Development of a prototype Chemical-Optical Sensor for the Detection of Organic Micro-Pollutants in Drinking Water

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KWR Watercycle Research Institute

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Summary

Water utilities take surface water in for the production of drinking water. As soon as Early Warning Systems (EWS) detect pollution, intake is stopped.
This report describes the research activities on the development of the direct detection of micro-pollutants with an opto-chemical based sensor. Such an opto-chemical sensor can be part of a EWS.

The opto-chemical sensor used is the Mach-Zehnder Interferometer (MZI), an evanescent field measurement based system. Refractive index changes of 10^{-7} can be detected, corresponding with the detection of molecules of 100 - 200 D at a concentration of 0.1 – 1 µg/L.
The measurement of refractive index does not indicate the type of compound responsible for the pollution. Therefore it is needed to introduce a selective coating on top of the sensor surface that contains selective elements like antibodies. The development of an interface layer for the detection of atrazine is described as well as the direct measurements of atrazine on a functionalised MZI system. Within this research a detection limit for the direct detection of atrazine of 5 µg/L is achieved. The reproducibility of these measurements is low indicating that additional research efforts are needed before a MZI-based measurement system can be part of an early warning system.

Contents

List of figures and tables

Abbreviations

Ab	Antibody
AFM	Atomic force microscopy
Ag	Antigen
D	Dalton
DCDMS	Dichlorodimethylsilane
HR-SEM	High resolution scanning electron microscopy
kD	kilo Dalton
$MgCl_2$	Magnesium chloride
MZI	Mach Zehnder interferometer
MαBiot	Mouse antibody against biotin
NaOH	Sodium hydroxide
PBS	Phosphate buffered saline
SEM	Scanning electron microscopy
ShαAtr	Sheep antibody against atrazine
Si_3N_4	silicon nitride
SPR	Surface Plasmon Resonance
T	Temperature
XPS	X-ray photon spectroscopy

1 Introduction

Sensors utilising optical chemical detection techniques for in situ measurements are not yet commercially available. However, demonstrators of various systems are under development. Optical chemical techniques offer the possibility to design highly sensitive and selective sensors for on-line detection of organic micro-pollutants, something which is not possible with currently available monitoring devices [Lambeck, 2006].

Based on the physical properties of the sensor systems within this project we used the Mach-Zehnder Interferometer (MZI) supplied by the company OptiSense. The MZI-sensor has a good noise to signal ratio and makes use of silicon based technology which offers the possibility of a small and low cost sensor in the future.

This project tested the specificity of the sensor by using antibodies to detect a specific contaminant with the sensor. Antibodies are a group of proteins that have a high affinity for it corresponding antigen. These antibodies are commonly in use for ELISA techniques. By immobilisation of these antibodies to the sensor surface the sensor, can be selective for a contaminant of choice.
A protocol to functionalise the sensor surface for the detection of atrazine has been developed within the project. To reduce analyses costs it is decided to develop an assay for the direct detection of atrazine. The physical sensitivity of the MZI-sensor makes such a measurement feasible. Two assay formats for direct detection are developed and tested. The first format is based on the direct immobilisation of the anti atrazine antibody (ShαAtr) to the sensor surface. The second is based on a two-stage-rocket principle. First an antibody against biotin (MαBiot) is immobilised. This MαBiot recognises the biotinilated antibody ShαAtr. The advantage of the second format is the simplification of the regeneration procedure that is necessary to prepare the sensor for reuse.
Results from both methods to detect atrazine in a direct way are presented.

2 Background

2.1 Evanescent field detection principles

"Evanescent" means "tends to vanish" and this is actually happening at the surface of an optical chemical sensor. Figure 1 shows the electrical field distribution of light guided by a (planar) waveguide. The major part of this mode energy is located in the waveguide layer but a small part of the energy mode is located outside the waveguide causing a so called "evanescent field". The intensity of evanescent field decays exponentially with the distance from the waveguide at which they are formed.

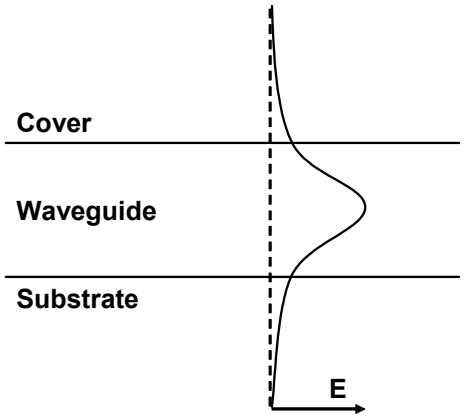

Figure 1: Electrical field distribution of light guided through a planar waveguide structure consisting of three media. The evanescent fields are the exponentially decaying fields outside the waveguide layer.

The propagation of the electrical field through the waveguide is proportional with the refractive index on top of the waveguide. In opto-chemical sensors the cover as shown in Figure 1, is removed and replaced by a biological or chemical interface. At such an interface molecular interactions can occur causing a change in refractive index. Molecules like an antibody can be immobilised to the waveguide surface. Such antibody will bind its pairing antigen, with possibly a refractive index change as result. In the case of this report an antibody that is recognizing atrazine is immobilised, and atrazine is used as the antigen.
Evanescent field measurements are of interest because the detection of molecular interaction can be monitored in real-time without the use of any reporting molecule (fluorescent probes, enzymes, radio-isotopes).

Two optical techniques based on evanescent field measurement for the detection of molecular interactions are of interest: Surface Plasmon Resonance (SPR) and Mach-Zehnder Interferometry (MZI) With SPR the refractive index changes causes a change in the intensity of a reflected laser beam at a gold surface while MZI makes use of phase shift changes of the light. The sensitivity of the analyte measurements depends strongly on the signal to noise ratio of the instrumentation used. The best ratio is obtained with MZI [Lambeck, 2006]. As a consequence Mach-Zehnder Interferometry is used for the detection of atrazine.
OptiSense markets the Mach-Zehnder Interferometer (MZI). Their system consists of an integrated optical waveguide, an electronic unit for the modulation of the waveguide and signal detection and a fluidic system for sample transport over the sensor surface. A detailed description is given in paragraph 4.1.
In this system the chemically induced changes of the effective thickness of the interface layer are probed by an optical beam leading to a concentration dependent change of the beam velocity in the sensing

window [Heideman, 1993]. A serrodyne modulated Mach Zehnder Interferometer circuit translates these changes into a periodic signal of which the phase difference with a periodic reference signal finally determines the concentration change.
With MZI it is possible to observe a change in refractive index as a result of binding to antibodies of antigens with a mass of over hundred Daltons. This means that atrazine can be detected with a direct binding assay format.

2.2 S_A-layer protein

2.2.1 What are S-layer proteins

S-layers are two-dimensional paracrystalline arrays usually found as the outermost layer of the cell envelope of bacteria. These layers are composed of single (glyco)proteins. S-layers self-assemble in an entropy-driven process during which multiple, noncovalent interactions between individual S-layer protein monomers and the underlying cell surface take place [Smit, 2002; Sleytr, 1996].

2.2.2 Choice of S_A-layer protein

In this report S_A-layer protein of *Lactobacillus acidophilus* (ATCC 4356) is used. *Lactobacillus acidophilus* is easy to culture and the collection and purification of the proteins are straight forward. A detailed description is provided in protocol SEN-005 (paragraph 7.4).

2.2.3 Uses of S_A-layer protein

The interaction between the outer layer of bacteria and S_A-layer protein is based on hydrophobic interaction between the molecules. S_A-layer proteins also form self-assembly monolayers on hydrophobic surfaces. The sensor surface of the MZI can be made hydrophobic by silanisation. The S_A-layer proteins will form a self assembled layer at this hydrophobic surface. The interaction strength is very high and only strong acids are able to oxidise the proteins off from the surface. Changes in salt composition and strength have no influence on the interaction.
The S_A-layer protein has a tetra ether form of which the bottom part is highly hydrophobic and the top is highly hydrophilic. The hydrophobic part of the molecule interacts with the hydrophobic sensor surface. The hydrophilic top is pointing in to the liquid on top of the sensor surface. During assembly multi layers of S_A-layer proteins are formed. These multi layers are removed by a washing step with high concentrations of $MgCl_2$ and NaOH.

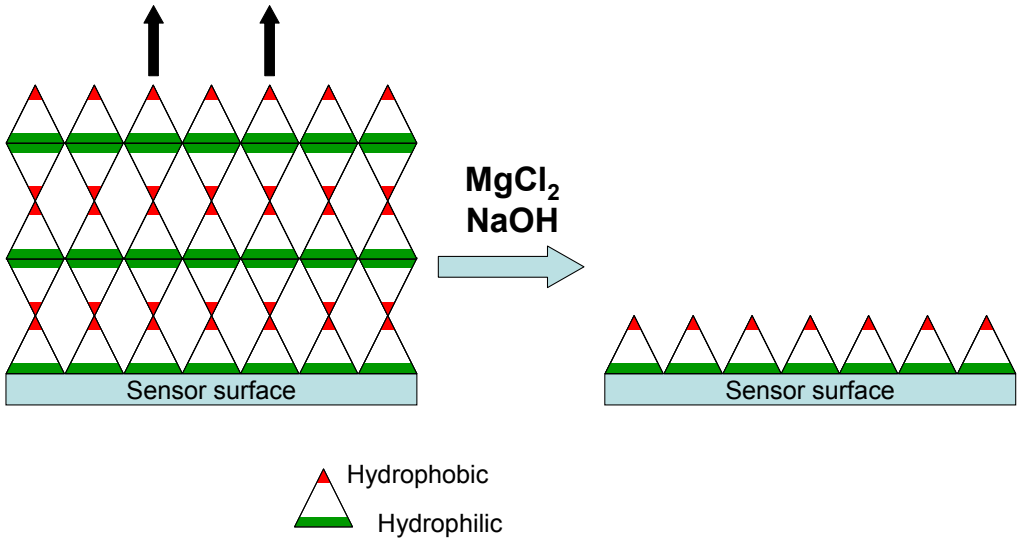

Figure 2: Formation of S_A-layer proteins and removal of formed multi layers by $MgCl_2$ resulting in a S_A-layer protein modified sensor surface

On top of the S_A-layer proteins different interface layers can be build up by covalent immobilisation of crosslinkers and proteins of choice. The procedures of cleaning, silanisation and S_A-layer protein assembly are described in protocol SEN-001, SEN-002 and SEN-003 (Paragraph 7.1, 7.2 and 7.3)

2.3 Cross-linkers

2.3.1 What is crosslinking

Crosslinking is the process of chemically joining two or more molecules by a covalent bond. The reagents used contain reactive ends to specific functional groups like primary amines, sulfhydryls, carboxyls, etc. on proteins or other molecules. Crosslinkers are commonly used to modify nucleic acids, drugs, proteins or solid surfaces. An example is the preparation of immunogens for the production of antibodies against low molecular weight compound. Crosslinkers are in use to bind the haptens to carrier proteins which induce immunogenic reactions. In this report crosslinking is used to immobilise an antibody of choice to a protein modified sensor surface. The antibody is raised against the herbicide atrazine.

2.3.2 Types of crosslinkers

Different types of crosslinkers are available differing in their chemical reactivity, water solubility, length and cleavability. Table 1 shows a list of reactive groups and their target functional groups. The choice for a crosslinker depends on the combination of chemical groups to link and the system to work in. The goal is to achieve an optimal binding between the substrate and target molecules with a minimum lost of functionality.

Table 1: Reactive crosslinker groups and their functional group targets.

