functions are fitted on a credit card-sized platform. They are built by stacking several layers of individually cut fluidic circuits, yielding a complex three-dimensional microfluidic structure with the size of the functional elements ranging from 100 micrometres to a few millimetres. The different layers can be bonded using adhesive or thermal binding processes. This stacking technique allows rapid sensor fabrication and testing (13).

Paper-based microfluidics are another option to manipulate small amounts of liquids. Whitesides and co-workers started to work with paper in microfluidics by producing channels on paper to detect analytes in 2007. They developed 2D and 3D micro-assays by patterning paper with different designs (9, 14). Since then, this material has become more interesting to work with and to incorporate into these lab-on-chips due to its low cost and disposability. The cellulose material moves the liquid sample due to capillary forces, making power and mechanical pumps unnecessary. A paper-based device is easy to use, has a rapid response, is sustainable and is also compatible with many medical applications (15). The devices are based on lateral flow tests, with the pregnancy test as the most well-known example (fig. 1). Such a test consists of a sample pad, where the sample is administered, a reagent pad and a test line. Once the sample is added to the administration zone, it will migrate forward to the reagent pad where antibodies are present that have conjugates specific for the antigen of the target molecule. This entity flows further along the paper strip towards the test lines, that contain immobilised antibodies specific to the target molecule-antibody complex. The entity will then bind to the antibodies on the test line, creating a sandwich-assay and showing a positive result (4).

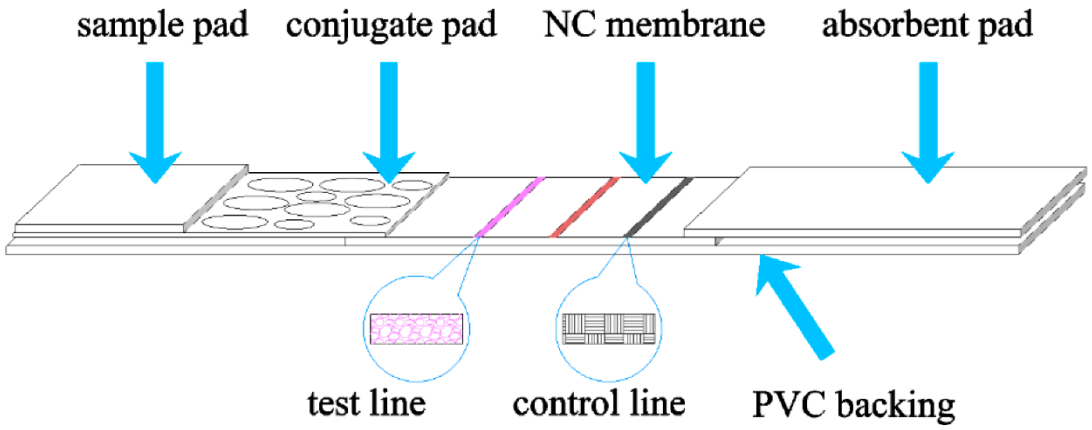

Figure 1: The pregnancy test is a well-known lateral flow test (16).

1.2 Detection methods

Analytes can be detected in several ways (fig. 2). The pregnancy test for example uses colorimetric detection, a technique that is mostly used in laboratory tests and industrial applications (fig. 2A). A calibration chart is used to acquire semi-quantitative readouts. Different analytes can be detected by creating different detection zones. Once the sample is administered, the presence of target analytes can be detected by colour changes in these detection zones. However, this technique has a low selectivity and sensitivity. Chemiluminescent and electrochemiluminescent detection makes use of light that is generated due to a chemical or electrochemical reaction between two reactants (fig. 2B). These reactions can be oxidation, enhancement or inhibition of luminescent effects or electron transfer (17). Fluorescent detection has a higher sensitivity and selectivity than colorimetric detection (fig. 2C). Target analytes are identified by receptors that are bound to a fluorescent agent with a characteristic wavelength. A fourth method is electrochemical detection which needs a three-electrode system to carry out a measurement. It is a technique with a faster response and higher sensitivity, yielding better quantitative results. Another advantage of this technique is the possibility to detect label-free (18, 19).

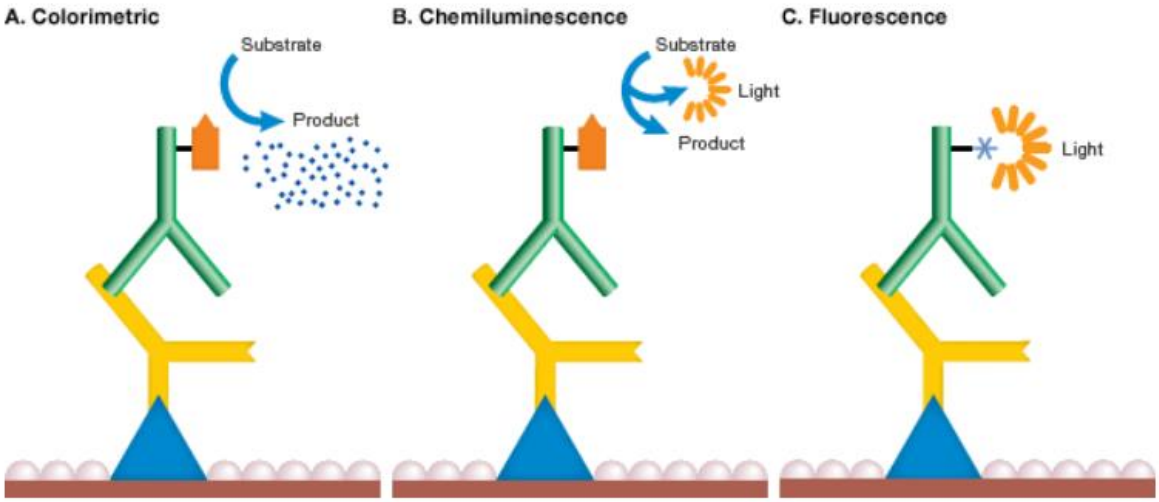

Figure 2: The different detection techniques (20).

1.3 Detection with antigens or antibodies

Enzyme-linked immuno sorbent assay (ELISA) is an enzyme immunoassay that makes use of the previously mentioned detection techniques. It can be used as a homogeneous or heterogeneous detection tool. The working principle of the first uses the inactivation of the enzyme when it binds to the antibody, making washing and thus separation of the antigen from the medium unnecessary. Only the unbound enzyme-labelled analytes will react with the substrate, producing a colour (21). This detection method is primarily used for substances in small quantities, but it is expensive and has a low sensitivity. In the latter method, interference of other non-specific molecules after binding of the antibody or antigen is prevented by washing away anything other than the antibody – antigen complex. The sensitivity is severely increased by doing this, causing it to be more used. The heterogeneous type of ELISA is split up into multiple subtypes because of the variety in structure and characteristics of the substances. The direct assay (fig. 3A) is mostly used to determine the amount of high molecular weight antigens. Antigens or antibodies are immobilised onto a surface and a washing step occurs to remove the unbound molecules. An enzyme is then administrated, followed again by washing and the addition of a substrate. A signal is produced through the reaction of the enzyme with the substrate and can be detected as a change in colour, light production or other detectable signals (22, 23). This signal is then measured to determine the amount of antigen or antibody. A new labelling antibody is necessary for each ELISA however because it only recognizes one specific analyte (24, 25). The indirect assay (fig. 3B) gets its name from a secondary antibody or antigen which is added to the medium to measure the antigen. Antibodies are then added to the plate that is functionalised with immobilised antigens and an antibody-antigen complex is formed. A secondary antibody, tagged with an enzyme, and the substrate are then administered to render this complex visible and measure the concentration of the antigen. The third subtype of this enzyme immunoassay is the sandwich ELISA (fig. 3C). The plate is now coated with antibodies and blocked. Subsequently, a sample is added before incubation and washing. When the antigen binds to the antibody, an unbreakable complex is formed while the washing removes the unbound analytes. Secondary antibodies that are tagged with specific enzymes for these target molecules are then added, followed by incubation and washing. A substrate is added to the medium to create a colour reaction that can be visualised and measured. This method has a sensitivity that is two to five times higher than other ELISAs. Competitive ELISAs (fig. 3D) are the last type, having the wells coated with antigen-specific antibody or antibody-specific antigen. The sample and the enzyme tagged antigen or antibody are added to the medium at the same time. The enzyme-functionalised and non-tagged molecules then compete to bind to the wells. After washing and the addition of the substrate, the coloration can be used to quantify the concentration of the target analyte (26).

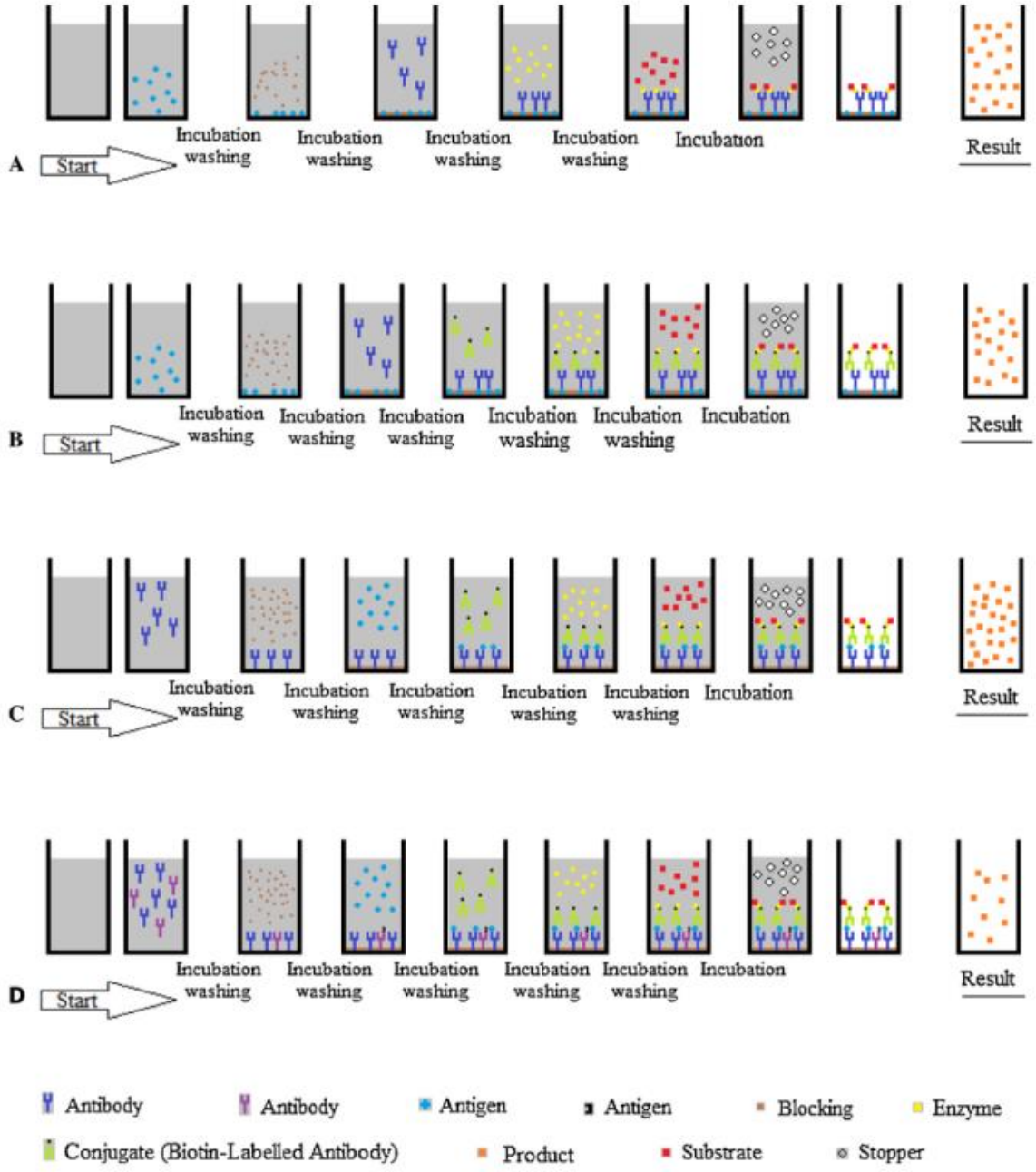

Figure 3: Different types of ELISA (26).

1.4 Electrical impedance spectroscopy

Impedance is the frequency dependent resistance to the current flow of a circuit element and is measured by applying an AC potential and then recording the current or applying a current and recording the voltage. It can be expressed as a complex number with a real and an imaginary part (27, 28). Electric impedance spectroscopy is a label-free method and stands out to the current detection techniques in several ways. The electric field lines are created in such a way that the analytes themselves distort them. While the current techniques require additional instruments and data processing, a direct readout of the signal is gained by plotting the impedance in function of time and no post processing is necessary in this label-free method (29, 30). This makes it interesting to use in developing countries. The technique is based on a Randles circuit (fig. 4), that consists of R_s (resistance of the solution), R_{ct} (charge transfer resistance) and C_{dl} (double layer capacitance). These circuits can be more complex, but this is a simple circuit to explain the concept. Electric impedance spectroscopy measures the frequency dependent resistance at different frequencies. From this data, a bode plot can be created where the frequency is plotted to the magnitude of the impedance. At low frequencies the current will go through the R_s, R_{ct} and C_{dl}, so the value of the latter two can be determined. At high frequencies however, a short-circuit will occur at the double layer capacitance. In this case, only the resistance of the solution will be measured but this is often not the property of interest (27). When a sample is administered to the device, the target analytes can bind to the receptors that are present in the sensor. This changes the value of the double layer capacitance and thus also the impedance. That change is visible on the bode plot, making it possible to conclude that an analyte has bound to the receptor (30).

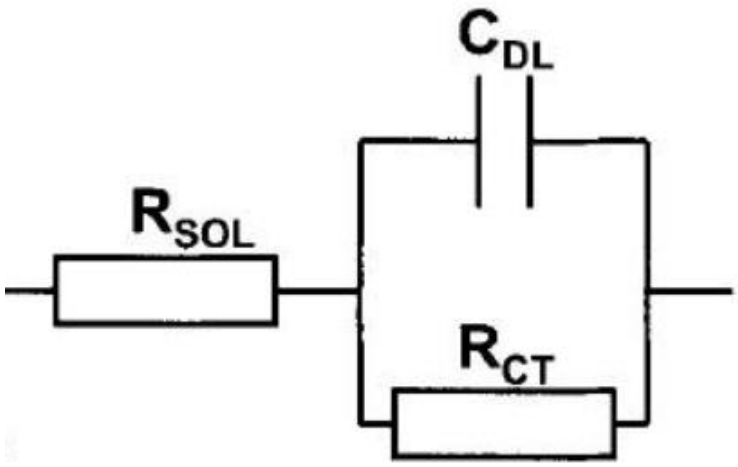

Figure 4: A general Randles circuit (27).