Reactive Group	Target Functional Group
Aryl Azide	Nonselective (or primary amine)
Carbodiimide	Amine/Carboxyl
Carbonyl	Hydrazine
Diazirine	Nonselective
Hydrazide	Carbohydrate (oxidized)
Hydroxymethyl Phosphine	Amine
Imidoester	Amine
Isocyanate	Hydroxyl (non-aqueous)
Maleimide	Sulfhydryl
NHS-ester	Amine
PFP-ester	Amine
Psoralen	Thymine (photoreactive intercalator)
Pyridyl Disulfide	Sulfhydryl
Vinyl Sulfone	Sulfhydryl, amine, hydroxyl

2.3.3 Crosslinker choice

For the immobilisation of an antibody to the sensor surface the choice is made to crosslink an amino group to a carboxylic group.
Carboxylic groups can be activated by a carbodiimide. This activated group is able to react with a nucleophilic group. Examples are amino-, sulphate-, phosphate groups and water. If carboxylic groups of a protein are activated the amino groups of the protein has the tension to react, causing internal crosslinking. By using an excess of crosslinker the internal crosslinking can be avoided.
Figure 3 shows the reaction scheme of the EDC/NHS-ester driven amide binding reaction.

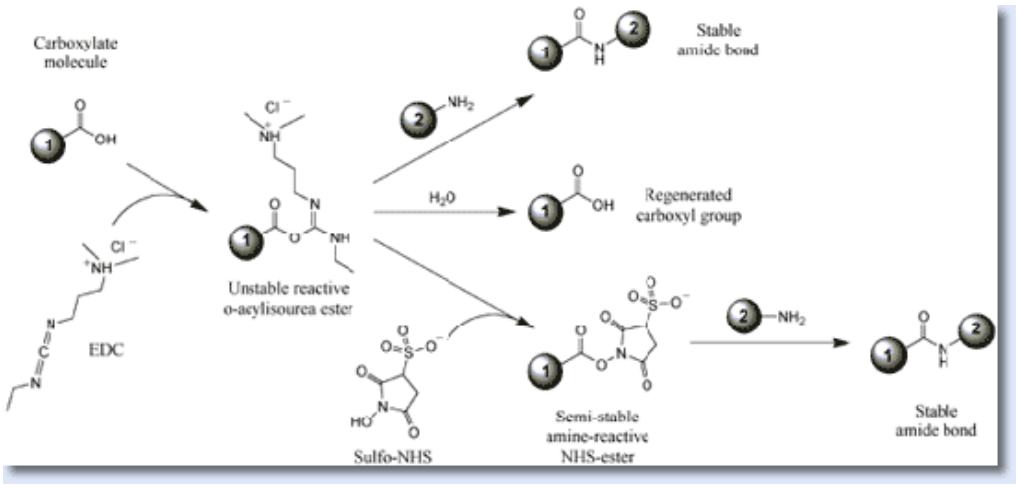

Figure 3: Reaction scheme of EDC/NHS-ester driven amide binding

The N-hydroxysuccinimide (NHS) ester group reacts with the ε-amine of lysine residues and the α-amine at the N-terminus of each peptide to produce a stable product. NHS esters react with deprotonated primary amines, therefore, the reaction requires neutral to basic pH values to proceed. Primary amines react with NHS esters by nucleophilic attack and NHS is released as a by-product. Hydrolysis of the NHS-ester competes with the reaction in aqueous solution and increases with increasing pH.

Therefore pH driven immobilisation is introduced. At each protein there are two types of amino groups, the α- and ε-amines, differing in their pK_a value. The α-amines at the N-terminus of a protein have a lower pK_a then those of lysines, l-arginine, l-asparagine and l-glutamine. This difference in pK_a can be used for preference immobilisation. By adjusting the pH of the coupling buffer under or near the pK_a of the α-amines the reactivity for activated carboxylic groups of these groups will be higher then those of the other amino groups.
The N-terminus of an antibody is located at the opposite position of the antigen recognizing site of the heavy chain of an antibody. In this way it is avoided to bind the antigen binding site of the antibody to the sensor surface.

A more detailed description is given in the protocols described in paragraph 3.2.
Additional information on crosslinkers can be found in reference
(http://www.piercenet.com/Objects/View.cfm?Type=Page&ID=FE7F690D-58AE-4342-AE85-BA94DCA642F8)

3 Materials and Methods

3.1 Equipment

3.1.1 Introduction to the Mach-Zehnder Interferometer

OptiSense has developed a sensitive integrated optical sensing system for measuring chemical concentrations. In this system the chemically induced changes of the effective thickness of the interface layer are probed by an optical beam leading to a concentration dependent change of the beam velocity in the sensing window [Heideman, 1993]. A serrodyne modulated Mach Zehnder Interferometer circuit translates these changes into a periodic signal of which the phase difference with a periodic reference signal finally determines the concentration change. Figure 4 shows the periodic signal caused by a modulated triangle signal.

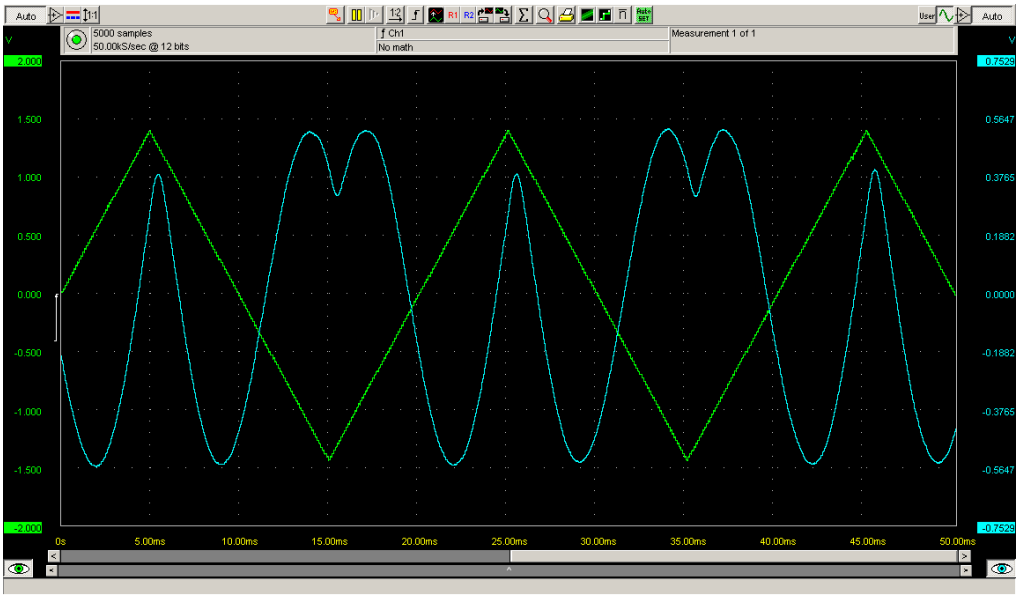

Figure 4: Modulation and resulting periodic signal of a Mach-Zehnder Interferometer.

In this system effects of many disturbing factors such as fluctuations of the temperature or of the intensity of the probing beam are strongly reduced leading to a resolution about one order of magnitude higher than of optical systems of comparable nature. Such a detector enables the determination of a change of effective thickness of the interface (this change being a consequence of filling originally empty sites in the interface) of about 2 x 10^{-5} nm, corresponding to an increase in weight of that interface smaller than 0.1 pg/mm2. As an example: using as a chemical interface a monomolecular antibody layers specific for aflatoxine B_1 concentrations lower than 4 x 10^{-7} weight percent can be detected.
Capture of molecules of all chemical species results into a change of the effective thickness and direct measurements are possible enabling response times of several minutes. This discriminates this type of detectors positively from e.g. the also very sensitive luminescent detectors. Most analytes themselves are not luminescent and for luminescence based sensing time consuming indirect methods (e.g. sandwich assays or competition assays using luminescent labelled chemicals) are required leading to appreciable (e.g. 15 minutes) time intervals in between two successive measurements. Direct methods as applied in the MZI system can be used in a continuously monitoring mode as very sensitive alarming detectors.

3.1.2 *Construction of the Mach-Zehnder Interferometer*

The serrodyne modulation MZI-based sensing system (see Figure 5) consists of four subsystems. The chemo-optical interface applied within the sensing window of the integrated optical readout circuit, the optical 'chip' having lateral dimensions of about 0.1 x 4 cm. The optical chip is connected to an electronic subsystem, which on one side delivers the electrical modulation signal to the chip and on the other side converts the optical output signal into an electrical signal and processes the signals properly for deriving from them the values of concentrations. For enabling a controlled transport of sample and if required other chemicals to the chemo-optical interface a small micro fluidic system is applied on top of the optical chip.

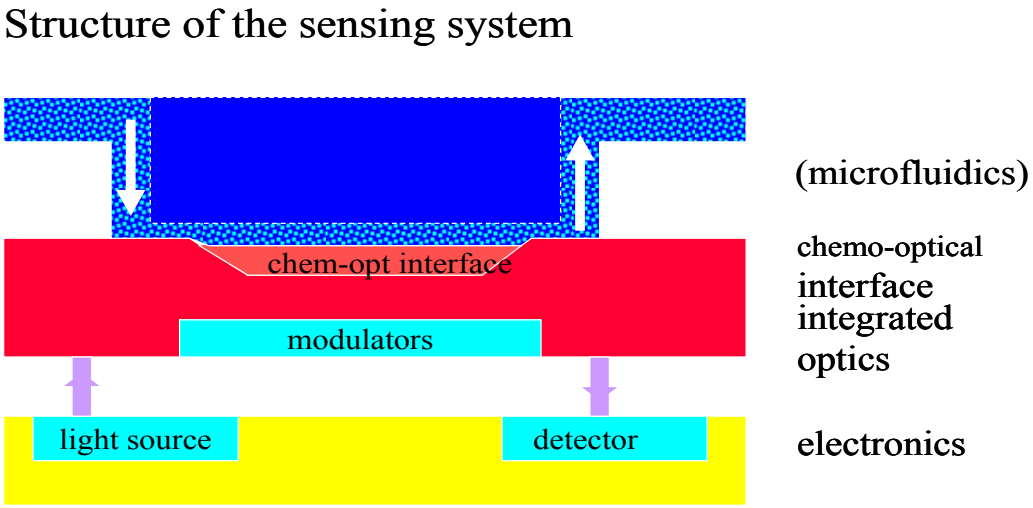

Figure 5: Structure of the sensing system

The current MZI platform contains an optical chip in which 5 MZI structures are implemented (see Figure 6). Only one structure is connected and used for the measurements. One structure consists of two windows (sensing and reference) at each side of the modulator section. One sensing window is 4 mm long x 80 µm wide and is 2 µm deep.
The special feature of these MZI's is that are serrodyne modulated affording for very high resolution.

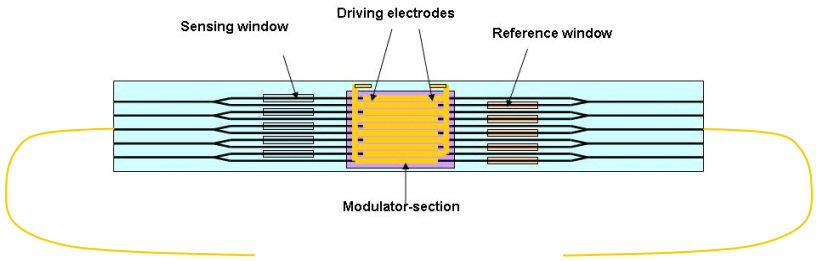

Figure 6: Schematic representation of the optical chip containing 5 individual Mach-Zehnder interferometers of which one is optically connected

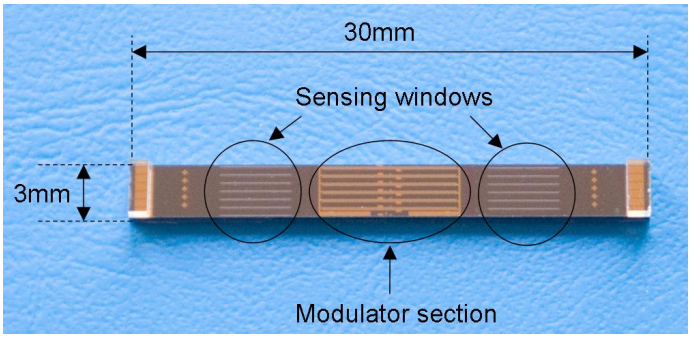

Figure 7: Photo of the MZI optical chip

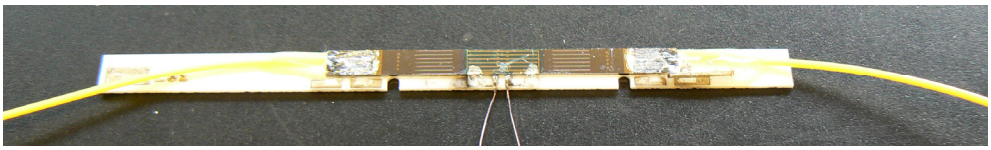

Figure 8: Picture of the MZI-chip mounted on ceramic support. The yellow wires at both ends are the optical-fibers and in the middle of the chip are the electric connections for the modulator.

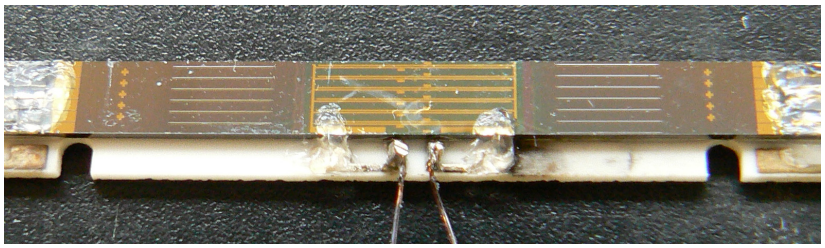

Figure 9: Detail of the MZI-chip with the modulator and connections in the centre and the 5 etched sensing and reference windows at both sides.

On top of the optical chip a micro fluidic system (flow cell) is applied for transporting the sample to the sensitive area of the chip in a controlled way. (See Figure 10).

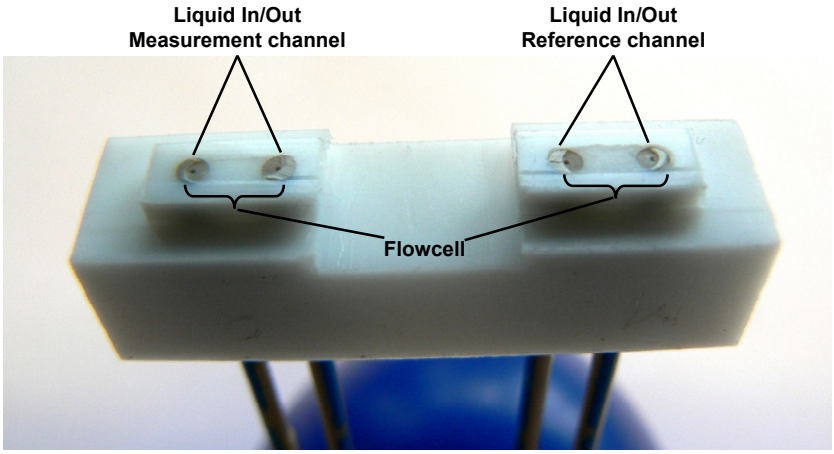

Figure 10: Photo of the flowcell

Before measurements the different parts are mounted together resulting in a complete measurement system. Figure 11 shows the mounted MZI-system.

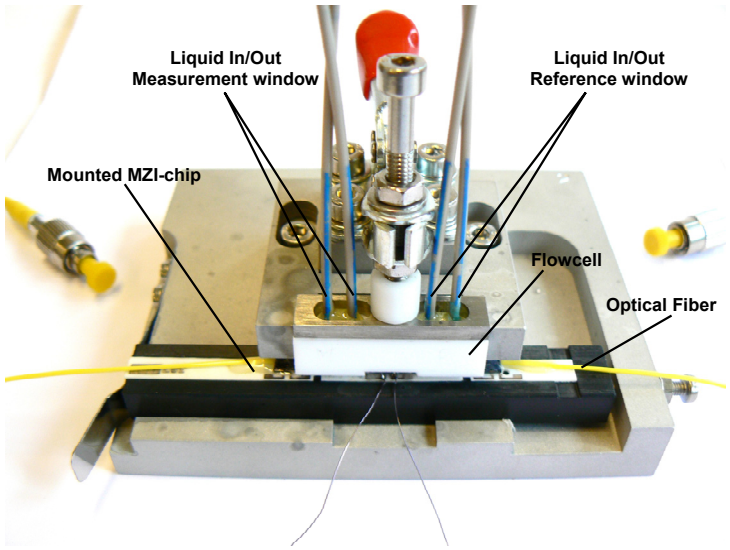

Figure 11: Mounted MZI-system.

The optical fibers and the wires of the modulating system are connected to the electronic unit. Figure 12 is a photo of this electronic unit.

Figure 12: Electronic unit of the Mach-Zehnder Interferometer.

3.2 Protocols

Before performing atrazine measurements the sensor surface has to be cleaned and functionalised. The protocols SEN-001 to SEN007 are describing the methods to be performed. These protocols can be found in appendices 7.1 until 7.6. A short description of the procedure is described below.

3.2.1 Cleaning of the sensor surface

The sensor surface has to be thoroughly cleaned to be free from organic-, anorganic material and fat. The organic material will be removed by piranha acid followed by a treatment with sodium hydroxide to remove fat. Before drying, the surface will be treated with hydrochloride to dissolve anorganic material.

3.2.2 Silanisation of the sensor surface

After cleaning, the sensor surface will be dried in an incubator to remove all remaining water. Water disturbs the forming of silane layers, the next step to prepare a functional sensor chip. For drying a temperature range has been tested from 50°C up to 120°C. A solution of 5% Dichlorodimethylsilane (DCDMS) will be used to silanise the sensor surface resulting in a very hydrophobic layer with a contact angle of over 80 degrees. The removal and washing steps after the incubation of DCDMS is performed with and without sonification. Sonification helps to remove DCDMS-particles formed during incubation. To stabilise the DCDMS layer the sensor chip is cured in an incubator at 50°C for 1 hour.

3.2.3 Formation of S_A-layer protein interface layer

The sensor chip is treated with S_A-layer protein from *Lactobacillus acidophilus* (ATCC 4356) after the sensor chip has reached room temperature. S_A-layer protein has a hydrophobic and a hydrophilic site. By self-assembly the S_A-layer protein molecules are forming a monolayer of protein. The hydrophobic site is orientated to the DCDMS layer while the hydrophilic site sticks into the aqueous solution on top of the protein layer.
During formation of the monolayer, multi-layers will be formed. The multi-layers are removed with a strong solution of magnesium chloride and sodium hydroxide subsequently, resulting in a perfect orientated monolayer of S_A-layer protein.

3.2.4 Antibody immobilisation

The self-assembled S_A-layer proteins expresses amino and carboxylic groups at there surface which can be used for further immobilisation of proteins. The proteins of interest in this research are the antibody against atrazine (ShαAtr) and the antibody against biotin (MαBiot).
Within this project the choice has been made to used the bi-functional cross-linker 6-aminohexanoic acid for the immobilisation of the antibody. 6-Aminohexanoic acid links an amino group to a carboxylic group by forming an amide binding.

Figure 13 shows the structure of 6-aminohexanoic acid.

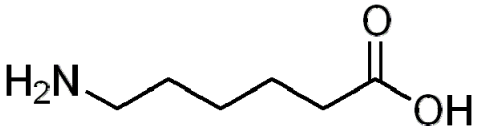

Figure 13: Molecular structure of 6-aminohexanoic acid.

The length of the spacer arm is of importance for the immobilisation efficiency of the antibody layer. The spacer length introduces a freedom of movement of the immobilised antibodies resulting in an increasing density of antibody molecules per square area.

The reaction scheme of the carbodiimide reaction is presented in Figure 14.

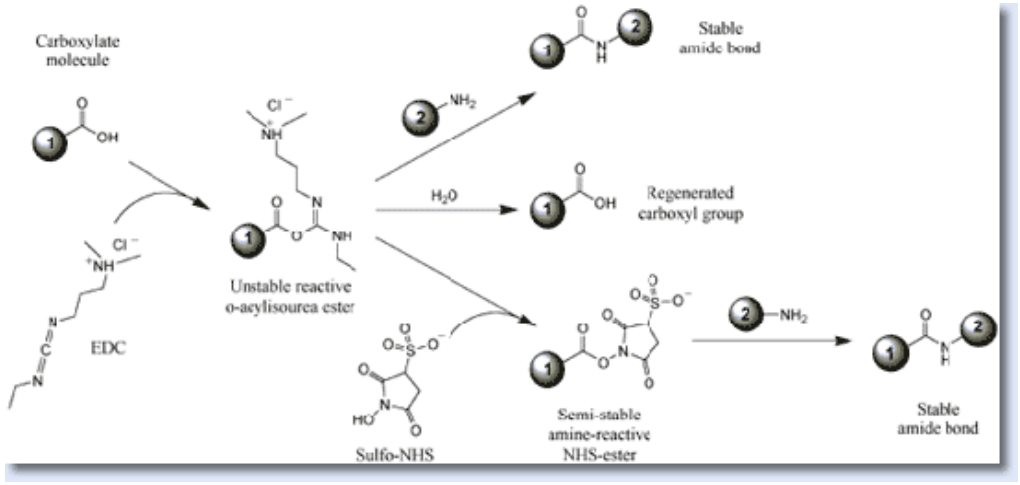

Figure 14: Reaction between carboxylic and amino groups

The result of the sequential procedure described before is schematically presented in Figure 15.

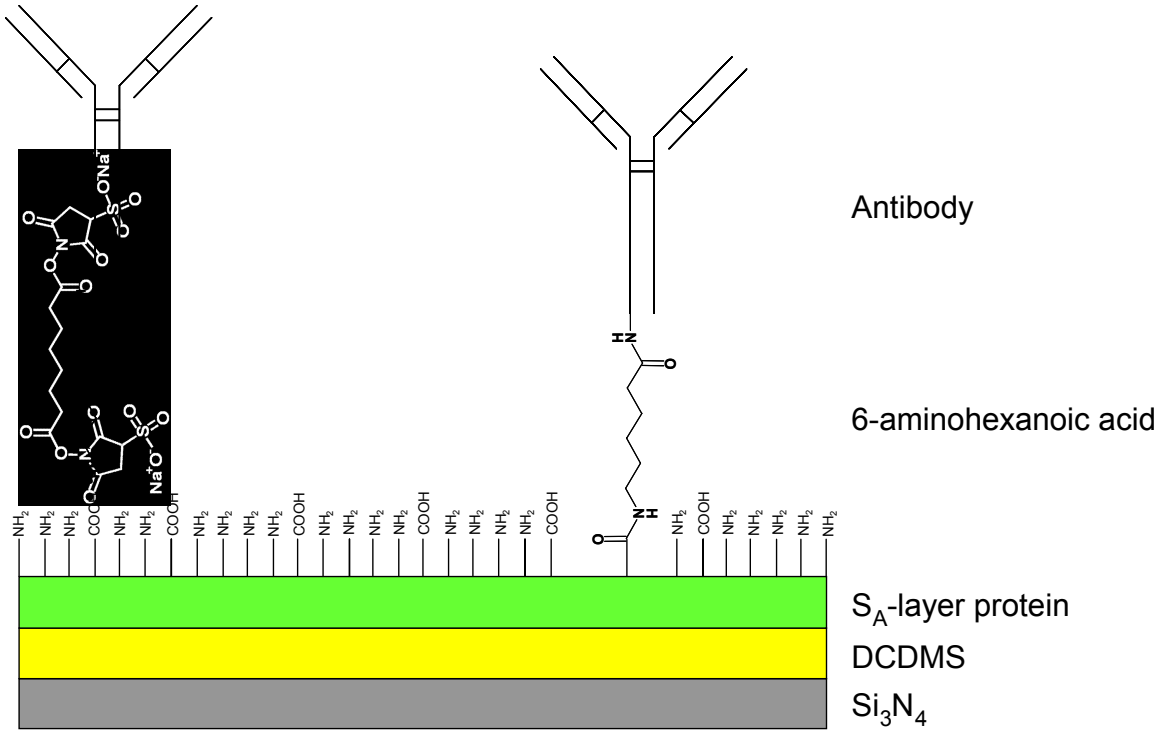

Figure 15: Schematic representation of the layer build-up on the MZI sensor chip.