1.5 Polymeric detection

Molecularly imprinted polymers (MIPs) are used as the receptors to detect the analytes. They are constructed of polymers and their affinity and selectivity approaches that of antibody-antigen complexes. Their advantages over the real binding complexes are an intrinsic stability and robustness, due to the high number of cross-links, making them useful in tough conditions such as high temperature or pressure. Additionally, production is cheap, and they have a lengthy shelf-life. MIPs are created by introducing functional monomers and cross-linkers to a template/target molecule (fig. 5). Afterwards, polymerization occurs, and the functional groups of the monomers are fixated due to the heavily cross-linked structure. Once the template molecule is removed, these recognition particles gain a molecular memory to this molecule due to the specificity of the created binding sites (31).

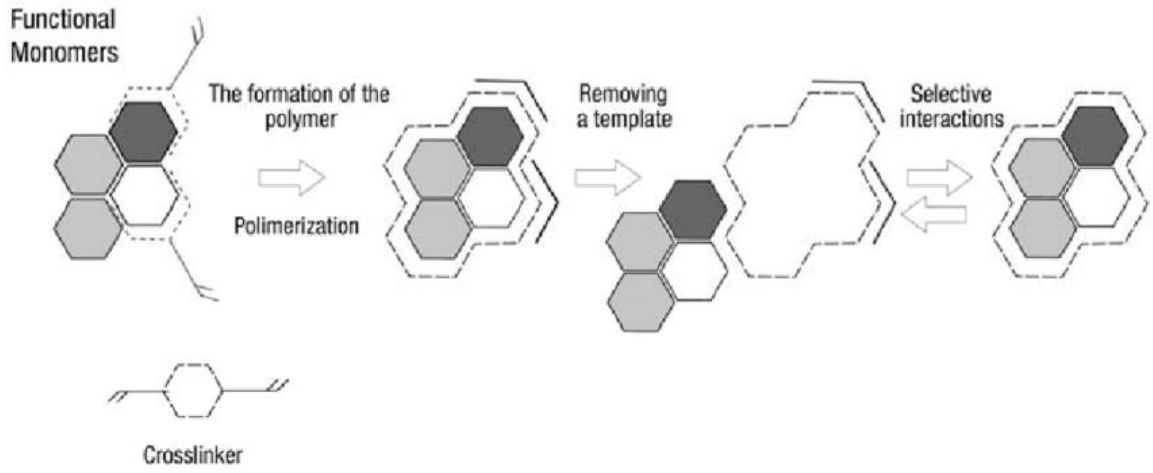

Figure 5: The molecularly imprinted polymer synthesis process (32).

1.6 Purpose

In this work, microfluidic lab-on-cards are created that detect molecules by electrical impedance spectroscopy using two types of receptors. The devices will be created from several layers of PET, double-sided tape and in one case paper. Fluid is guided through these different layers by making gaps in the double-sided tape and creating channels when this layer is adhered to a PET layer. A first device will make use of MIPs and non-imprinted polymers (NIPs) as receptors for nicotine and electrical impedance spectroscopy as detection technique. Once this sensor is formed, electrical impedance spectroscopy measurements can be performed. The base impedance values will be determined in a plexiglass flow cell first by administration of phosphate-buffered saline and MilliQ. Afterwards, paper will be added to test the influence of the cellulose material. Next, MIPs and NIPs are added in between two pieces of paper. The impedance is monitored while nicotine is administered to determine if there is a change in impedance. Once these values are acquired, the impedance is measured in the LOC when nicotine is added. The creation of an automated immunoassay platform will be the second application of the lab-on-card. Antibodies will be used as receptors for the target analyte. A lab-on-card will be made first where the bottom layer of the well will be treated to allow binding of antigens. These layers can intervene with impedance measurements, so the right balance between the ability to bind antigens and the ability to measure impedance will be an important factor. Afterwards, an adjusted protein SPAG16 ELISA protocol will be used to measure the impedance while different ELISA steps are executed. If the background signals are found, the enzymatic antibodies will be added to combine the colorimetric detection with electrical impedance spectroscopy to detect molecules. To validate the sensor where the immunoassay principle is integrated, the screening of humoral autoimmune response in Multiple Sclerosis is taken as an application. If a functional lab-on-card is created, it can be used for detection of molecules that show the presence of a disease.

1.7 Application

A future purpose of this type of sensor can be the detection of *Mycobacterium tuberculosis* in sputum (33). This bacterium is responsible for tuberculosis, a disease that still causes 1.7 million deaths worldwide each year (1). The high death count is due to not being able to control the spread of this disease. The infectious character of the disease cannot be easily detected and treated, allowing the bacterium to spread (34). The transmission happens via aerosol droplets that are present in the air for a prolonged period. When someone inhales these particles, the active form of tuberculosis can develop but this probability is very small. The symptoms are fever, weight loss and the symptoms of the affected organs (35). Most people who inhale these droplets eliminate the bacterium or have a latent bacterium which is in equilibrium with the host after the immune system has contained the bacilli. However, reactivation of this latent bacterium is possible depending on the strength of the immune response due to chronic diseases. An LOC could be made where primers would be the receptors for certain DNA strands of the bacterium. Fluorescence measurements would be done with SYBR Green I to detect the presence of the strands.

2. Materials & methods

2.1 Hardware

The cutting of the different materials such as PET, double-sided tape, Kapton tape and plexiglass were performed with a Trotec Speedy100R laser cutter (fig. 6A). It uses a laser beam, which is a column of high intensity light, of a certain wavelength or colour. Once the laser goes through the nozzle bore, the focused beam hits the plate. The focusing of the laser takes place in the laser cutting head and is done by a special lens or curved mirror. This process is very important because the shape of the focus spot and the density of the energy in that spot need to be perfectly round and consistent. That single pinpoint has an extreme heat density. This results in rapid heating, melting and partial or complete vaporizing of the material. By using a vector drawing program, the designs can be uploaded to the laser cutter and the desired products can be acquired. In this case Inkscape was used. PET, double-sided tape and Kapton tape were also cut with a Silhouette CAMEO blade cutter which uses a blade and an adjustable force, depth, speed and amount of repetitions to get through the material (fig. 6B). Impedance measurements were carried out by a Keysight 4194A impedance/gain-phase analyser in combination with a Keithley 7002 switch system, or with an Ivium technologies Iviumstat standard in combination with an Ivium technologies HiMUX electrochemical multiplexer. A small potential was administered to the sensor and the current was recorded at frequencies ranging from 1 Hz to 1 MHz.

Figure 6: A. Trotec Speedy100R laser cutter. B. Blade cutter

2.2 Software

A MATLAB script was used to process the impedance data acquired from the measurements and to create files that could serve to form an equivalent circuit from the plexiglass flow cells that were used to measure the impedance (fig. 7A). An Iviumstat was used to accomplish this by recording the real and imaginary part of impedance from 1 Hz to 1 MHz. The data that was acquired from these measurements was processed and converted into an equivalent circuit (fig. 7B), which helps understand the behaviour of the impedance in the flow cell. The number of elements in the circuit and which element took each spot could be chosen. Subsequently, the program would fit the created equivalent circuit to the data and visualise the similarity between the actual data and the data that would be gained from this circuit. The lower the determination coefficient of the amplitude, the better the fitting of the equivalent circuit. The different devices that were used were controlled with LabView programs created by the BDE group.

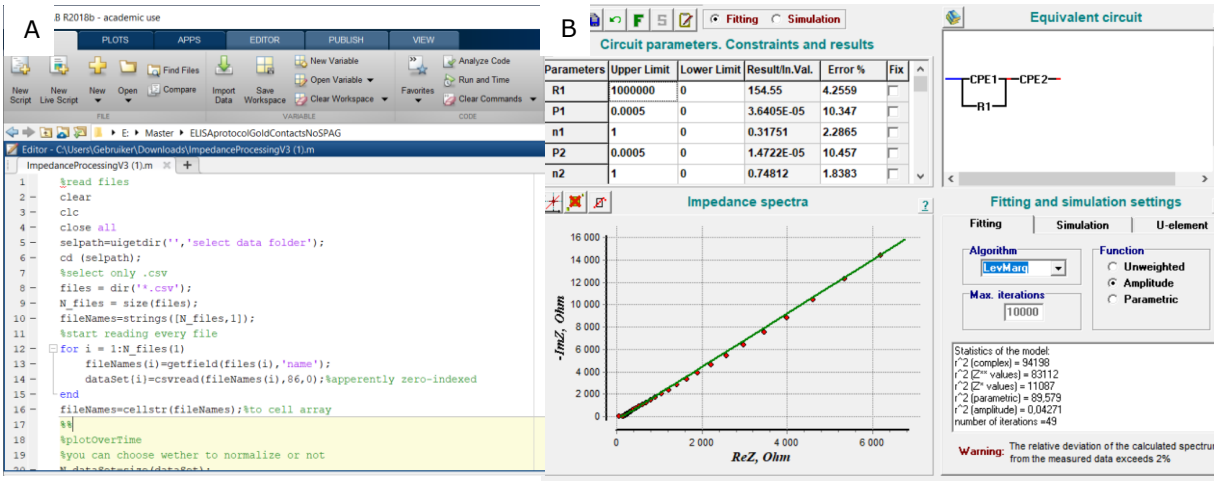

Figure 7: A. MATLAB script. B. Equivalent circuit of a flow cell filled with PBS.

2.3 Plexiglass flow cell characterisations

A plexiglass flow cell (fig. 8A) was prepared with two paper pieces and MIPs or NIPs between them enclosed by the two halves of the flow cell. Each plexiglass flow cell contained a thermocouple, present in the centre of each half, where a clamp was attached to serve as electrical contacts (fig. 8B). The fluid entered in the bottom half and passed through the paper to the top half (fig. 8C) to decrease the amount of trapped air. The electric field lines pass through the paper in a vertical manner. Firstly, PBS and MilliQ were administered to the plexiglass flow cell to create a stable signal. MilliQ was obtained from an atrium comfort system from Sartorius and PBS was acquired by dissolving BupH™ Phosphate Buffered Saline packs from Thermo scientific in MilliQ. Afterwards, nicotine was added to monitor the detection of these molecules by the MIPs. The signal of the NIPs served as a background signal.

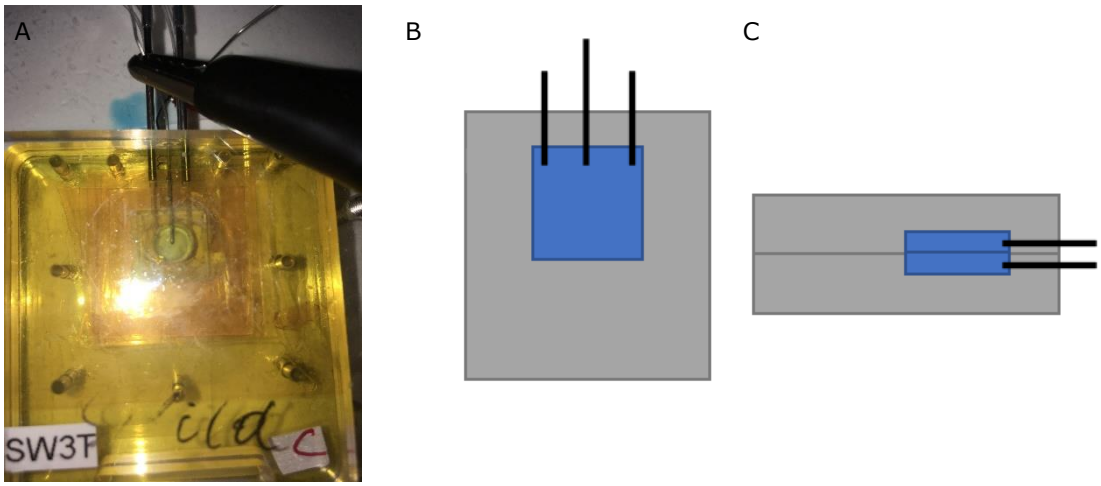

Figure 8: A. Flow cell, view from above. B. Schematic representation of 8A. The centre bar visualises the thermocouple, while the two outer bars exemplify the inlet and outlet of each half. The blue square represents the space where the paper pieces are placed. C. Schematic representation of the side of the flow cell.

2.4 Immunoassay

2.4.1 Well coating for antigen binding

The bottom of the immunoassay lab-on-card needed to be functionalised to allow antibody detection. Polystyrene was chosen to create an attractive layer for proteins, as these molecules did not attach to PET. The treated surface was first coated with aluminium to create electrical contacts. Subsequently, the polystyrene coating was formed by spin coating for 40 seconds at an acceleration of 4000 rpm/s and a speed of 2000 rpm. To validate the thickness of the polystyrene layer, a scratch was made on this coating and DektakXT from Bruker was used. This device made use of a thin needle with a diamond head that scratched the surface and measured the change in height (fig. 9). The thickness of the polystyrene coating was determined by the height difference.

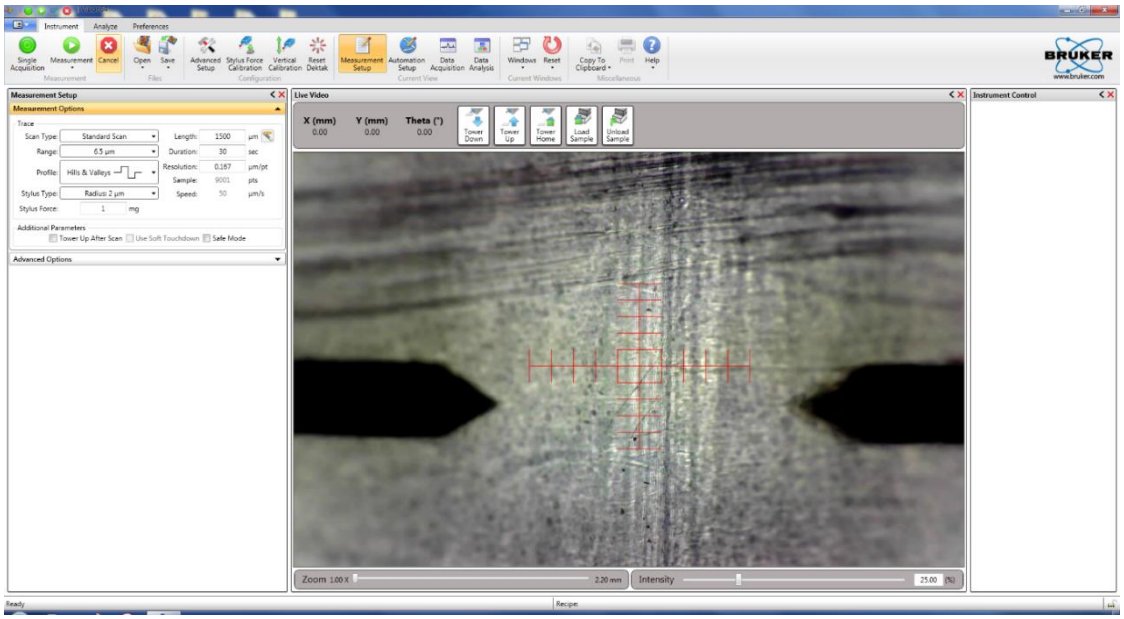

Figure 9: Positioning panel for the needle of the DektakXT. Pathlength, duration of measurement and scan type can be changed.