In the case of the immobilised MαBiot antibody the ShαAtr has to be biotinylated to make it recognisable for the MαBiot. The procedure for biotinylation can be found in protocol SEN-008 in paragraph 7.7. Additional information on biotinylation can be found in the supplement of SEN-008 in paragraph 7.8.

Figure 16 presents the two different ways of immobilisation of ShαAtr antibodies to a sensor surface. Option "A" is the direct immobilisation of ShαAtr to the SA-layer protein. Option "B" makes use of the intermediate antibody MαBiot.

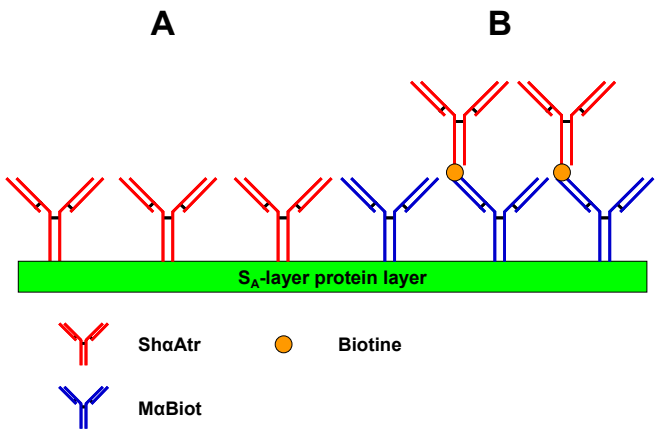

Figure 16: Immobilisation of ShaAtr in two ways. Option A is direct immobilisation. Option B makes use of the intermediate antibody MaBiot and biotinylated ShaAtr

3.2.5 *Atrazine measurements*

After immobilisation of the antibodies against atrazine the MZI system has been mounted and the fluidic system purged with running buffer (Phosphate Buffered Saline (PBS)).
Sequentially samples and regeneration buffers have been passed over the sensor surface. The sensor data per analysis are presented in a so called "sensorgram" from which the interaction strength and level can be determined.
Figure 17 presents a theoretical sensorgram with corresponding terminology.

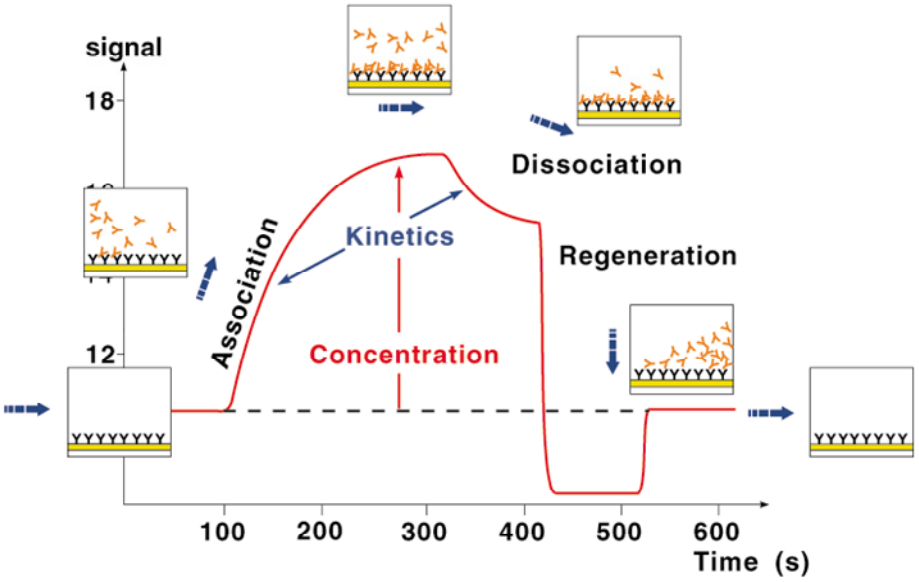

Figure 17: Theoretical sensorgram with corresponding terminology

The first part of the sensorgram is called "baseline". Only running buffer is transported through the fluidic system. At 100 seconds sample is injected and binding of atrazine to the immobilised antibody takes place. This phase is called the "association phase". The "dissociation phase" starts as soon as sample injection stopped. Running buffer is flowing over the sensor surface and the bound molecules will be released from the sensor surface into the liquid. This release depends on the affinity strength between the antibody and the atrazine. By increasing affinity, dissociation will decrease. The ratio between the association and dissociation is a measure for the affinity strength between antibody and atrazine. The signal level at the end of the association phase is a measure for the concentration of atrazine in solution. By increasing concentration of atrazine in the sample the signal level will increase. The dissociation of atrazine can take some time, hours to days. This time frame is too long and therefore we like to give a helping hand to the system. This is called "regeneration". During the regeneration phase a liquid is injected that disturbs the equilibrium between the antibody and atrazine in favor of the dissolved atrazine. In the end regeneration results in the same sensor surface composition as before sample analysis. The sensor is now ready to receive the next sample.
In case of the use of MαBiot as first antibody followed by the ShαAtr the first association phase will be caused by the interaction between MαBiot and ShαAtr. On top of this interaction the association phase of the atrazine is placed.
The protocol for the measurement of atrazine is presented in paragraph 7.10 (SEN-015)

4 Results and Discussion

4.1 Preparation of sensor surfaces

To qualify the effectiveness and reproducibility of the different process steps measurements and visual observations have been performed. To determine the hydrophobicity contact angle measurement have been performed. Elipsometry has been used to assess the layer thickness of the layers.
A stereomicroscope, interference microscoop, high resolution scanning electron microscope (HR-SEM), scanning electron microscope (SEM) and atomic force microscope have been used for visualisation of interface layers.

4.1.1 Cleaning

The cleaning of the Si_3N_4 surface with piranha acid is very effective and robust. After each cleaning a visual quality control has been performed. When drops of water remain at the sensor surface the cleaning procedure has been repeated until a complete hydrophilic surface has been obtained. The visual examination has been validated by contact angle measurements.

A thorough cleaning avoids problems with the deposition of the DCDMS on the Si_3N_4 surface.

4.1.2 Silanisation

The influence of temperature and sonification on the silanisation performance has been investigated. The heating step in 0b of protocol SEN-002 is meant to evaporate the bound water molecules at the Si_3N_4 surface. Remaining water influences the rate of crosslinking of the applied DCDMS. An anhydrous surface provides a stable DCDMS layer. For the same reason anhydrous toluene has been used for dissolving the DCDMS.
The washing step of 0c of protocol SEN-002 is performed with and without sonification. The results of this combined experiment are presented in Table 2.

Table 2: Contact angle and layer thickness measurement of silanised surfaces

Sample	Temperature (°C)	Sonification	D* silane (nm)	Contact angle (°)
1	50	-	2.2	99.0
2	50	+	0.3	96.0
3	60	-	0.6	100.0
4	60	+	1.3	98.1
5	75	-	3.5	96.9
6	75	+	2.1	93.9
7	120	-	1.2	96.6
8	120	+	0.6	95.9
Blank	-	-	-	22.9

* Indicative thickness measurements.

HR-SEM pictures are made of the corresponding substrates presented in Figure 18 and Figure 19. The temperature (T) and if sonification took place (+/-) is state in right up corner of the photos.

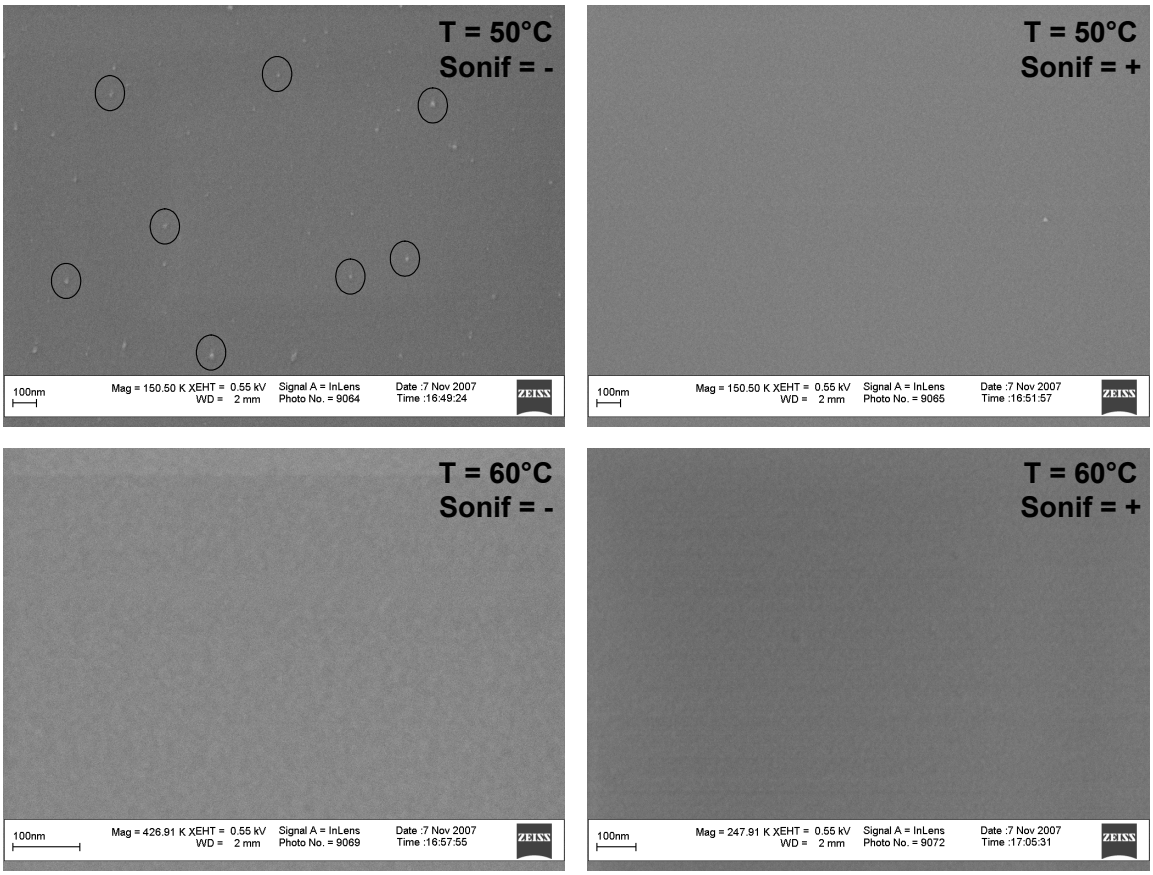

Figure 18: HR-SEM photos optimisation silanisation (1)