2.4.2 ELISA protocol

All reagents and buffers for the ELISA protocol were supplied by the Biomedical Research Institute. The ELISA process that followed the protocol from de Bock et al. (36) was started by administering a certain concentration of SPAG16 into the well (protocol in SI). After the dilution of 10xPBS to 1xPBS and the creation of 2% marvel PBS (MPBS) and PBSTween (PBST), a washing buffer, the coated well was washed with 1xPBS. Then, the well was blocked with 2% MPBS for two hours at 37°C. Afterwards, the blocked well was washed three times with PBST and one time with PBS. Next, dilutions of the standard were made in 2% MPBS and the samples were diluted 1/100 plasma: MPBS. Thereafter, the plasma was administered to the sensor and thus in the well to incubate for 2 hours at room temperature. Once the 2 hours were over, the well was washed three times with PBST and one time with PBS. Rabbit anti-human-HRP was then added and incubated for one hour at room temperature. During this incubation time, 3,3',5,5'-Tetramethylbenzidine (TMB) was brought to room temperature and used to stain the well after washing. The reaction that was started with rabbit anti-human-HRP is stopped after ten minutes with $2NH_2SO_4$.

2.5 Protocol for impedance spectroscopy measurements of SPAG16

A protocol was also necessary for the impedance measurements of the ELISA protocol (table 1). It was slightly altered by stopping before the rabbit anti-human-HRP would be added. A stable impedance signal for the coating of SPAG16 and the blocking with MPBS was required first. Multiple steps of PBS administration at a temperature of 20°C were added to the protocol after SPAG16 coating and blocking to determine if and how the impedance changed after a temperature change and after addition to the bottom layer of the well.

Table 1: Protein ELISA SPAG16 impedance spectroscopy protocol.

Temperature (°C)	Medium	Contents	Importance
20	PBS 1X	/	Baseline for the whole measurement
4	Coating buffer (protocol)	SPAG16	Coating of the PS substrate
20	PBS 1X	/	Measure the layer in the same conditions as the baseline
37	PBS 1X	Blocking: Marvel skimmed milk	Fill up non-occupied zones
20	PBS 1X	/	Measure the additional presence blocking layer in the same conditions as the baseline
20	PBST 1X	(Tween 0.1%)	Wash excessive blocking agent
20	PBS 1X	/	Wash excessive Tween (Measuring not required)
20	Sample (MBPS 2% 1X)	Analyte: AntiSPAG16	Analyte to be detected
20	MBPS 1X (2%)	Blocking: Marvel skimmed milk	Measure the layer with the same conditions as the sample
20	PBS 1X	/	Measure the layer with the same conditions as the baseline
20	PBST 1X	Tween (0.1%)	Wash excessive analyte
20	PBS 1X	/	Measure the layer with the same conditions as the baseline

2.6 Antibody detection set-up

Two set-ups were used for the antibody detection. First, the lab-on-card was put on a 15 cm high plexiglass table in Styrofoam box filled with ice to create an environmental temperature of 4°C. A thermocouple was attached to the surface of the table to monitor the temperature inside the box. Once the tubes were put into the PDMS slabs that sat on top of the sensors' inlets and outlets, the box was closed. The inlet tubes were connected to the different concentrations of SPAG16, while the outlet tubes were connected to a valve, which in turn was linked to a syringe pump. The desired concentration of SPAG16 was administered by activating the syringe pump and choosing which channel of the valve was used. Afterwards, the rest of the ELISA protocol discussed in section 2.4.2 was done. An oven was used to raise the temperature to 37°C to allow blocking (fig. 10A). The second set-up was used when employing golden contacts that were formerly part of an ELISA microplate. This was put on a thermoblock from Qinstruments, which makes the measuring process easier, by regulating the temperature between 4°C and 37°C. Iviumstat is used to record the impedance over time (fig. 10B).

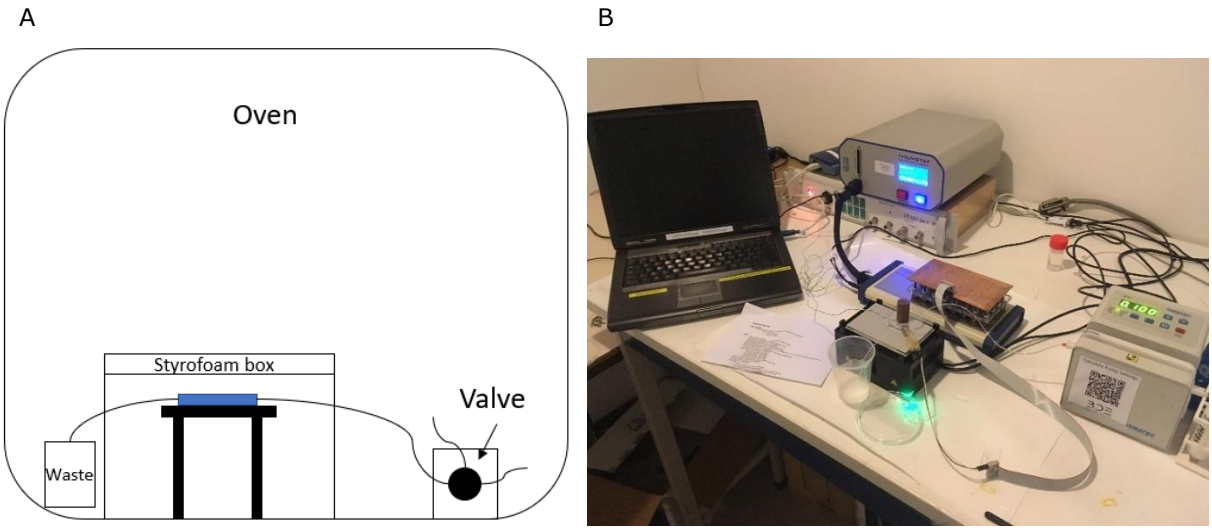

Figure 10: A. Set-up with the oven and styrofoam box. The blue slab represents an LOC position on a plexiglass table. B. Set-up with a Coldplate.

3. Results

3.1 Plexiglass flow cell

The decision was made to start with a golden standard plexiglass flow cell so that the background impedance signals of PBS and MilliQ were already known before tests with lab-on-cards were performed. It was also used to test the influence of paper on impedance and to see if the binding of nicotine to the MIPs could be distinguished in the impedance signal.

3.1.1 Phosphate-buffered saline (PBS) and MilliQ

The base impedance values were determined by filling a flow cell with phosphate-buffered saline first, then adding MilliQ after approximately four minutes. After five minutes, 1xPBS is added again. Impedance is measured at the same time and the values of the two fluids are compared. The impedance levels differed between the liquids in the measurement (fig. 11). 1xPBS has a lower impedance than MilliQ. This is expected due to the lower number of ions present in MilliQ, raising the resistivity.

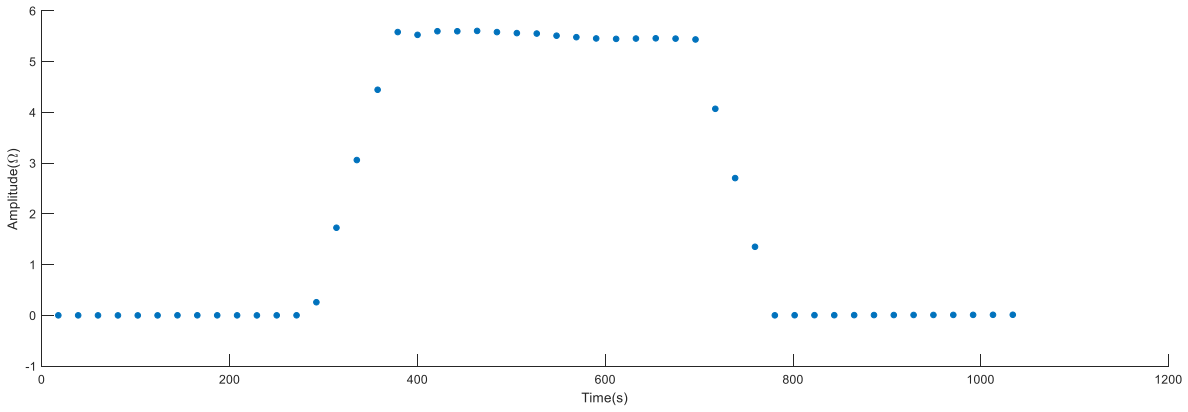

Figure 11: Normalised impedance of 1xPBS and MilliQ plotted over time at a frequency of 126 Hz.

3.1.2 Addition of paper

Whatman paper type 1 from GE Healthcare was used in the lab-on-card (LOC) sensors. It has a pore size of 11 µm and it is widely used in many applications. The material is cut into squares of 9 mm by 9 mm and used to fixate MIPs and NIPs. Once the base impedance values were acquired, paper with a 11 µm pore size was added to the flow cell. The impedance values of the previously mentioned fluids were determined in the presence of paper (fig. 12). The overall base impedance increases as can be seen in the rise in impedance signal.

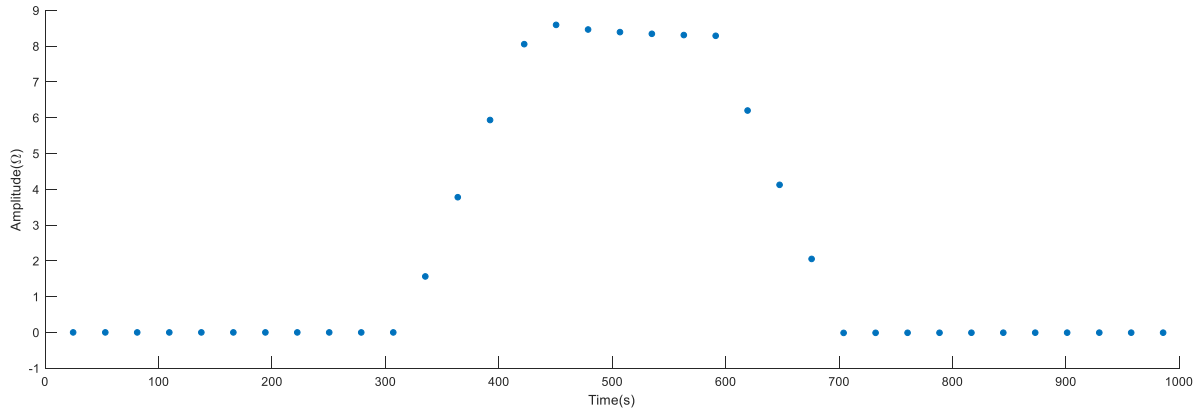

Figure 12: Normalised impedance of 1xPBS and MilliQ after the addition of paper, plotted over time at a frequency of 126 Hz.

3.2 Detection in the flow cell

After the determination of the impedance values of 1xPBS and MilliQ, MIPs and NIPs are sandwiched between two paper pieces and put into a flow cell. 1xPBS and nicotine are added to the flow cell to acquire impedance values of both fluids. All measurements were different however (fig. 13), which raised the question whether the pore size of the Whatman paper type 1 was too big to contain the recognition particles.

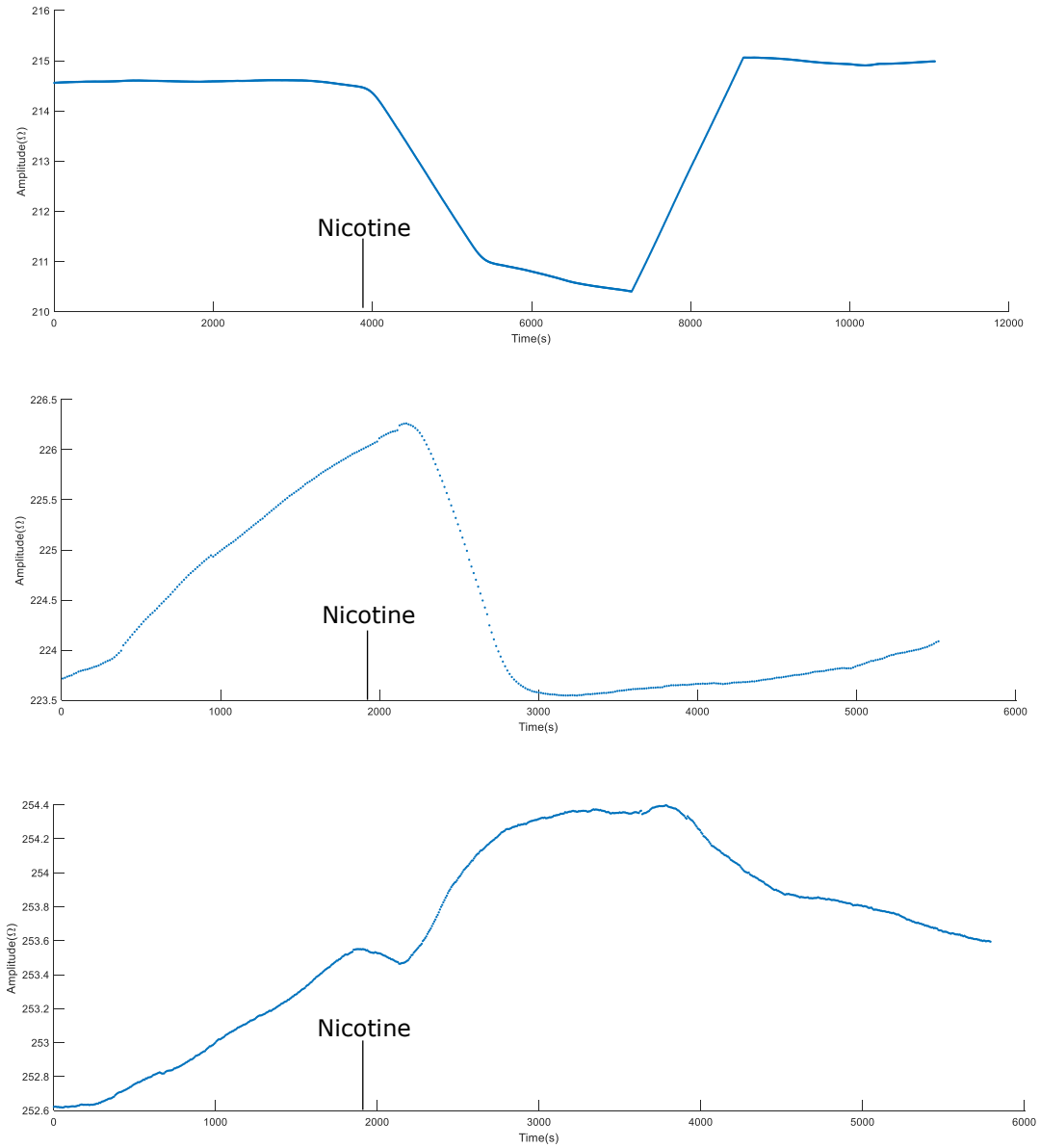

Figure 13: Measurements of sensor with Whatman paper type 1 at 624 kHz, which has a pore size of 11 µm.

The measurement where the substrate was changed to a paper with a smaller pore size of 200 nm gave a more promising result, with an increase in impedance when the nicotine was administered around 2400 s (fig. 14A). The signal from the NIP-functionalised flow cell differed from the signal of the MIP-functionalised sensor, confirming that the change in signal is due to nicotine binding to the MIPs (fig. 14B). The bode plot shows that the change in the capacitive double layer part happens at a low frequency range (fig. 14C).

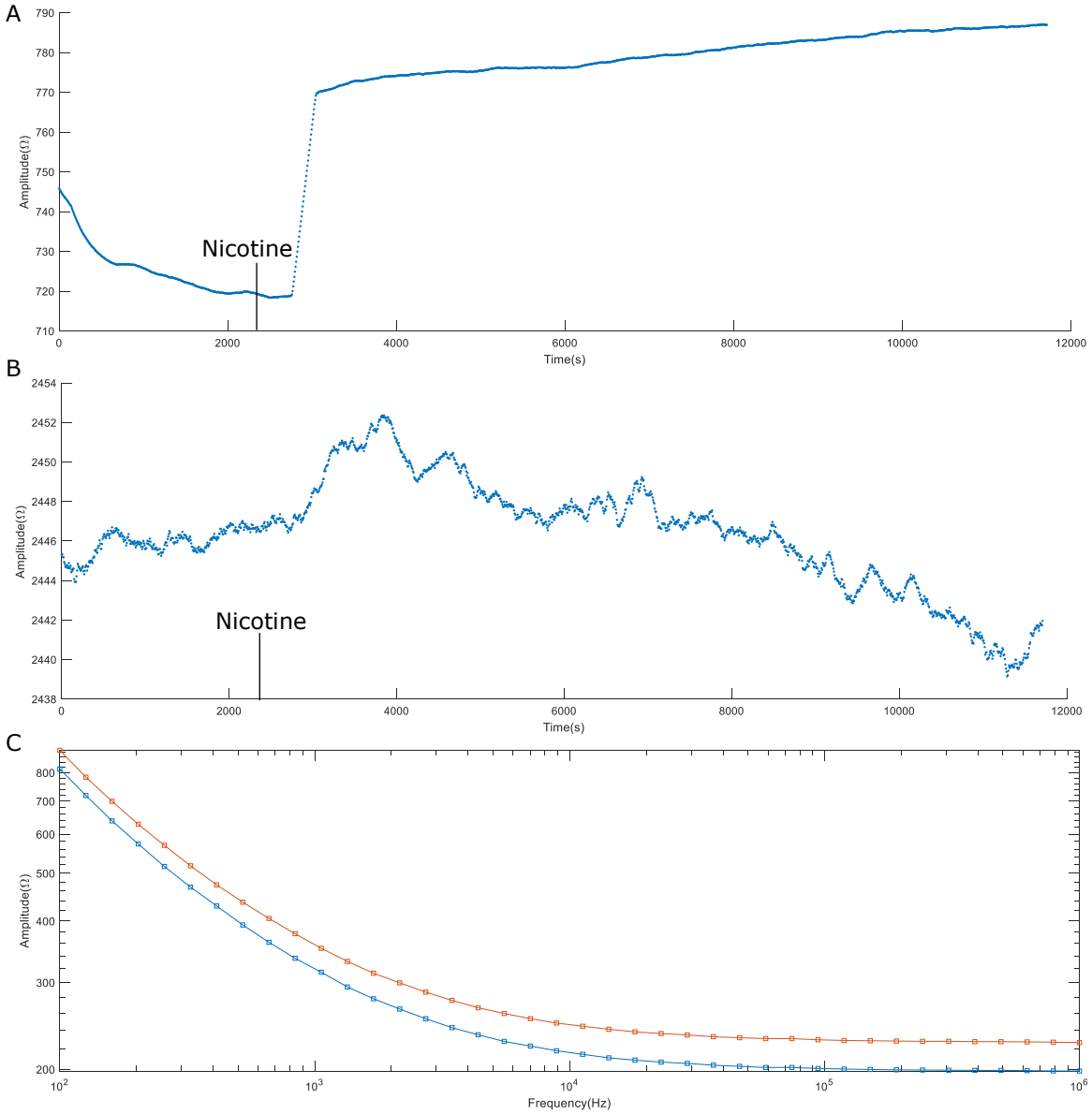

Figure 14: A. Measurement of the sensor at 126 Hz with paper that had a pore size of 200 nm containing MIPs. B. Measurement of the sensor with paper containing NIPs. C. Bode plot of the same measurement to illustrate that the graph with amplitude over time is taken at a frequency where capacitance determines the impedance. The blue curve gives the impedance before nicotine addition, the red curve gives the impedance after nicotine is added.

3.3 Lab-on-card

3.3.1 Heat pressing the lab-on-card

Due to the time-consuming process of screen printing the electrodes on paper-based designs, the decision was made to create lab-on-cards. Carbon electrodes were not necessary anymore because PCB contacts could be used, or aluminium contacts could be made by sputtering aluminium on PET and then laser cutting this product. The first lab-on-card that was created consisted of PET and double-sided tape to monitor the behaviour of flow in such a device after heating with various temperatures (fig. S1). This design had one lane that led to a well where the liquid then continued in one lane to the outlet. The LOCs were heated by putting them in a hot press for 30 minutes at the desired temperature (fig. 15).

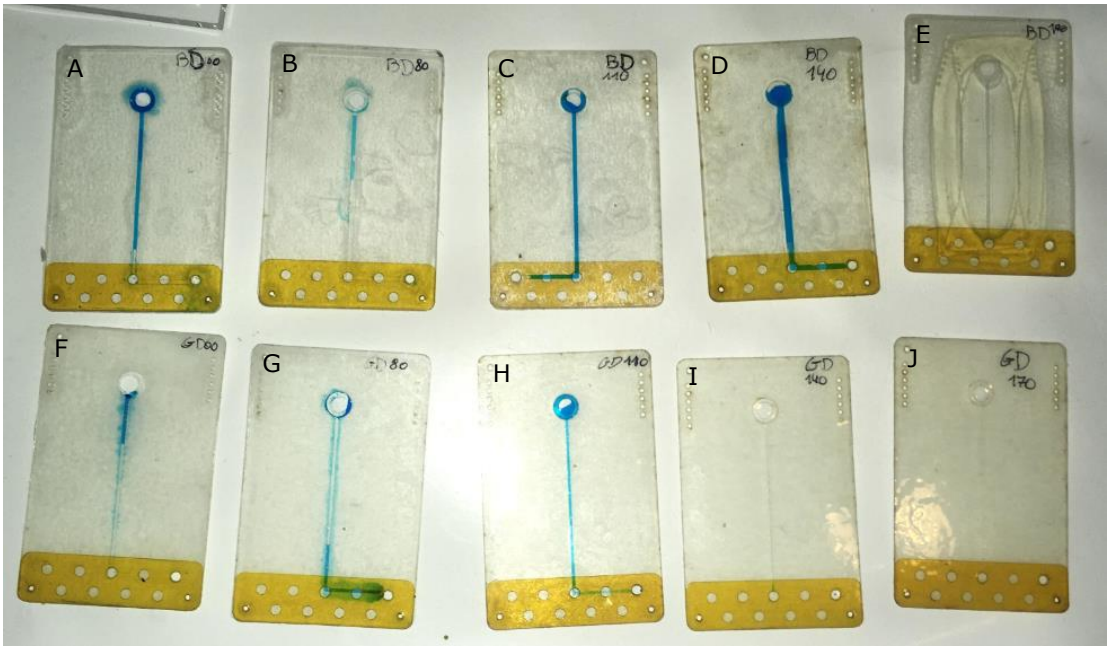

Figure 15: Sensors that were heated at different temperatures for 30 minutes. BD = 150 µm thick double-sided tape. GD= 174 µm thick double-sided tape. Going from A to E the temperature goes from 60°C to 170°C during the heat sealing of thin double-sided tape. Going from F to J the temperature goes from 60°C to 170°C during the heat sealing of the thicker double-sided tape.

The sensors were tested by administering blue salt, dissolved in MilliQ, to their inlet and monitoring the flow of this liquid. From 60°C to 140°C the liquid was not interrupted, and the flow remained. A morphological change was seen with the 150µm thick double-sided tape at 170°C. The material probably shrunk due to the heat (fig. 15E). The sensors with the 174 µm thick double-sided tape had a flow until the sensor was exposed to 140°C (fig. 15I). A flow was no longer possible from this point. This is probably due to the liquifying of the glue in the double-sided tape that clogged the channels. These assumptions were confirmed by microscopic images (fig. 16).

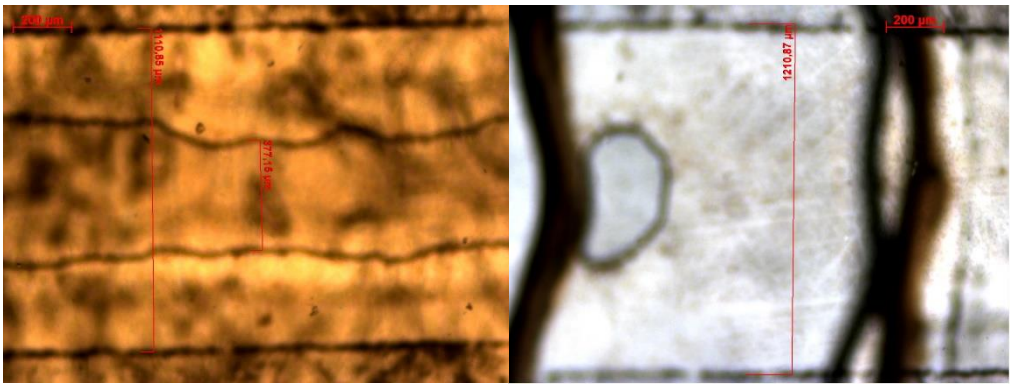

Figure 16: Microscopic images of the channels of the sensors that were heated at 140°C (left) and at 170°C (right).

3.3.2 MIPs and NIPs as detection tools

The sensor consisted of multiple alternating layers of PET and double-sided tape (fig. 17). The fluid needed to be directed through the different layers because the contacts were situated next to each other on the bottom of the sensor. MIPs or NIPs were sandwiched between two paper squares and fixed by double-sided tape on both sides. Electrical contacts on PCBs are added between the layers. Once the sensor is built, it is pressed together to increase the connection. Afterwards, the sensors were heat pressed to melt the glue and to increase the attraction between layers. All the layers were cut out by a blade cutter first, but the laser cutter quickly became the preferred cutting tool because of its precision. The inlet and outlet of the sensor were covered with a PDMS slab to increase the flow of the fluid during the measurement and to prevent leaking.

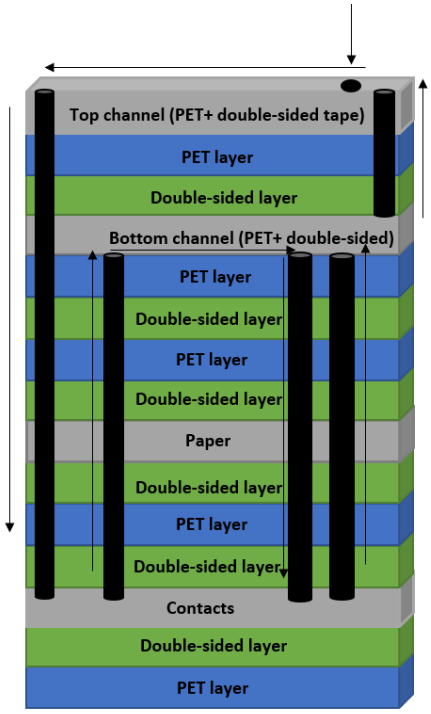

Figure 17: Layer schematic of the lab-on-card design to test flow.

The measurement set-up first consisted of a lab-on-card where the contact pins were at the bottom of the card. The sensor is then fixed on a breadboard with those pins. Afterwards, the pins were put at the top of the card to avoid the use of the breadboard and thereby decreasing the amount of noise. The top sensor contained NIPs in between two paper pieces while the bottom sensor contained MIPs (fig. 18A). The gaps in the layers were used to administer the connection between the contacts and the measuring equipment (fig. 18B). Both sensors leaked however due to swelling of the paper. This phenomenon separated the layers and allowed the fluid to flow into different parts of the sensor.