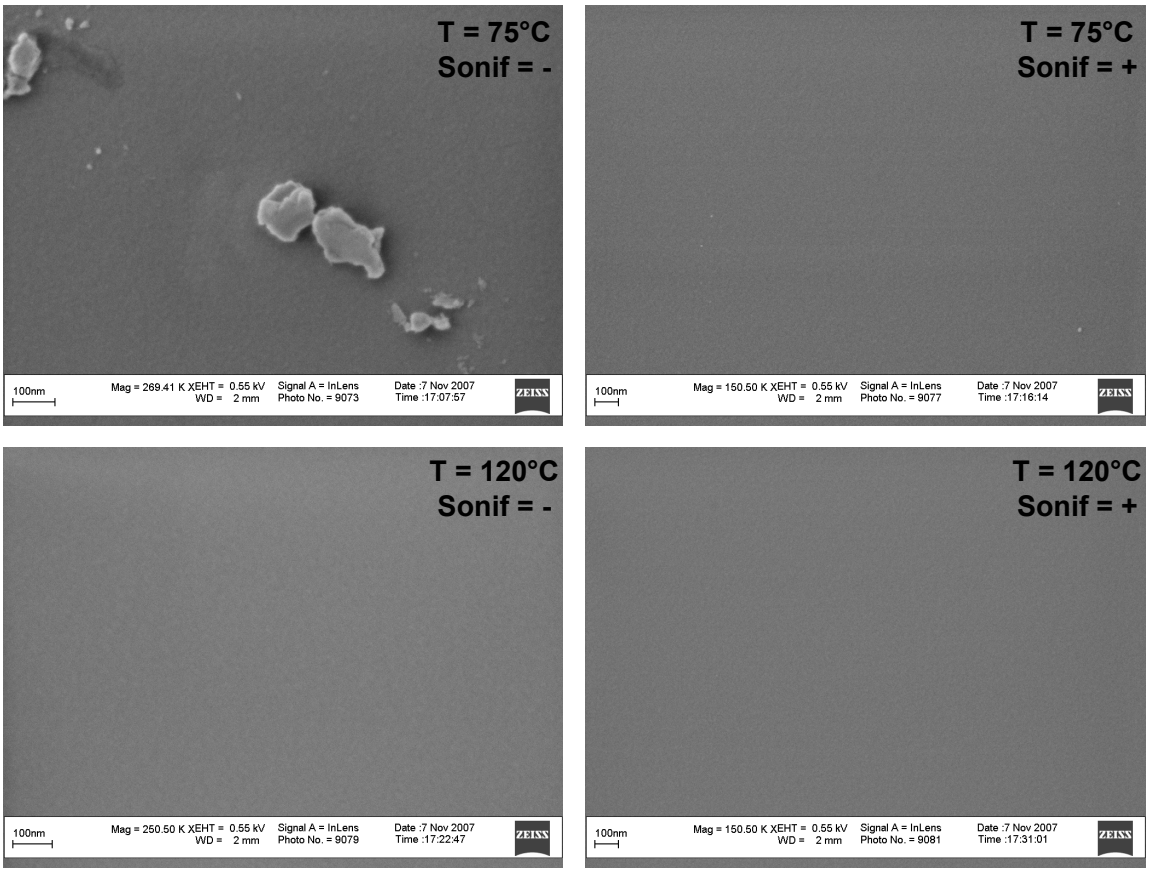

Figure 19: HR-SEM photos optimisation silanisation (2)

From Table 2 it is shown that there are no major differences between the different treatments. The significance of the layer thickness measurements is under discussion because only once per substrate a measurement was performed. The data show no relation to the treatment of the substrate.
The deviation in the contact angle shows no big differences either. All surfaces have an angle of over 80° what is necessary for the S_A-layer protein to form a self assembled layer. This also proves the deposition of the DCDMS on the sensor surface.

Although the photos of Figure 18 and Figure 19 show a positive effect of the sonification treatment the differences are not that big to decide to incorporate sonification to the protocol.
Artifacts can be found on the substrates treated at 50°C and 75°C without sonification. Those of 75°C are not from the silanisation process itself. The rinsing solution used was polluted with dust. The artifacts of 50°C are of direct consequence of the silanisation procedure. The size of these artifacts is so small that they will not have any influence on the forming of the self assembled S_A-layer protein layer.
Further experiments are performed at 50°C without sonification. The decision is based on two considerations. The first is dealing with the sonification. This is a laborious step and has influences on the bonding of the optical fiber to the MZI-sensor chip. The fibers are glued to the surface and the bonding is destroyed by the vibrations of the sonification.
The glue is also the reason to work at a temperature of 50°C. By increasing temperature the aging of the glue increases. By increasing aging the position of the fiber is influenced resulting in a decreasing performance of the optochemical measurements.

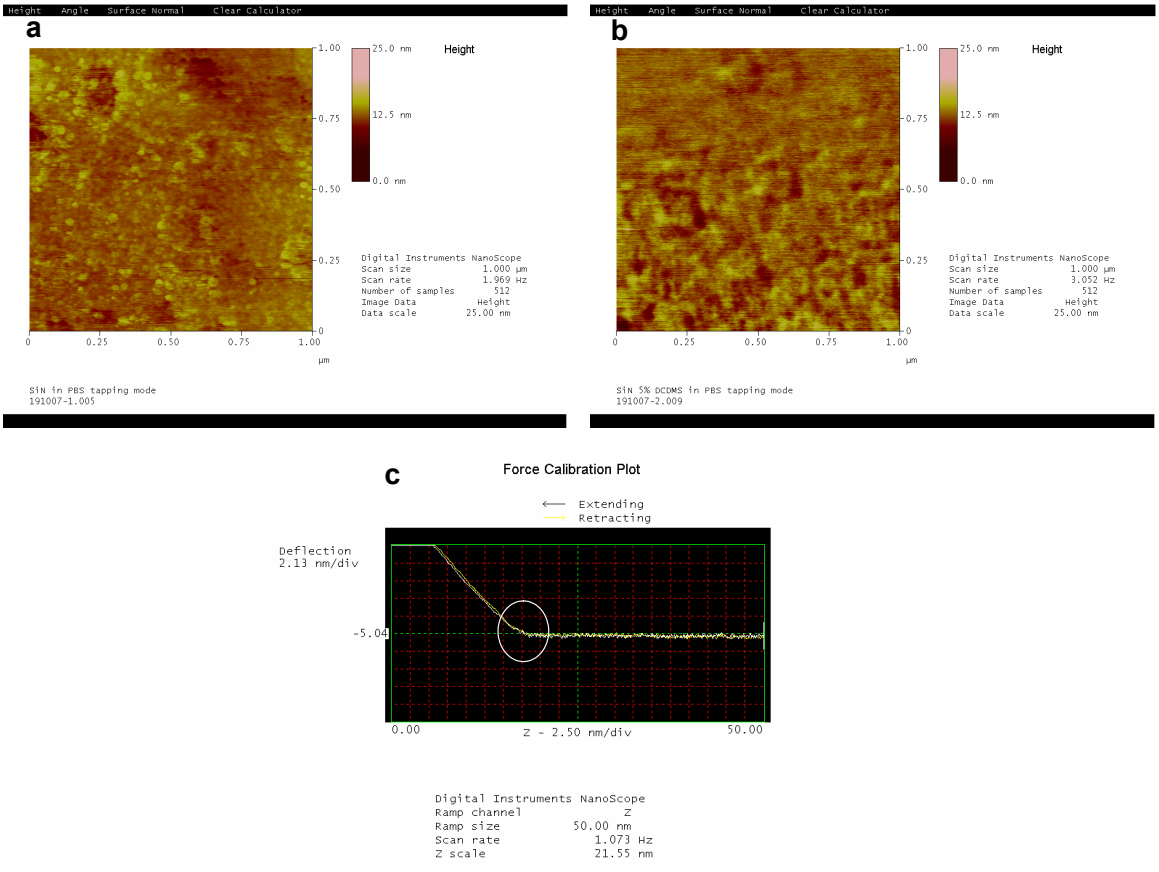

Figure 20: AFM representation of a Si_3N_4 surface(a), a DCDMS silanised surface (b). Photo c shows the "Force calibration plot"of the silanised surface.

The effect of the treatment with DCDMS is shown in Figure 20. Photo "a" is an AFM picture of a Si_3N_4 surface while photo "b" presents the surface after silanisation with DCDMS. The structure difference is obvious.
Photo "c" is "the force calibration plot" of the DCDMS silanised surface. To obtain such a plot the AFM-tip is pressed against the surface of the substrate. After a while the tip is removed from the surface. During pressure and release the force on the AFM-tip is measured. The overlapping forces as presented in photo "c" are evidence of an inelastic force between the AFM-tip and the surface. This is in accordance with the nature of a silanised surface.
A protein layer will give an elastic force between the surface and the AFM-tip. This is discussed in paragraph 4.1.3.

4.1.3 S_A-layer protein interface

The arrangement S_A-layer protein on a DCDMS silanised surface depends strongly on the anomaly of the DCDMS layer. Irregularities cause an unstable protein layer that is washed away with the $MgCl_2$, GuCl or NaOH rinsing steps. In those cases the surface becomes hydrophobic instead of hydrophilic. This is visual examined and confirmed by contact angle measurements. As soon as the contact angle becomes over 60° drops of water remains on the surface. When the S_A-layer protein layer sticks to the surface a film of water is monitored.
Hydrophilic S_A-layer protein layers are obtained when rinsed with 4M $MgCl_2$ or 20 mM NaOH. Rinsing with 6M GuCl provided always hydrophobic surfaces in the end.

Efficiency of the self assembly of S_A-layer protein is judged based on HR-SEM, SEM and interference microscopy. Figure 21 and Figure 22 show examples.

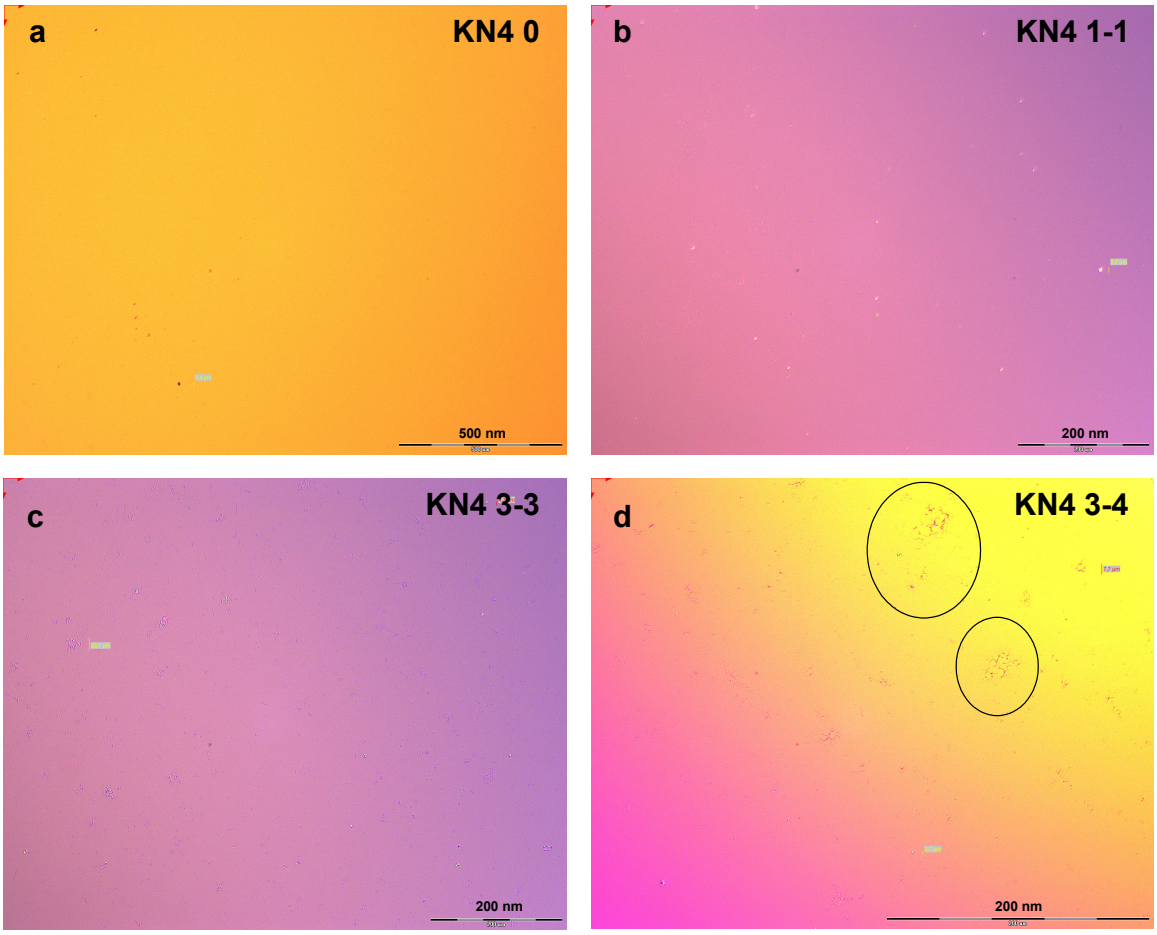

Figure 21: Interference microscopy photos of S_A-layer protein layers. Photo a shows an untreated Si_3N_4 surface. Changes in colour represent changes in layer thickness. Photo b, c and d are shots of S_A-layer protein treated surfaces.