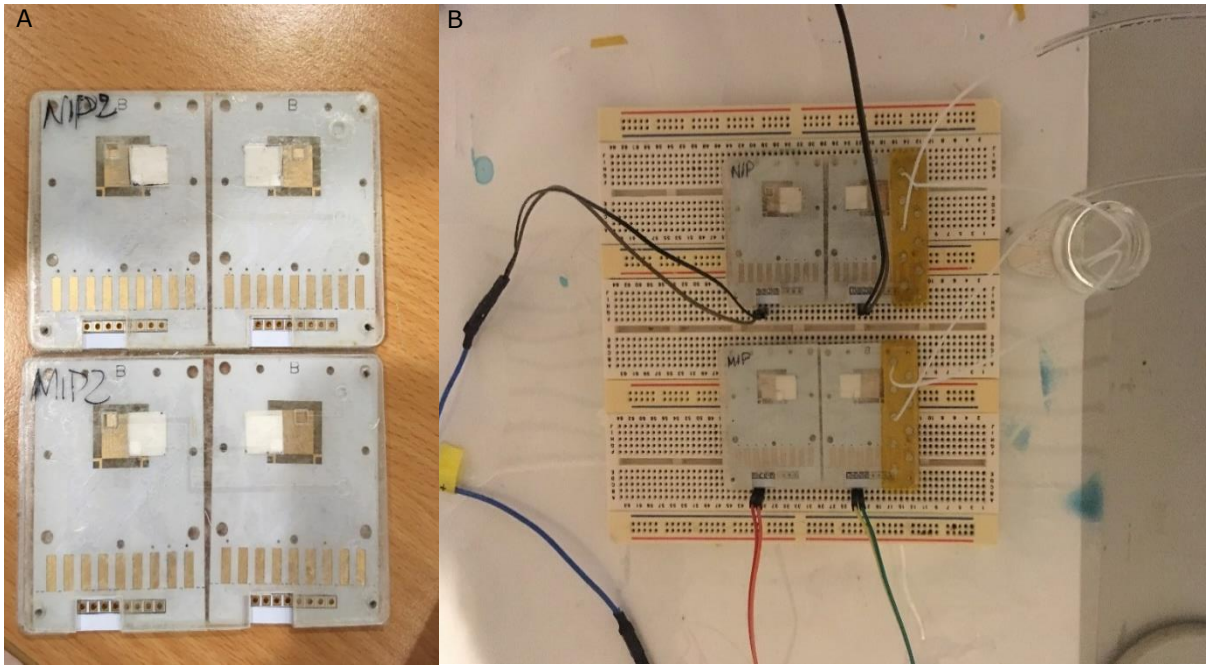

Figure 18: A. One sensor contains MIP, while the other one contains NIP. B. The connections of the sensors.

This problem was solved by adding three more layers around the paper (fig. 19) and thus compensating the increase in size of the cellulose material.

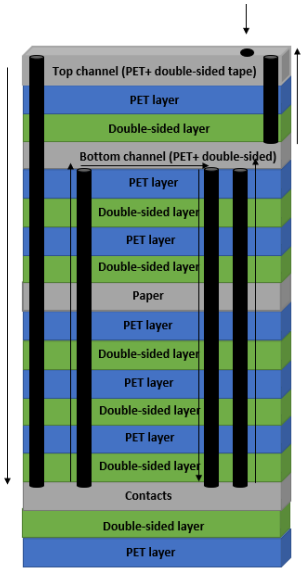

Figure 19: Updated layer schematic.

When the paper with a smaller pore size was used, six additional layers were introduced to prevent the fluid from leaking on top of the PCB slabs (fig. 20A). A background signal was first determined by administering 1xPBS and nicotine to a sensor that had no paper in between and thus was not functionalised with MIPs or NIPs. Nicotine is administered around 1800 s, visualised by the drop in impedance amplitude around 2000 s. 1xPBS is added around 3000 s and 200 seconds later a rise in impedance can be seen (fig. 20B).

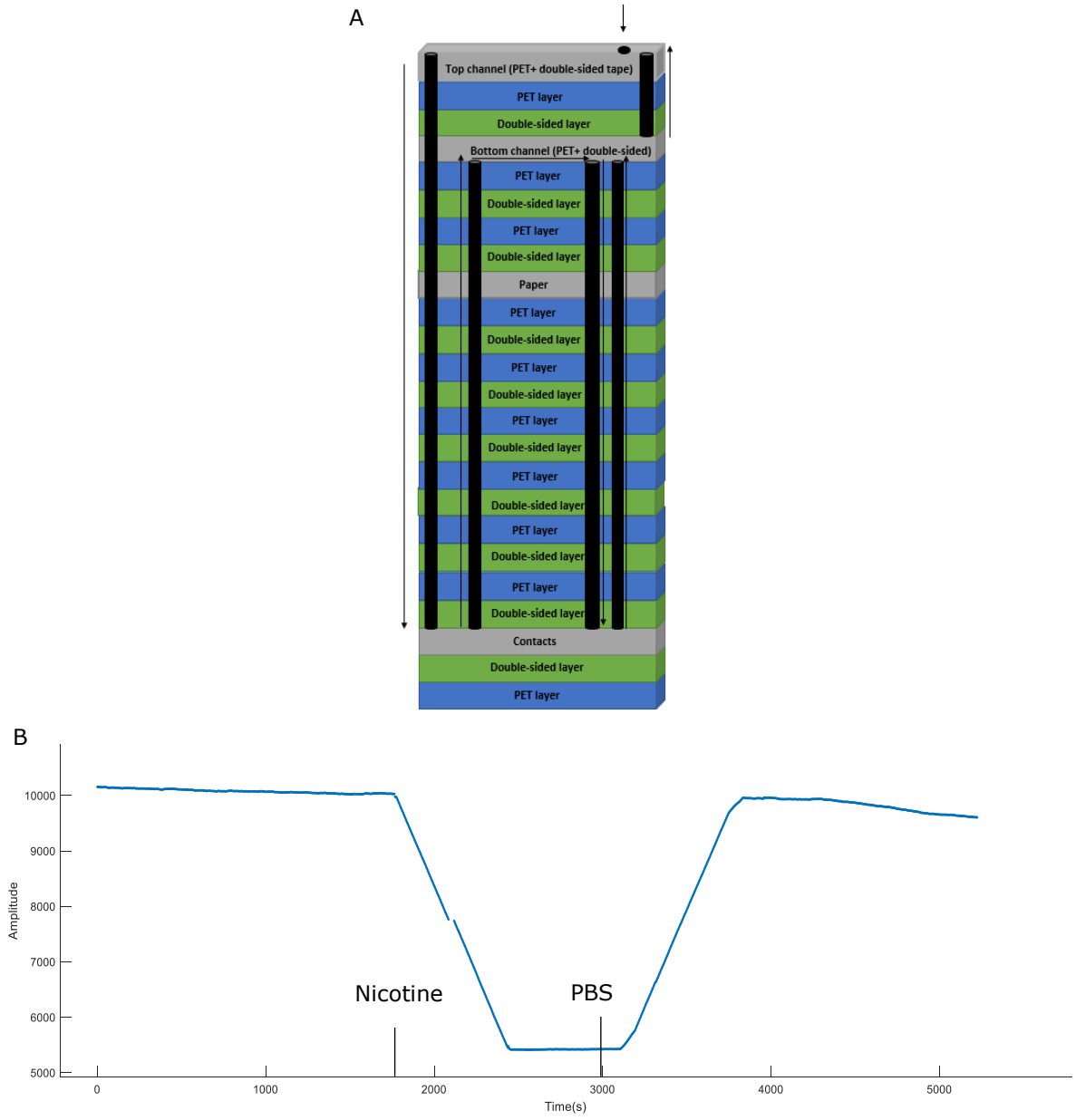

Figure 20: A. Sensor with six additional layers above the contacts to avoid leaking. B. Plot of amplitude over time for an LOC without paper.

Next, MIPs and NIPs were added to the lab-on-card to test if the binding of nicotine could be seen in the impedance signal. Nicotine was added around 3900 s (fig. 21A) as can be seen in the graph below as the impedance is decreased around 5000 s. Nicotine was also added in the NIP LOC after 4400 s but no change could be seen (fig. 21B). There was a difference in signal between MIPs and NIPs, but the measurements were done in different lab-on-cards.

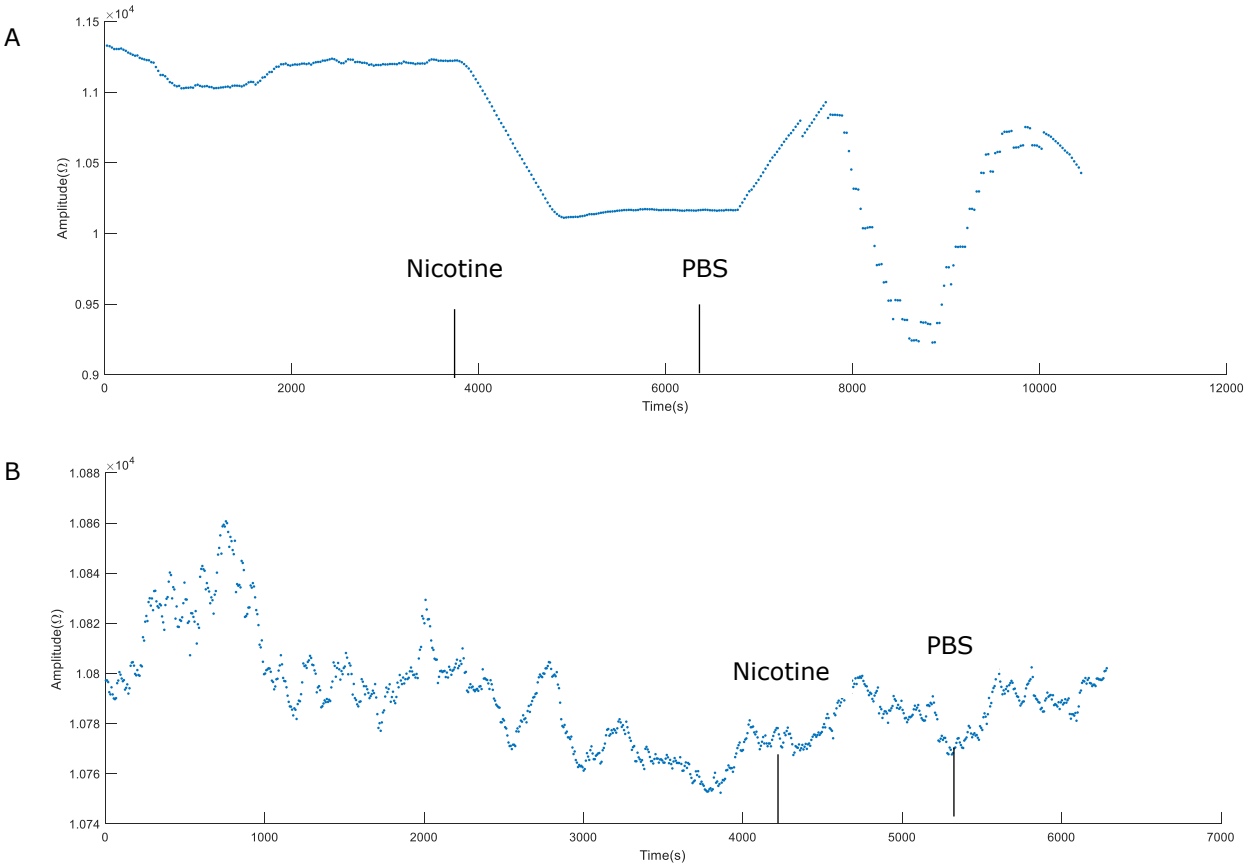

Figure 21: A. Impedance measurement of MIP in a lab-on-card. B. Impedance measurement of NIP in a lab-on-card. Both at 624 kHz.

3.4 Impedance as a detection tool for antibodies

3.4.1 Design for the immunoassay

An ELISA test starts with the immobilisation of target molecules that can bind primary antibodies. Afterwards, the antibodies are added, and they will bind to the targets. Finally, an enzyme-bound antibody is added to cause a colorimetric change that can be measured by a photospectrometric device (37). This means an indirect ELISA is used in the lab-on-card. This principle is combined with electric impedance spectroscopy in a lab-on-card by altering the basic design (fig. 22A). An opening was created in the bottom PET layer where two different sized squares, treated with polystyrene, could be placed (fig. 22B).

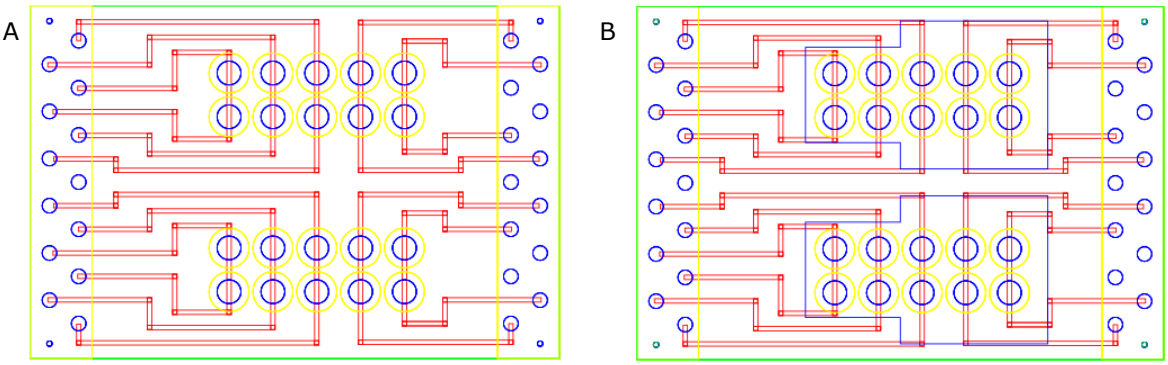

Figure 22: A. Design for ELISA. B. Design for ELISA was altered to incorporate impedance spectroscopy.

After the polystyrene was spin coated on the bottom layer of the well a DektakXT measurement was done. The layer on top of the PET was found to be 600nm thick for a polystyrene concentration of 12%. Afterwards, SPAG16 was the molecule that needed to be attached to the PET layer. THIO could be administered as a control as it can bind to polystyrene too, but these contact angles were not measured. SPAG16 was added by filling the sensor with this protein and letting it settle in the different wells for the night. Afterwards, the wells were dried up and the polystyrene squares were removed. These coated surfaces were then rinsed with MilliQ and dried. Contact angle measurements were done to confirm whether the proteins were bound to the PET layer (fig. 23). If SPAG16 had bound, the contact angle should be smaller than the contact angle with just a polystyrene layer or with a coating of lower concentration because of the hydrophilic character of this protein. A PET layer with a polystyrene coating had a higher contact angle than the PET layer with coating buffer. A downward trend was observed with increasing concentration, as was expected. The concentration of 10 µg/l of SPAG16 had large error bars due to the imperfect coating of polystyrene on the PET layer.

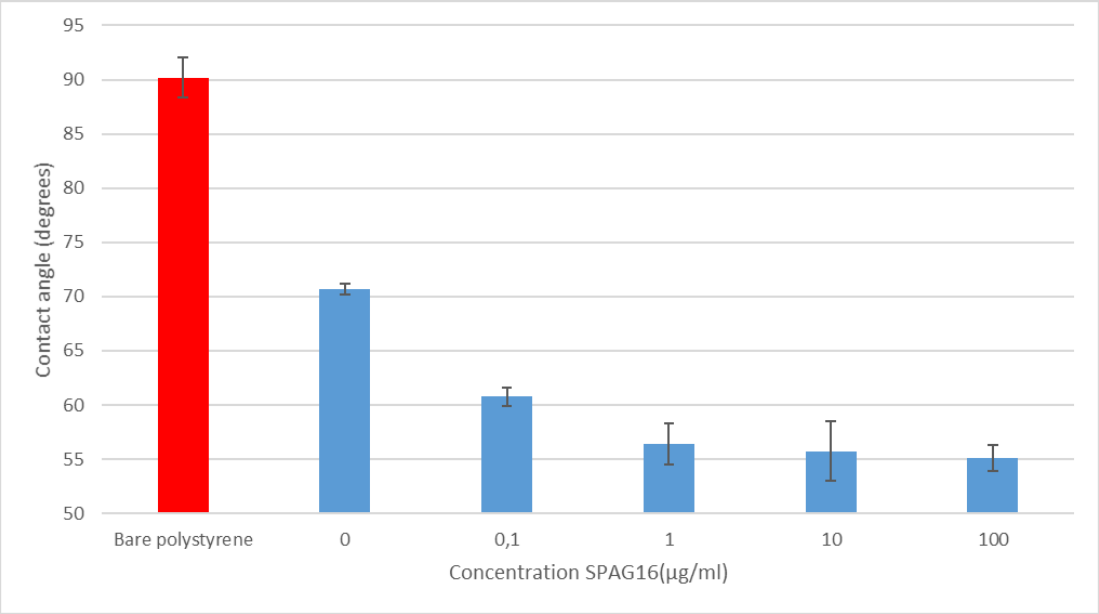

Figure 23: Contact angle measurements for coating buffer (0) and SPAG16 for 0.1, 1, 10 and 100 µg/l.

However, it was not possible to implement contacts to measure impedance in this lab-on-card without having to remove certain lanes and thus reduce the number of wells. So, a lab-on-card was then made (fig. 24) to use the ELISA principle for one concentration only. A gap was created to add a square with polystyrene coating. The ELISA process from de Bock et al. (36) was then started by administering a certain concentration of SPAG16 into the well.

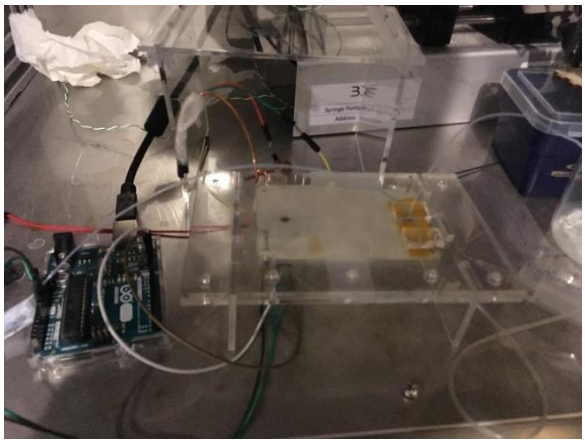

Figure 24: The lab-on-card is built in such a way that the well is aligned with the sensor.

The intensity measurement took place while the TMB was added and the enzymatic reaction was happening. The violet wavelength was used to measure the change because the energy that was released with the reduction of hydrogen peroxide to water created wavelengths that are present in the violet range. The first measurement with this technique gave no significant result. This could be due to the design of the LOC which had the outlet channel underneath the inlet channel, not allowing the fluid to fill the well entirely (fig. S2). The antibodies could not attach to the bottom of the well and the enzymatic reaction could not take place. A second measurement with a design that had opposing inlet and outlet channels (fig. S3) was done but this time only the colour change was looked at. There was a change to blue when the TMB was administered, indicating the presence of the enzyme-bound antibodies. The tubes to the inlet and from the outlet were also blue, indicating that there were antibodies present as well. No conclusion can be formed because there is no certainty that the blue coloration in the well is due to the presence of the antibodies in the well or due to the liquid reacting with the antibodies present in the tubes.

An ELISA reaction was also completed in a microplate, followed by optical density measurements. The reaction product of this ELISA was then introduced into the LOC and optical density was measured again. The two measurements were compared, and the determination coefficients showed that the sensitivity of the LOC is approximately the same as the sensitivity of the microplate (fig. 25). The optical density is lower in the lab-on-card because of its smaller well size, causing less absorption of the incoming light.

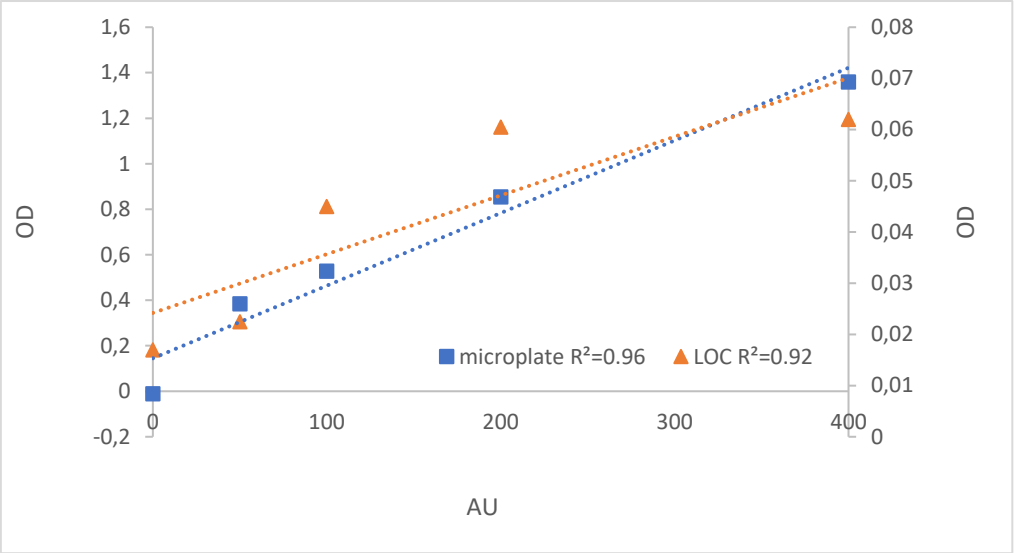

Figure 25: Optical density measurements of the microplate and the LOC.

3.4.2 Addition of impedance spectroscopy

To implement the contacts for impedance measurements, a part of the bottom PET layer needed to be removed to create a vacancy. This hole would then be filled by a Polystyrene coated PET piece, functionalised with aluminium contacts. These contacts were covered with Kapton tape during the spin coating process to maximise the connection between the probes and the aluminium on the PET layer. The red circle (fig. 26A) marks the well with interdigitated contacts at the bottom. The other layers were also tweaked to allow the connection between these contacts and contact pins (fig. 26B).

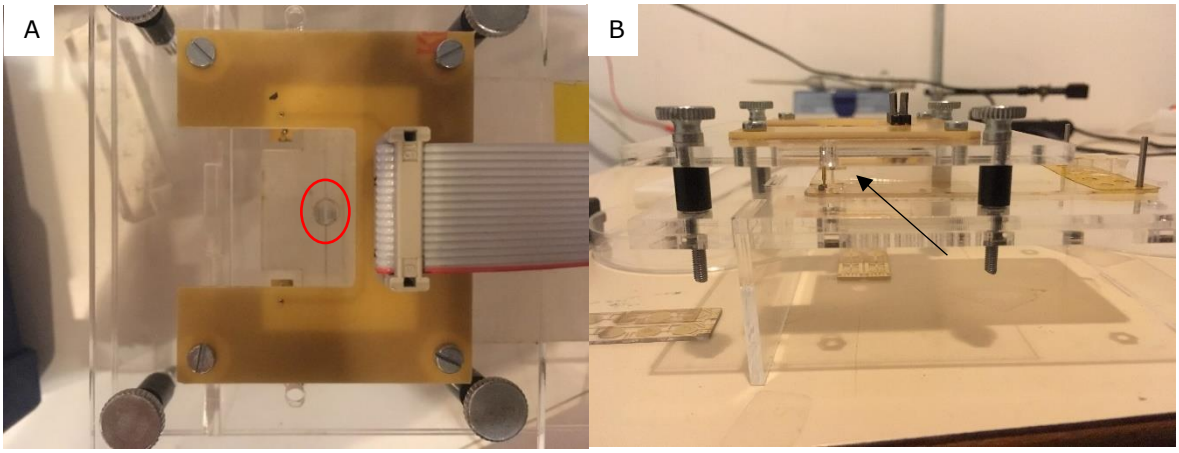

Figure 26:A. Measuring device used to record impedance. B. Side view of the measuring device, with the arrow pointing at the pogo contacts.

The initial concentration of 12% polystyrene in toluene gave a layer with a thickness of around 600 nm. However, this seemed too thick to measure impedance through. A PET layer without polystyrene coating was measured to see what type of signal would be acquired (fig. 27A). Other concentrations of 10, 8, 6 and 4% were used as well with the latter present in a layer of 200 nm. The spin coat parameters were changed, increasing the rotation speed to 5000 rpm. This created a layer that was still not thin enough to allow the fluid to touch the interdigitated aluminium contacts and create connection, only an open-circuit was measured (fig. 27B). Even smaller concentrations of 2, 1 and 0.5% were then spin coated to see if this could work but a stable impedance signal could still not be measured.

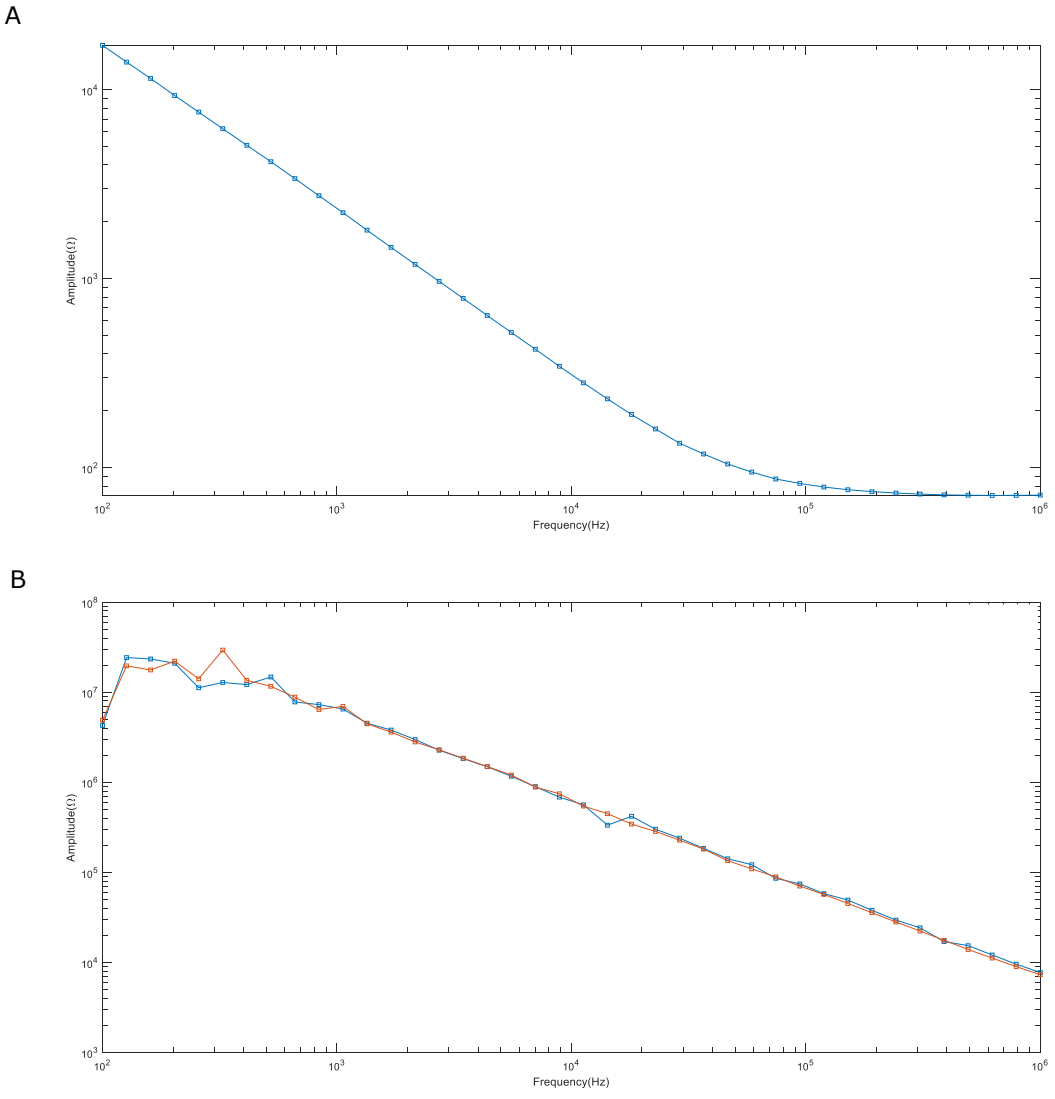

Figure 27: A. Bode plot of the sensor without polystyrene coating. B. Bode plot of the sensor with a polystyrene coating of 4 and 6% after raising the rotation speed to 5000 rpm. The blue plot is 4%, the orange one is 6%.

Concentrations of 0.25%, 0.1% and 0.05% were created to test if an acceptable layer could be spin coated. All three concentrations showed the same impedance behaviour (fig. 28). 0.25% was from then on chosen to be the concentration that was used to spin coat.