Figure 21a shows a cleaned Si_3N_4 surface as reference. The levelled colour indicates a very flat surface. Figures Figure 21b, c and d represents surfaces with self assembled S_A-layer protein layers. The tapered colour of Figure 21d is an indication of an irregular protein layer (See also the structures in the circles). Colour indicates regular layers in photo Figure 21b and c. These results show that interference microscopy can be used to determine the assembly efficiency. Interference microscopy is much easier to perform then HR-SEM or SEM and therefore the preference technology.
The treatment of the examples differs. For KN4 1-1 and KN4 3-3 fresh prepared S_A-layer proteins are used while for KN4 3-4 an old batch proteins has been used (1 year old). The results show the effect of aging of the S_A-layer proteins.
The incubation time of the S_A-layer proteins differs for KN4 1-1 and for KN4 3-3 and KN4 3-4. For KN4 1-1 the incubation time was overnight while for the other two the incubation time was 1 minute. The results of photo b and c show that the self assembly of S_A-layer protein is very fast. The interference microscope does not show major differences.

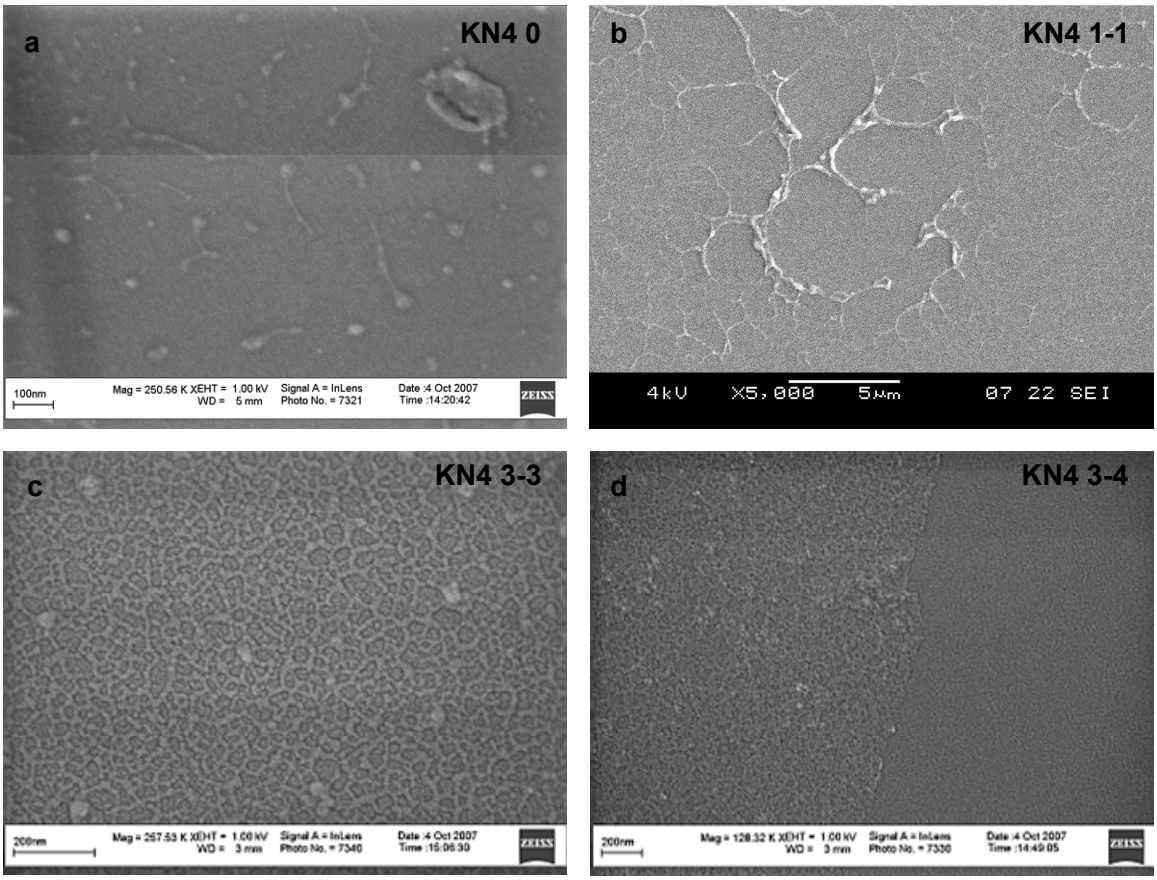

Figure 22: HR-SEM en SEM pictures of S_A-layer protein layers. Photo a is an HR-SEM picture of untreated bare Si_3N_4 surface. Photos b, c and d show the S_A-layer protein layers corresponding with KN4 1-1 (b, SEM), KN4 3-3 (c, HR-SEM) and KN4 3-4 (d, HR-SEM).

Figure Figure 22a shows a HR-SEM photo of untreated and unpurified Si_3N_4 surface. Figure 22b, c and d are presenting (HR)-SEM photos of self assembled S_A-layer proteins. The photos are from corresponding with the experiment previous described in Figure 21.
A clear difference is observed between the Si_3N_4 and S_A-layer protein treated surfaces. This counts too for the different treatments with S_A-layer proteins.
The irregularities indicate the inefficient removal of the multi layers S_A-layer proteins. In photo Figure 22d a clear bilayer of S_A-layer protein is visual.

Table 3 represents the measured layer thicknesses and contact angles of the substrates previous described.

Table 3: Layer thicknesses and contact angles of different interface layers

Sample	**Surface**	**Thickness** (nm)	**Contact angle** (°)
KN4 0	Si_3N_4	-	30.5
-	DCDMS	2.2	102.4
KN4 1-1	S_A-layer protein	6.1	78.5
KN4 3-3	S_A-layer protein	5.2	67.6
KN4 3-4	S_A-layer protein	2.6	78.1

The results of the thickness measurements are not consistent with the observations seen in the photos of Figure 22 and the expected layer thickness. Based on the dimensions of S_A-layer protein theoretically the thickness of a monolayer should be 6 nm. The thickness measured for the S_A-layer protein layers is always the sum of the thickness of the DCDMS-layer and the S_A-layer protein layer.
The lower protein layer thickness can be explained by the dimensions of the spot used for the thickness measurement and the spot of the (HR)-SEM measurements. The spot of the elipsometer has a dimension of 1 by 4 mm while those of the (HR)-SEM are either 20 by 25 µm (SEM) or 1 by 1.5 µm (HR-SEM). That are surface ratios of $4*10^6$: 500 : 1.5
The (HR)-SEM locations are chosen to show particular issues while the elipsometer measure is an average of a bigger surface. The values of the elipsometer measurements show that the fresh prepared S_A-layer proteins forms a thicker protein layer then the older batch of proteins. Almost no proteins have been assembled in the case of experiment KN4 3-4. This is also reflected in the contact angle results. For KN4 3-4 this angle is close to 80° reflecting a hydrophobic surface. The same counts for KN4 1-1 but in the photo b of Figure 22 it is clearly show that there are remaining fragments of the multilayer after rinsing. When a bilayer still exists the surface will be come more hydrophobic due to the fact that the hydrophobic side of the S_A-layer protein is exposed to the outside.
Sample KN4 3-3 gives a significant drop in hydrophobicity and is almost hydrophilic. From photo c of Figure 22 it is clearly demonstrated that the layer thickness is not optimal. It seems that there are holes between walls of S_A-layer proteins. These holes might be filled up with increasing incubation time.

From substrates treated with S_A-layer proteins AFM shots are produced. The advantage of AFM above (HR)-SEM is the availability of a third dimension, height. The color indicates the thickness of the layer measured. Figure 23 shows three AFM shots and a "Force calibration plot" of a S_A-layer protein layer.

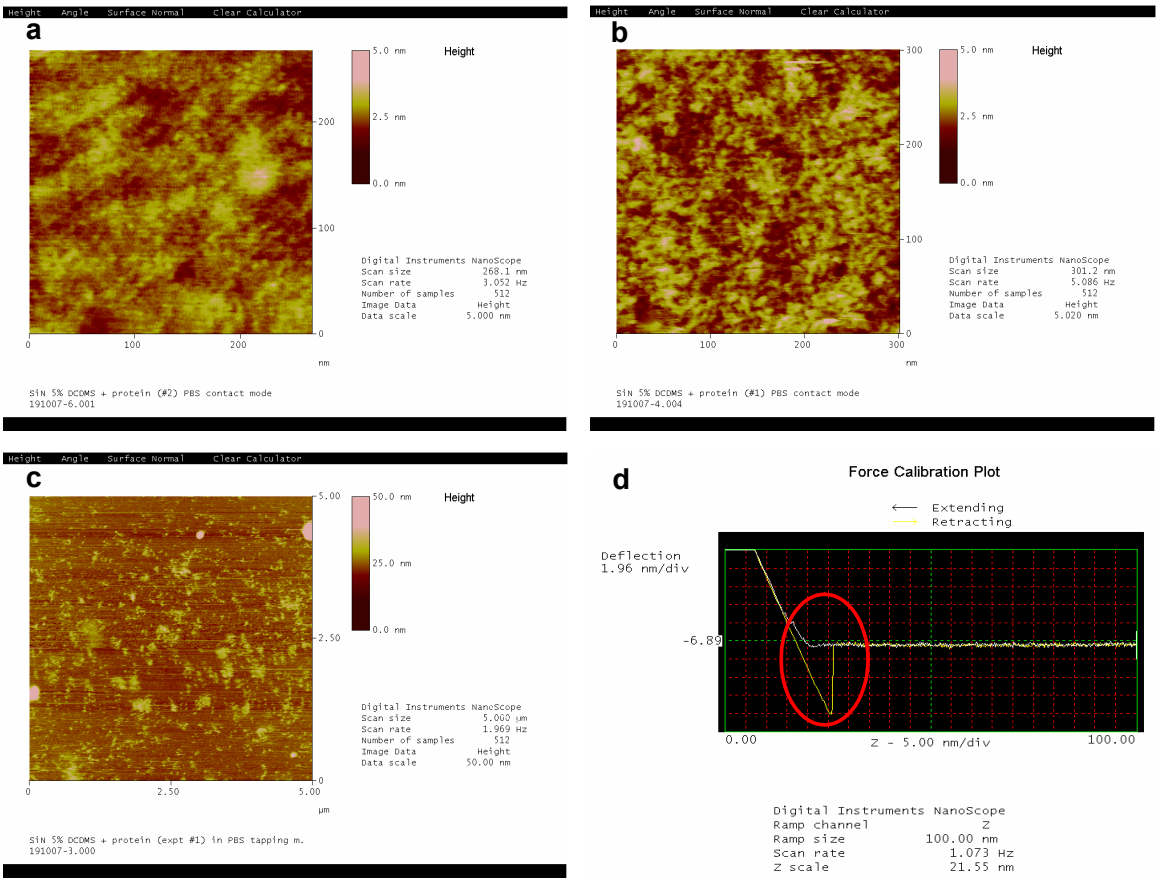

Figure 23: AFM pictures of three S_A-layer protein layers (photos a, b and c). Photo d is a "Force calibration plot" of one of het S_A-layer protein layers

The layer thicknesses in photo a and b correspond to the theoretical dimensions of a monolayer of S_A-layer protein.
Photo c represents a bigger surface area. In this photo a clear inhomogeneous S_A-layer protein layer is observed with fragments of 5 layers thick. This indicates an incomplete removal of the bilayers formed. This result is consistent with results obtained with HR-SEM.

The "Force calibration plot" from the S_A-layer protein layer shows a significant difference (See red circle) with the force calibration plot of the silanised Si_3N_4 surface. The curve of the up going AFM-tip (yellow) differs from the down going AFM-tip (white). This indicates that the AFM-tip "feels" an elastic layer. The difference is caused by the fact that proteins have a stronger interaction with the AFM-tip then DCDMS molecules. Therefore more energy is needed to break up the bonding between the AFM-tip and the protein. The force calibration plot of photo d of Figure 23 indicates that the S_A-layer protein has assembled at the surface.

4.1.4 Antibody immobilisation

Two ways of immobilising ShαAtr have been tested. Figure 16 of paragraph 3.2.4 shows the configuration of each immobilisation.
First the immobilisation with the intermediate MαBiot has been tested. Figure 24 presents the MZI-sensor signals of the sequential steps performed to immobilise the first antibody MαBiot.

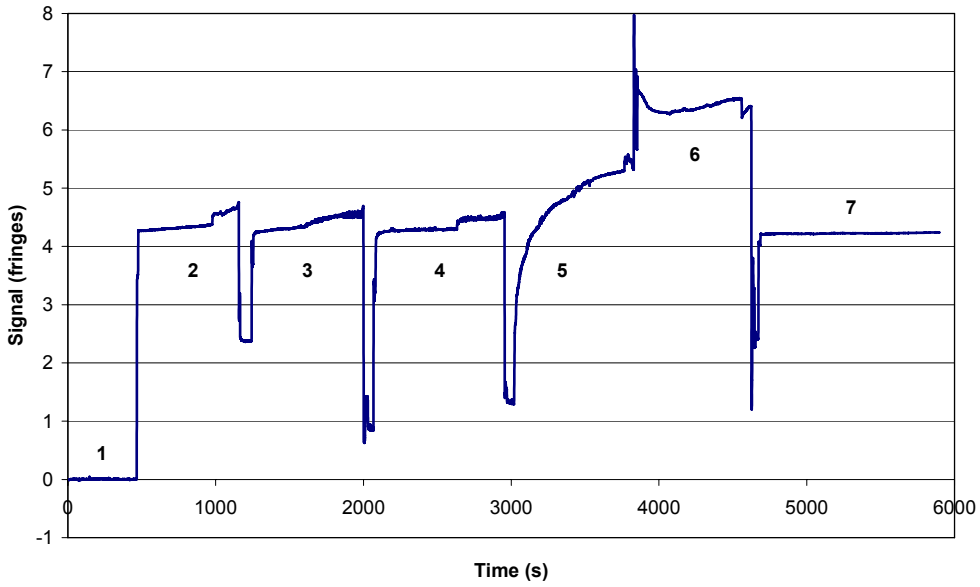

Figure 24: Sequential incubation steps for the immobilisation of MaBiot on a S_A-layer protein layer. 1=MES-buffer, 2=EDC/NHS, 3=6-aminohexonoic acid, 4=EDC/NHS, 5=MaBiot, 6=ethanol amine, 7=MES buffer

A total shift of 4.239 fringes has been obtained. Directly after biotinylated ShαAtr has been injected. Figure 25 shows the sensor signal of the interaction between the immobilized MαBiot and the biotinylated ShαAtr.

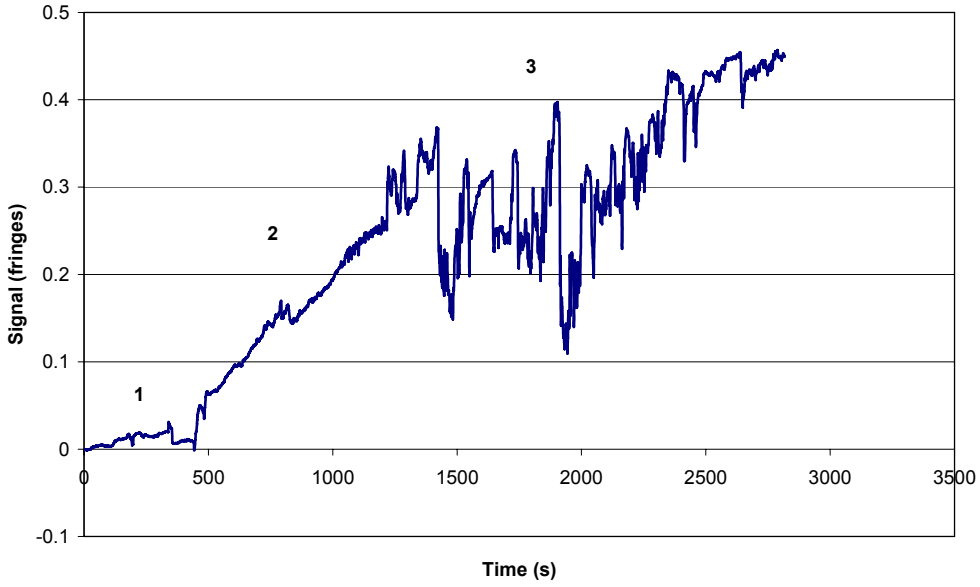

Figure 25: Interaction of biotinylated ShaAtr to a MaBiot functionalised sensor surface. 1=PBS-buffer, 2=biotinylated ShaAtr, 3=PBS-buffer

The total shift of this interaction is approximately 0.3 fringes. This is a factor 10 to 12 times lower than the signal obtained for the immobilisation of the MαBiot antibody. In the case of a one to one interaction

the signal of the biotinylated ShαAtr should have been comparable with the signal for MαBiot. The reduction in signal of the ShαAtr indicates a lost of recognising power. This can be caused either by the efficiency of the biotinylation of the ShαAtr antibody or by the side directed coupling of the MαBiot antibody. In this stage of research it is not identified which mechanism has been responsible. Theoretically two mechanisms might occur. The first is the random immobilisation of the MαBiot antibody to the surface. In this case the recognising side of the antibody can be bound to the surface loosing its recognising capacities. The second mechanism is the efficiency of the biotinylation of the ShαAtr antibody. By inefficient biotinylation the majority of the ShαAtr antibodies might not be biotinylated, resulting in a decrement of recognition by the MαBiot. A second effect can be the lost of recognition for atrazine due to the binding of biotin in the recognising side of the ShαAtr antibody. Based on the high noise level that is observed from second 1250 and further it is decided not to proceed with the direct measurement of atrazine. This is explained by the fact that an antibody has a molecular weight of approximately 150 kD while atrazine has a molecular weight of 187 D. The noise of the signal is around 0.1 fringes. The maximum signal to be expected will be 150,000 / 187 = 802 times lower than the signal of the biotinylated ShαAtr, 0.0004 fringes. This signal of 0.0004 fringes is much lower than the noise of 0.1 fringe and will not be detected.

Based on the previous results is has been decided to switch to the second immobilisation procedure in which ShαAtr has been directly immobilised to the S_A-layer protein layer. Figure 26 shows the MZI-sensor signals of the sequential immobilisation steps for the immobilisation of ShαAtr.

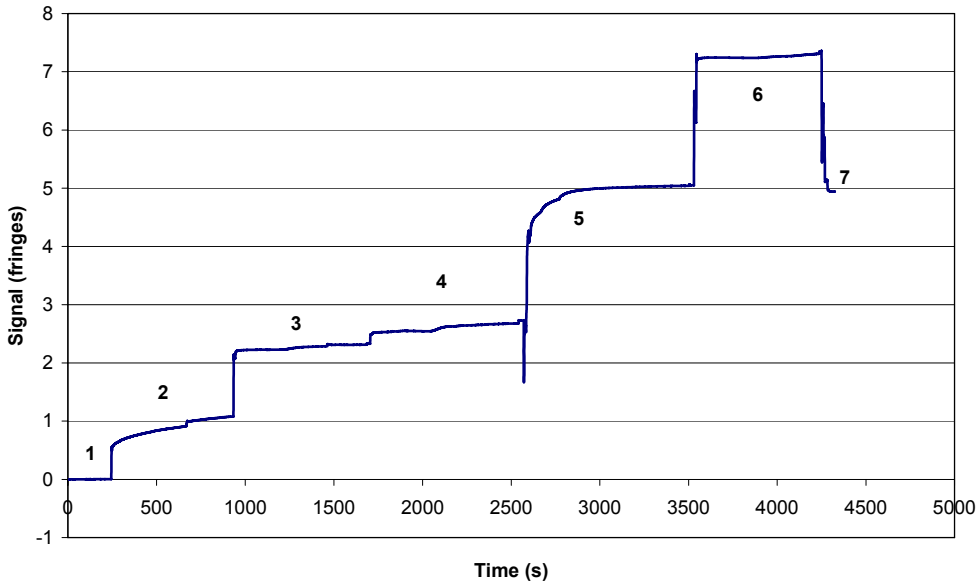

Figure 26: Sequential incubation steps for the immobilisation of ShαAtr on a S_A-layer protein layer. 1=MES-buffer, 2=EDC/NHS, 3=6-aminohexonoic acid, 4=EDC/NHS, 5= ShαAtr, 6=ethanol amine, 7=MES buffer

The obtained signal shift after the immobilisation procedure is 4.943 fringes. This is even more than the shift obtained for the immobilisation of MαBiot.

4.2 Atrazine measurements

As discussed in 4.1.4 it had no sense to perform atrazine measurements on the MαBiot + biotinylated ShαAtr coating. No results will be shown for this coating.

For the direct immobilisation of ShαAtr a lot of atrazine analyses have been performed. After each atrazine interaction a regeneration step is included. A complete sequence of analysis is presented in Figure 27.

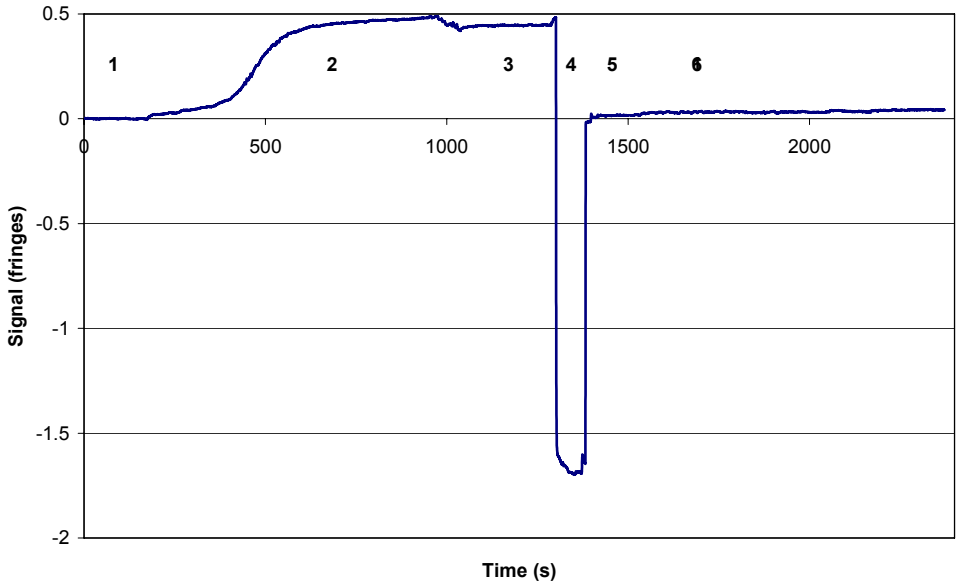

Figure 27: Complete atrazine analysis sequence. 1=PBS, 2=5 µg/L atrazine in PBS, 3=PBS-buffer, 4=20 mM NaOH, 5=PBS-buffer, 6=5 µg/L atrazine in PBS

The results show a good signal, 0.446 fringes, for the first atrazine analysis with a concentration of 5 µg/L. However after regeneration with 20 mM NaOH no interaction with atrazine can be observed. Different regeneration procedures have been tested but no proper regeneration procedure could be developed.