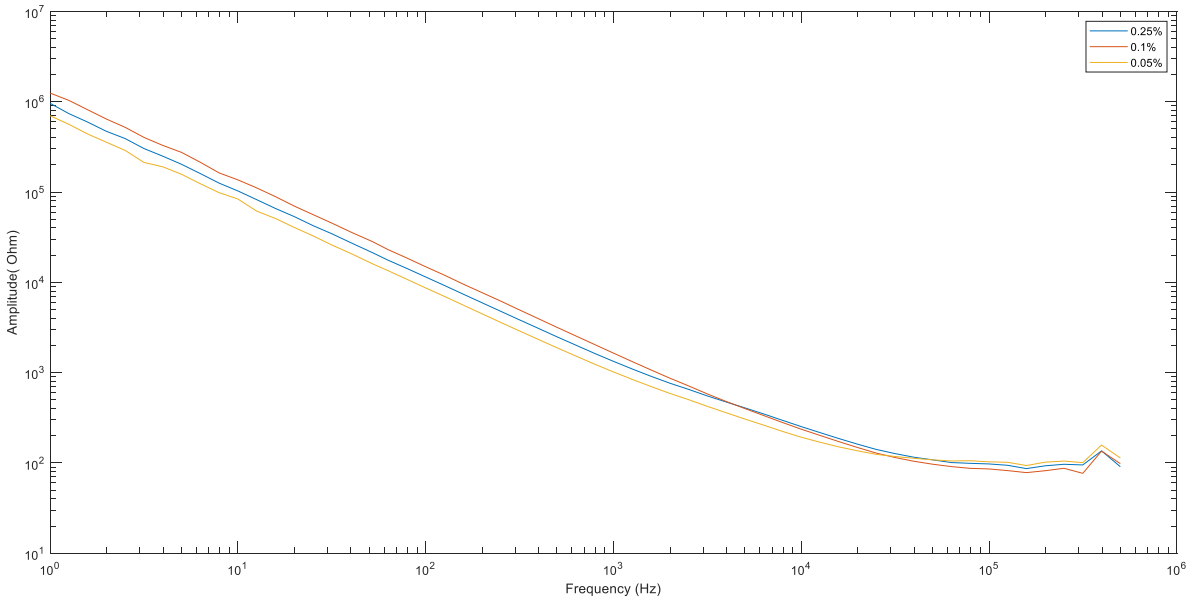

Figure 28: Bode plot of the different concentrations and a PET layer without polystyrene coating.

Commercial flexible sensors were used as well to test if impedance could be measured through the coating layer on gold interdigitated contacts. It was seen that the polystyrene coating, the PVC coating (fig. 29A) and a heater without coating (fig. 29B) showed a continuously decreasing signal that so far cannot be explained. The choice was then made to change to gold interdigitated contacts on glass to measure impedance.

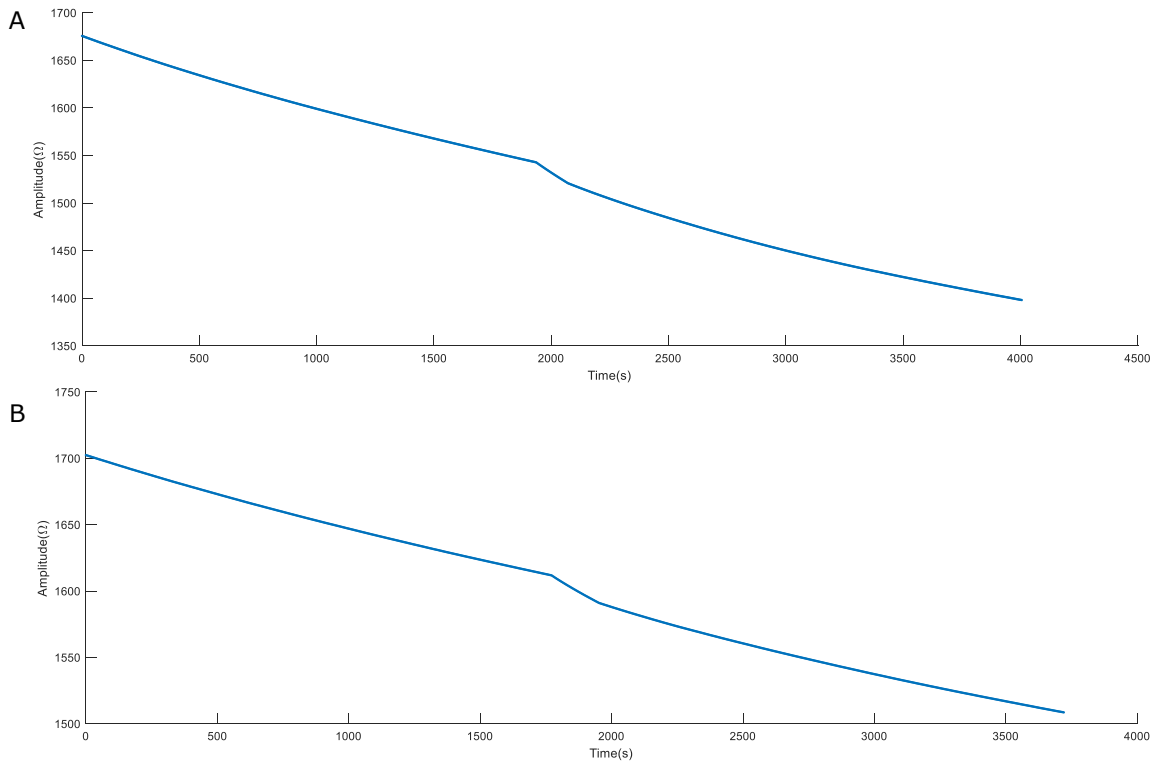

Figure 29: A. Impedance measurement of a commercial flexible sensor with a coating of 1% PVC. B. Impedance measurement of a flexible sensor with no coating.

3.4.3 Impedance measurements with gold contacts

After the stability tests, impedance measurements were done with gold contacts on glass plates. These are used in the original micro plates used for ELISA. Once the glass was coated with 0.25% polystyrene, a PDMS slab with an inlet and outlet tube was stuck onto a microfluidic channel, cut out in double-sided tape. The protocol mentioned in table 1 was followed and impedance was measured. It began with SPAG16 coating of the well, followed by washing with PBS. Afterwards, 2% MPBS was added to block any unbound antigen and washing took place after two hours with PBS and PBSTween. Finally, the sample, in this case the standard, was added and incubated for four hours. With every step in the protocol, the impedance changed (fig. 30). The impedance is higher at the end than at the beginning as expected because an extra layer has formed on top of the bottom of the well consisting of antigens, blocking agent and the sample.

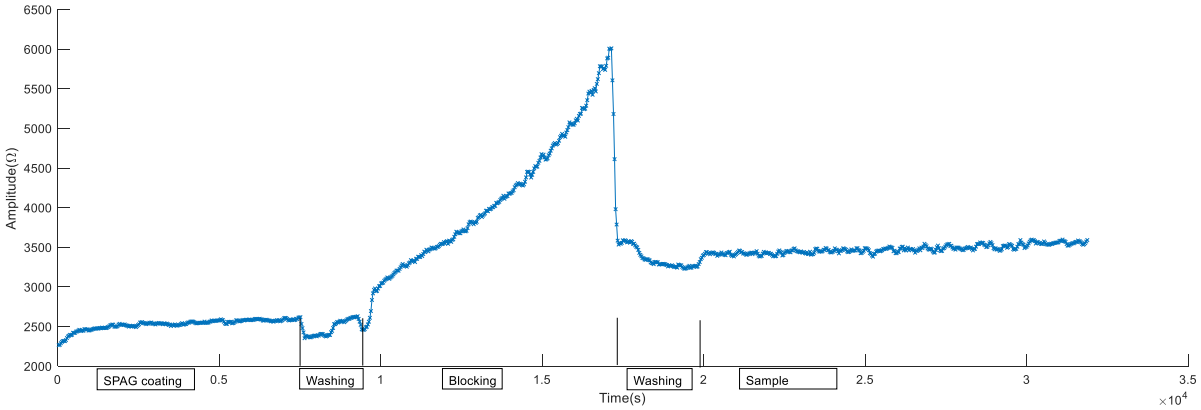

Figure 30: Impedance measurement of the ELISA impedance spectroscopy protocol at 126 Hz.

A second measurement was done where the protein ELISA SPAG16 impedance spectroscopy protocol from chapter 2.6 was employed (fig. 31). This time, SPAG16 was replaced by coating buffer to monitor if the impedance level of PBS would change after the different steps. It was expected that the PBS level after the addition of coating buffer would be from the same magnitude as the level before coating buffer was added because there were no antigens to bind to the bottom of the well. A difference between the two levels was observed however, probably due to drift. After blocking occurred, a difference in PBS levels was present as expected, showing that the bottom of the well had bound the blocking agent.

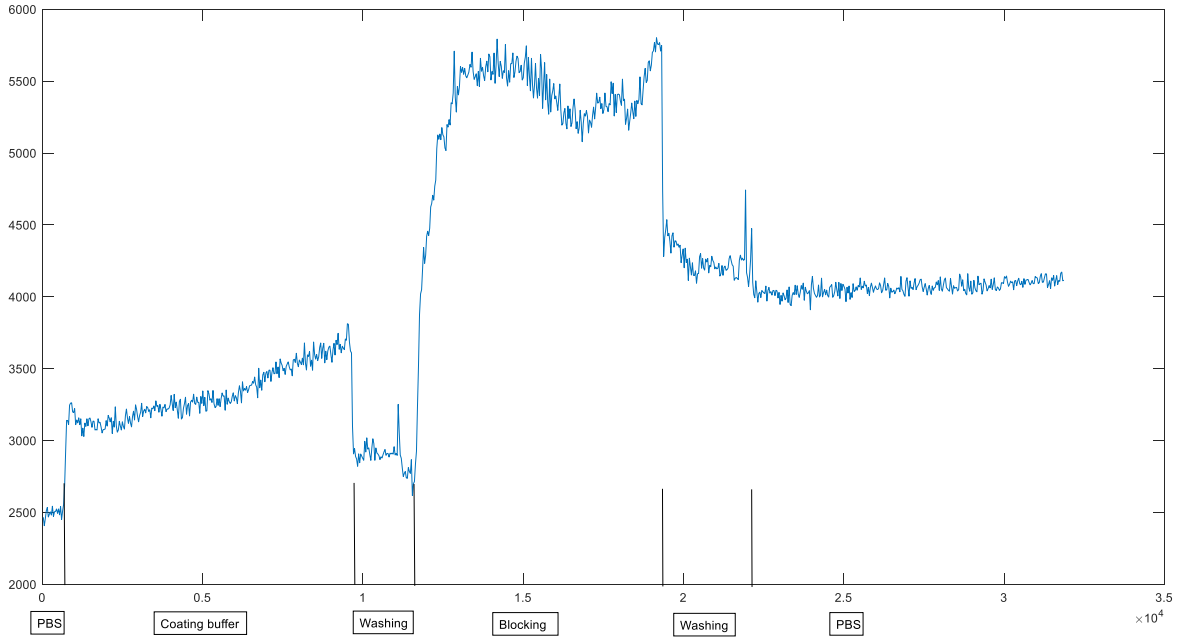

Figure 31: Impedance over time of the ELISA SPAG16 impedance spectroscopy protocol with coating buffer instead of SPAG16 and no sample added.

4. Conclusion

The research question for this work was to find out whether it was possible to create an electrical impedance platform that could harbour different receptors such as MIPs, DNA and proteins. This sensor could be useful to solve the problems of the current detection techniques. The high cost, educated personnel and extra equipment would no longer be necessary with this platform. The sensor employs microfluidics, meaning only a small amount of fluid is needed to detect analytes. This technique also reduces the analysis time and has already numerous applications in point of care. A lab-on-card was chosen to function as the impedance platform because of their layering technique. By stacking several layers of individually cut fluidic circuit this multi-purpose tool can be rapidly fabricated and tested.

Before the LOCs were made, impedance behaviour was monitored in a flow cell. It was first filled with PBS and MilliQ to get the base impedance values, which showed a distinct difference between the two fluids. This difference is due to the lower number of ions present in MilliQ, raising the resistivity. Next, paper was put in the flow cell to monitor its influence. The impedance differed from the impedance without the presence of paper. After these findings, lab-on-cards were made.

These credit card sized sensors are an innovative tool that can be used for various applications. They are built by alternating PET-layers with double-sided tape layers and PCB contacts at the bottom are used to measure impedance. But first, the influence of heat was tested on this lab-on-card because it was thought that heat would increase the attraction between the different layers. Two types of tape were heated from 60°C to 140°C. The double-sided tape of 150 µm was functional until 170°C, while the tape of 174 µm showed a flow until 140°C was reached. Once these temperatures were reached, morphological changes were present that inhibited the flow. Paper was still used in these cards as a harbouring platform for MIPs. Two paper pieces were added with MIPs or NIPs sandwiched in between them and nicotine was administered. There was a difference in impedance between both receptors, but these measurements were done in different lab-on-cards. To form a conclusion, a lab-on-card needs to be made where MIPs and NIPs are present, removing the possible influence.

Due to the layering technique, the choice was made to shift focus to antigens as receptor and make an LOC without paper where an immuno assay and electrical impedance spectroscopy were combined. An LOC was made that mimicked the lay-out of a microplate used for ELISA. Different concentrations of SPAG16 were added to test if they would stick to the polystyrene coating and to determine if the LOC could be used for immunoassays. This was confirmed by contact angle measurements where a decrease in contact angle was observed with increasing SPAG16 concentration and thus increasing hydrophilicity. The optical density was measured to test the sensitivity of the LOC and compare it to the sensitivity of a microplate. This resulted in a R=0.92 for the LOC and 0.96 for the microplate, meaning that their sensitivity was largely the same. Afterwards, the ELISA protocol was carried out to see if the antibodies had bound to the antigen receptors. A colour reaction could be seen when the TMB was added, confirming the presence of the antibodies. The enzymatic colour reaction could not be stopped because there was no way of mixing the stop reaction with the TMB, since the TMB would be flushed out if we added the stop mixture.

To implement impedance measurements, contacts needed to be added to the card, but this was not possible without the removal of wells. It was then decided to create a credit-card sized LOC where just one well was present and thus one concentration could be introduced. Contacts were introduced to the well by laser cutting PET coated with aluminium. Once the aluminium residue was removed, this piece was spin coated with polystyrene to increase the bonding of the antigens to the bottom of the well. The concentration of the polystyrene, dissolved in toluene, was tested to see if impedance could be measured with this layer on top of the contacts. Concentrations up to 0.25% showed the expected impedance behaviour, while every concentration above that value showed the impedance values of an open-circuit. This result could be explained by the thickness of the layer depending on the polystyrene concentration. Above 0.25% polystyrene, the layer was too thick to allow measurement through the polystyrene surface. Afterwards, this PET layer was stuck at the bottom of the LOC and measurements could be done. Tests were also done on gold interdigitated contacts on glass coated with 0.25% polystyrene. An altered ELISA protocol was used to determine if SPAG16 and blocking had bound on the bottom of the well. A change in impedance was seen when both liquids were administered. The impedance levels of PBS before and after the addition of both liquids differed as well, confirming that the layer on the bottom of the well became thicker.

For the first time to our knowledge, the creation of a lab-on-card that made use of electrical impedance spectroscopy was successful. Maddipatla et al. (2016) already made a paper-based electrochemical impedance sensor but a lab-on-card was not used (38). A stable impedance signal was acquired while using the aluminium interdigitated contacts on PET. The preliminary results of the MIP-based LOC were promising and showed a change in impedance when nicotine was bound. The card that used antigens as receptors was not successful yet. In the future, this LOC should be optimised. The optimal concentration of polystyrene needs to be determined in such a way that enough antigens can stick to the bottom of the well. Other coating materials should be tested as well, such as PVC to make sure that polystyrene is the optimal choice. Afterwards, the enzyme-bound antibodies can be added to the ELISA protocol to test if a colour change can be detected. If a functioning lab-on-card is made, it can be reconfigured to implement more microfluidic functions such as a mixing chamber, allowing the stop reaction of the ELISA protocol to take place. Finding another way to implement the contacts into the lab-on-card could be interesting as well to further simplify the building process. Once this lab-on-card is validated, it can be used for different applications such as tuberculosis detection or the screening of humoral autoimmune response in Multiple Sclerosis as previously mentioned.

5. References

1. Lawn SD, Zumla AI. Tuberculosis. The Lancet. 2011;378(9785):57-72.
2. Gong MM, Sinton D. Turning the Page: Advancing Paper-Based Microfluidics for Broad Diagnostic Application. Chemical Reviews. 2017;117(12):8447-80.
3. Moazeni M, Karimzadeh F, Kermanpur A. Peptide modified paper based impedimetric immunoassay with nanocomposite electrodes as a point-of-care testing of Alpha-fetoprotein in human serum. Biosensors and Bioelectronics. 2018;117:748-57.
4. Yetisen AK, Akram MS, Lowe CR. Paper-based microfluidic point-of-care diagnostic devices. Lab on a Chip. 2013;13(12):2210-51.
5. Jiang H, Weng X, Li D. Microfluidic whole-blood immunoassays. Microfluidics and Nanofluidics. 2011;10(5):941-64.
6. Seemann R, Brinkmann M, Pfohl T, Herminghaus S. Droplet based microfluidics. Reports on Progress in Physics. 2011;75(1):016601.
7. Shang L, Cheng Y, Zhao Y. Emerging Droplet Microfluidics. Chemical Reviews. 2017;117(12):7964-8040.
8. Teh S-Y, Lin R, Hung L-H, Lee AP. Droplet microfluidics. Lab on a Chip. 2008;8(2):198-220.
9. Liana DD, Raguse B, Gooding JJ, Chow E. Recent Advances in Paper-Based Sensors. Sensors (Basel, Switzerland). 2012;12(9):11505-26.
10. Li X, Ballerini DR, Shen W. A perspective on paper-based microfluidics: Current status and future trends. Biomicrofluidics. 2012;6(1):011301--13.
11. Rackus DG, Shamsi MH, Wheeler AR. Electrochemistry, biosensors and microfluidics: a convergence of fields. Chemical Society reviews. 2015;44(15):5320-40.
12. Gould P. Microfluidics realizes potential. Materials Today. 2004;7(7):48-52.
13. Weigl B, Bardell R, Schulte T, Battrell F, Hayenga J. Design and Rapid Prototyping of Thin-Film Laminate-Based Microfluidic Devices2001. 267-74 p.
14. Hu J, Wang S, Wang L, Li F, Pingguan-Murphy B, Lu TJ, et al. Advances in paper-based point-of-care diagnostics. Biosensors and Bioelectronics. 2014;54:585-97.
15. Dungchai W, Chailapakul O, Henry CS. Electrochemical Detection for Paper-Based Microfluidics. Analytical Chemistry. 2009;81(14):5821-6.
16. Lei H, Wang K, Ji X, Cui D. Contactless Measurement of Magnetic Nanoparticles on Lateral Flow Strips Using Tunneling Magnetoresistance (TMR) Sensors in Differential Configuration. Sensors. 2016;16(12):2130.
17. Lin Y, Gritsenko D, Feng S, Teh YC, Lu X, Xu J. Detection of heavy metal by paper-based microfluidics. Biosensors and Bioelectronics. 2016;83:256-66.
18. Furst AL, Muren NB, Hill MG, Barton JK. Label-free electrochemical detection of human methyltransferase from tumors. Proceedings of the National Academy of Sciences of the United States of America. 2014;111(42):14985-9.
19. Xu D, Xu D, Yu X, Liu Z, He W, Ma Z. Label-Free Electrochemical Detection for Aptamer-Based Array Electrodes. Analytical Chemistry. 2005;77(16):5107-13.
20. Bio-rad. Detection methods 2019. Available from: http://www.bio-rad.com/en-be/applications-technologies/detection-methods?ID=LUSQ6KKG4.
21. Ramakrishnan S. Textbook of Medical Biochemistry: Orient Longman; 2004.
22. Poli MA, Rivera VR, Hewetson JF, Merrill GA. Detection of ricin by colorimetric and chemiluminescence ELISA. Toxicon. 1994;32(11):1371-7.
23. Tie F, Pan A, Ru B, Wang W, Hu Y. An improved ELISA with linear sweep voltammetry detection. Journal of immunological methods. 1992;149(1):115-20.
24. Rao M, Kapila K, Gupta RM. ENZYME LINKED IMMUNOSORBENT ASSAYS REVISITED. Med J Armed Forces India. 1997;53(1):45-51.
25. Sakamoto S, Putalun W, Vimolmangkang S, Phoolcharoen W, Shoyama Y, Tanaka H, et al. Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. J Nat Med. 2018;72(1):32-42.
26. Aydin S. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. Peptides. 2015;72:4-15.

27. Lvovich VF. Impedance Spectroscopy : Applications to Electrochemical and Dielectric Phenomena. Somerset, UNITED STATES: John Wiley & Sons, Incorporated; 2012.
28. Barsoukov E, Macdonald JR. Impedance Spectroscopy : Theory, Experiment, and Applications. Hoboken, UNITED STATES: John Wiley & Sons, Incorporated; 2005.
29. Lei KF, Huang CH, Tsang NM. Impedimetric quantification of cells encapsulated in hydrogel cultured in a paper-based microchamber. Talanta. 2016;147:628-33.
30. Boonyasit Y, Chailapakul O, Laiwattanapaisal W. A multiplexed three-dimensional paper-based electrochemical impedance device for simultaneous label-free affinity sensing of total and glycated haemoglobin: The potential of using a specific single-frequency value for analysis. Anal Chim Acta. 2016;936:1-11.
31. Haupt K, Mosbach K. Molecularly Imprinted Polymers and Their Use in Biomimetic Sensors. Chemical Reviews. 2000;100(7):2495-504.
32. Gadzała-Kopciuch R, Sadowski R, Piwonska A, Buszewski B. Applications of Molecularly Imprinted Polymers for Isolation of Estrogens from Environmental Water Samples2015. 1-p.
33. Bentaleb EM, Abid M, El Messaoudi MD, Lakssir B, Ressami EM, Amzazi S, et al. Development and evaluation of an in-house single step loop-mediated isothermal amplification (SS-LAMP) assay for the detection of Mycobacterium tuberculosis complex in sputum samples from Moroccan patients. BMC infectious diseases. 2016;16(1):517.
34. McNerney R, Maeurer M, Abubakar I, Marais B, McHugh TD, Ford N, et al. Tuberculosis Diagnostics and Biomarkers: Needs, Challenges, Recent Advances, and Opportunities. The Journal of Infectious Diseases. 2012;205:S147-S58.
35. Dheda K, Barry CE, Maartens G. Tuberculosis. The Lancet. 2016;387(10024):1211-26.
36. de Bock L, Somers K, Fraussen J, Hendriks JJA, van Horssen J, Rouwette M, et al. Sperm-Associated Antigen 16 Is a Novel Target of the Humoral Autoimmune Response in Multiple Sclerosis. The Journal of Immunology. 2014;193(5):2147.
37. Lee J, Kwak YH, Paek S-H, Han S, Seo S. CMOS image sensor-based ELISA detector using lens-free shadow imaging platform. Sensors and Actuators B: Chemical. 2014;196:511-7.
38. Maddipatla D, Narakathu BB, Bazuin BJ, Atashbar MZ, editors. Development of a printed impedance based electrochemical sensor on paper substrate. 2016 IEEE SENSORS; 2016 30 Oct.-3 Nov. 2016.

6. Supplemental information

6.1 Figures

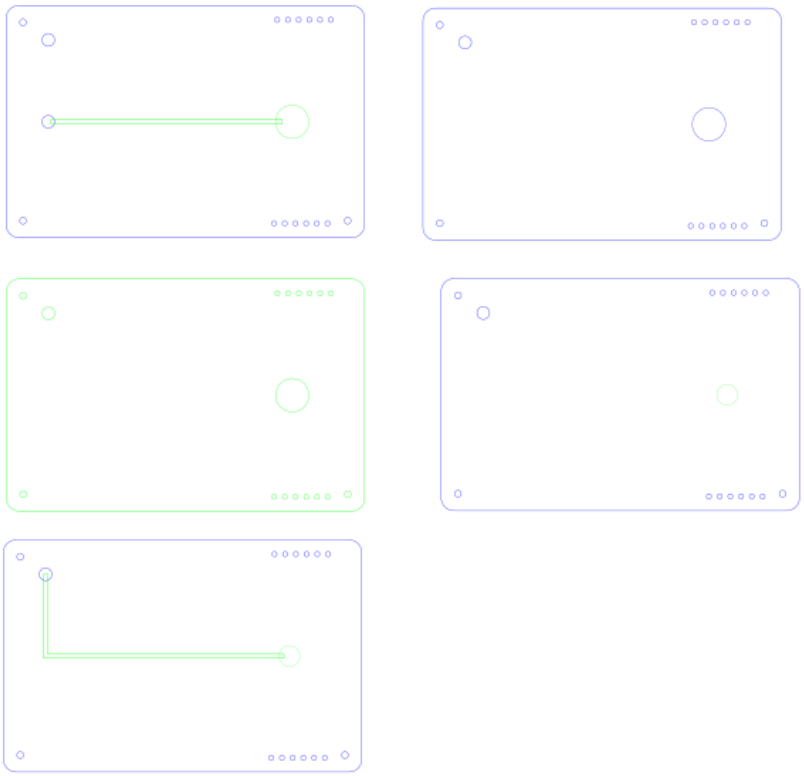

Figure S 1: Schematic of the different layers that the sensor consists of. Green stands for double-sided tape, blue stands for PET-layers.

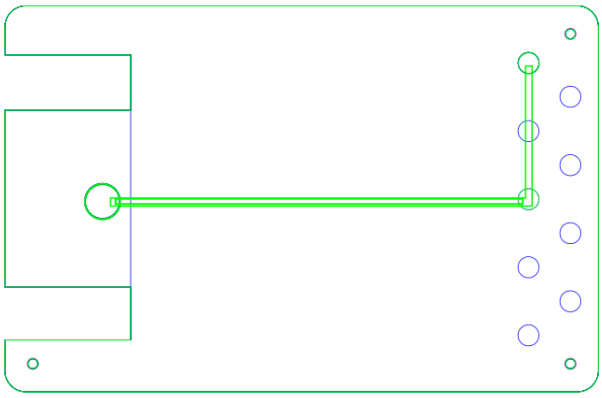

Figure S 2: First lab-on-card design for the immunoassay.

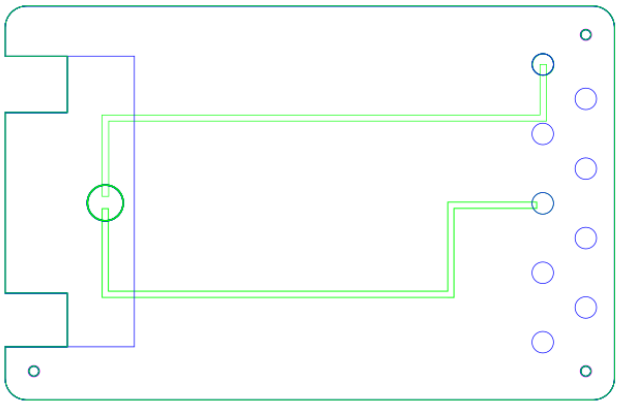

Figure S 3: Lab-on-card design for impedance spectroscopy combined with the immunoassay principle.

6.2 ELISA protocol

Day 1 (evening):

- Coat ELISA plates: 1µg/ml in coating buffer, overnight at 4°C, 50 µl/well
    - Stock concentration:
        - SPAG: 1,2 mg/mL
        - THIO: 965,6 µg/mL

Day 2:

- Dilute 10xPBS to 1xPBS
- 10% marvel in 1xPBS (MPBS): 5 g marvel (sterile) in 50 mL 1xPBS
- 2% MPBS: 10 ml 10% MPBS + 40 ml 1xPBS
- Washing buffer (PBSTween 0.1%): 1 ml Tween20/liter 1xPBS
- Wash coated plate 2 x 5 min with 1xPBS (shaking)
- Block with 100 µl 2% MPBS, 2h at 37°C (shaking, speed: 80)
- Wash blocked 96-well plate 3 x 5 min with PBST 0.1% (shaking) and 1 x 5 min with PBS (shaking), empty the plates on a paper towel between each washing step
- Make dilutions of the standard in 2% MPBS (1/25-1/50-1/100-1/200-1/400)
    - 1/25: 20 µl standard + 980 µl MPBS
    - 1/50: 500 µl (1/25) + 500 µl MPBS
    - 1/100: 500 µl (1/50) + 500 µl MPBS
    - 1/200: 500 µl (1/100) + 500 µl MPBS
    - 1/400: 500 µl (1/200) + 500 µl MPBS
- Dilute samples 1/100 (5 µl plasma + 495 µl MPBS) in 2% MPBS
- Use 50 µl plasma 1/100 or 50 µl standard
- Incubate 2h at RT (shaking)
- Wash plate:
    - 1 x with 1 x PBST 0.1%, empty immediately
    - 2 x 5 min with PBST 0.1% (shaking) and 1 x 5 min with PBS (shaking)
    - Empty the plates on a paper towel between each washing step
- Add 50 µl rabbit anti-human-HRP (1/2000 in 2% MPBS)
- Incubate 1h shaking at RT
- During incubation: bring the TMB-solution to RT
- Wash plate 3 x 5 min with PBST 0.1% (shaking) and 1 x 5 min with 1xPBS (shaking)
- Stain with 50 µl TMB/well (in the dark) for 10 min
- Stop with 25 µl 2N H_2SO_4
- ELISA-reader at 450 nm