Consequentially for each atrazine measurement the sensor surface had to be thoroughly clean and the sensor coating build up again. This has been a very laborious and time consuming process especially because not all immobilisations succeeded.

In the end two successful atrazine measurements could be compared. The comparison is presented in Figure 28.

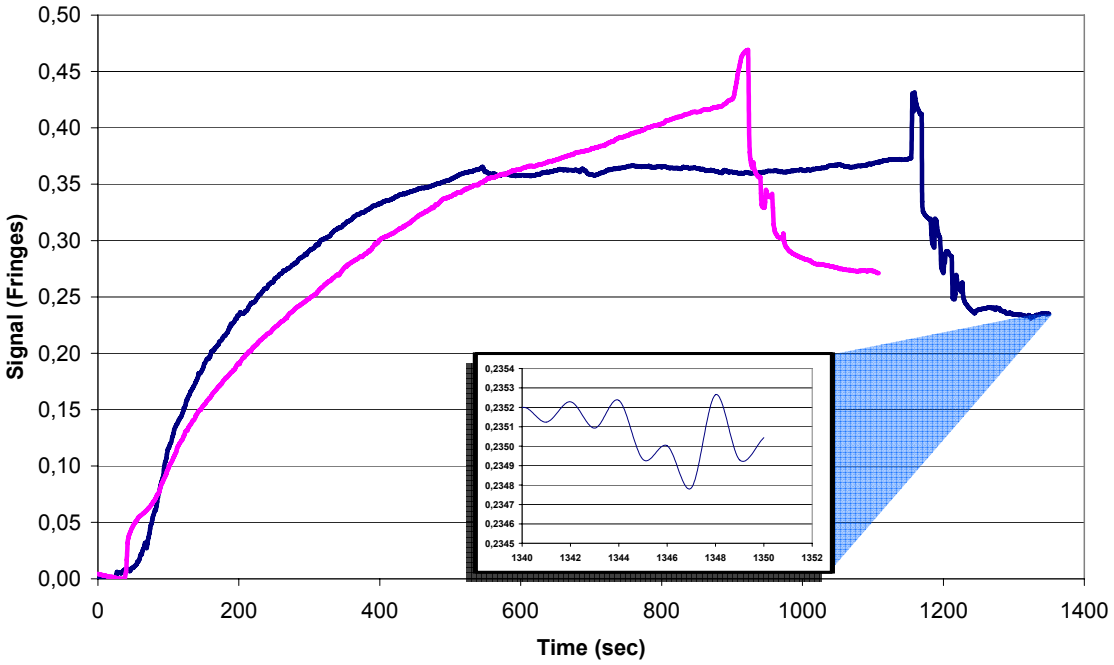

Figure 28: Comparison of two atrazine measurements at 5 µg/L. The insert shows the noise level of the signal.

The atrazine measurements have been performed on a coating of direct immobilized ShαAtr antibodies. The two interactions were performed on two independently prepared sensor surfaces. The concentration atrazine used was 5 µg/L. The insert shows the noise on the MZI-sensor signal. Based on this noise there is an expectation that the sensitivity of the direct measurement of atrazine may increase. No results are available yet to give a scientific underpinning.

5 Conclusions

5.1 Immobilisation procedures

Before antibodies could be immobilised on the sensor surface the surface had to be functionalised. Varying results were obtained with the chosen strategy. The deposition of DCDMS on the Si_3N_4 has a high reproducibility. The self assembly of the S_A-layer proteins on the DCDMS layer is less reproducible. Additional research is needed to understand the underlying factors. Nevertheless, frequently feasible S_A-layer protein layers were produced on top of which antibodies could be immobilised.
The immobilisation of antibodies by the carbodiimide reaction and the use of 6-aminohexanoic acid succeeded each time again. The same procedure has been used for both the MαBiot and ShαAtr antibodies. The results show that this immobilisation procedure is less efficient for the MαBiot immobilisation. The recognising side of the antibody might be bound to the surface giving steric hindrance for the binding of the biotinylated ShαAtr. This steric hindrance can also be expected for the binding between atrazine and direct immobilised ShαAtr. However, the size of atrazine is 800 times smaller as a biotinylated antibody resulting in reduced steric hindrance. Therefore the measurement of atrazine was possible due to the binding between atrazine and the ShαAtr antibody.

5.2 Atrazine measurements

Unfortunately, no results were obtained with the two-stage-rocket approach. No measurable results could be expected after the immobilisation of ShαAtr by the MαBiot.
The results obtained with the direct immobilised ShαAtr antibodies are encouraging. With this experimental setup the direct detection of a low molecular weight compound has been proven to be possible at a level of 5 µg/L atrazine. Drawback of this setup is the lack of regeneration possibilities. The procedure is usable for the development of a disposable device.
Based on the results the whole procedure for functionalising the sensor surface has to be optimised.

5.3 General

Based on the results described in this report the feasibility of the direct detection of atrazine is proven. However, the development of an early warning system for the in situ detection of contaminants has not been succeeded.

Developments have to be performed on both the hardware and chemistry of the MZI-system.
Noise reduction can be achieved by optimising the electronic unit. The reproducibility of the analyses can be increased by a redesign of the flow-through system which has to be coupled to an autosampler system. Temperature stabilisation of the whole hardware system will also have a positive contribution to the reproducibility of analysis. All together this might increase the physical sensitivity one or two orders of magnitude. A redesign of the sensor chip configuration can be taken in consideration because this will increase the physical sensitivity with one order of magnitude. The lower the noise level the more accurate the biochemical interactions can be visualised.
The selectivity and specificity of the sensor system relies on the performance of the biochemical interface layers on top of the sensor surface. Important is the orientation of each individual biochemical layer of the sensor interface. Therefore the protocol for the deposition of the S_A-layer proteins has to be optimised as well as the immobilisation procedure of the antibodies. Both optimisations will increase the reproducibility of the atrazine (organic compound) measurements. It is preferred to put effort in the development of a regenerabel interface layer. This increase the number of analyses per sensor surface resulting in a decreasing price per analysis.

6 References

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7 Appendix

7.1 SEN-001: Pre-cleaning of silica surfaces

7.1.1 Introduction

This protocol describes the pre-cleaning of silica surfaces. Piranha acid oxidises biological molecules on the surface. Strong Sodium hydroxide removes fat residues, resulting in a hydrophilic surface. Hydrochloric acid removes calcium residues.

7.1.2 Reagents

	CAS number	supplier	product number
Hydrochloric acid 30% (HCl)	7647-01-0	Merck KGaA	1.00313
Hydrogen peroxide (HOOH)	7722-84-1	Merck KGaA	1.08597
Sodium hydroxide (NaOH)	1310-73-2	J.T.Baker	0288
Sulphuric acid (H_2SO_4)	7664-93-9	J.T.Baker	6027
ultra pure water	MilliQ of the Millipore system		

7.1.3 Solutions

a) Piranha solution
   Mix 1 aliquot of hydrogen peroxide (30%) with 3 aliquots concentrated sulphuric acid.
b) 1M NaOH solution
   Prepare a solution of 40 g/L in MilliQ
c) 1M HCl solution
   Dilute a 30% HCl solution 10 times with MilliQ

7.1.4 Procedure

a) Incubate with piranha solution for 10 minutes
b) Rinse thoroughly with MilliQ
c) Incubate with 1 M NaOH for 30 minutes
d) Rinse thoroughly with MilliQ
e) Incubate with 1 M HCl for 30 minutes
f) Rinse thoroughly with MilliQ
g) Dry carefully with a medical swipe or with a gentle flow of nitrogen

7.1.5 Remarks

a) Caution: Piranha acid is a VERY STRONG and DANGEROUS acid. Handle with greatest cautiousness!
b) Prepare piranha acid just before use because the reactivity decreases during time.
c) Be aware that the temperature of the solution can increase up to 120°C causing bumping of the liquid! To avoid bumping stir the solution.
d) The glassware used has to be free of organic materials and solvents! Explosions will occur if during preparation organics are present.
e) After preparation do not add organic solvents to piranha acid! Explosions will occur.
f) After treatment the surface becomes hydrophilic. With a drop of water the treatment efficiency can be checked. The drop will not spread over the surface when the surface is still hydrophobic.

7.1.6 Literature

a) Popat K.C., Sharma S. and Desai T.A., "Engineered silicon surfaces for biomimetic interfaces", Business Briefing: Medical Device Manufacturing and Technology, World Markets Research Center, London, June 2002
b) http://en.wikipedia.org/wiki/Piranha_solution

7.2 SEN-002: Silanisation of silica surfaces with DCDMS

7.2.1 Introduction

This protocol describes the silanisation of silica surfaces with DCDMS. The silica surface becomes hydrophobic (contact angle > 80 degrees) after treatment.

7.2.2 Reagents

	CAS number	supplier	product number
Acetone	67-64-1	J.T.Baker	8142
Dichlorodimethylsilane (DCDMS)	25952-53-8	Fluka	40140
Hydrochloric acid 30% (HCl)	7647-01-0	Merck	1.00313
Sodium hydroxide (NaOH)	1310-73-2	J.T. Baker	0288
Toluene	108-88-3	J.T.Baker	8078
Ultra pure water	MilliQ of the Millipore system		

7.2.3 Solutions

a) 1M NaOH solution
   Prepare a solution of 40 g/L in MilliQ
b) 1M HCl solution
   Dilute a 30% HCl solution 10 times with MilliQ
c) 5% solution of DCDMS in toluene
   Add 25 µL of DCDMS to 475 µL toluene

7.2.4 Procedure

Before executing this protocol, execute protocol SEN-001

a) Rinse 3 times with acetone, and dry in a stove at 50°C for 1 hour
   Applying the silane on the silica surface
b) Incubate with 5% DCDMS for 10 minutes
c) Rinse 5 times with toluene
d) Rinse 5 times with acetone
e) Dry and cure the surface for 1 hour in a stove at 50°C

7.2.5 Remarks

a) Make sure that the DCDMS does not get in to contact with moisture, store under nitrogen
b) When a silica surface is not clean; it must be pre-cleaned prior to the silanisation of the surface. Direct after this pre-cleaning (protocol SEN-001), the surface is rinsed with acetone and dried in a stove at 50°C for 1 hour. Proceed with this protocol at the 'applying of the silane on the silica surface' step.

7.2.6 Literature

a) Dr Tejal A Desai, Ketul C Popat and Dr Sadhana Sharma, Engineered Silicon Surfaces for Biomimetic Interfaces, University of Illinois at Chicago.

7.3 SEN-003: S_A-layer protein self-assembly on hydrophobic surfaces

7.3.1 Introduction

S-layers, two-dimensional paracrystalline arrays usually found as the outermost layer of the cell envelope of bacteria, are composed of a single (glyco)protein. S-layers self-assemble in an entropy-driven process during which multiple, noncovalent interactions between individual S-protein monomers and the underlying cell surface take place.
S-layer proteins also form self-assembly monolayers on hydrophobic surfaces. Sensor surfaces can be made hydrophobic according to protocol SEN-002.

In this protocol the S_A-layer protein of *Lactobacillus acidophilus* (ATCC 4356) is used. The S_A-layer proteins are produced according to protocol SEN-005.

The S_A-layer protein has a tetra ether form of which the bottom part is highly hydrophobic and the top is highly hydrophilic. The hydrophobic part of the molecule interacts with the hydrophobic sensor surface. The hydrophilic top is pointing in to the liquid on top of the sensor surface.

7.3.2 Reagents

	CAS number	Supplier	Product number
Hydrochloric acid (HCl) (30%)	7647-01-0	Merck	1.00313
Magnesium Chloride ($MgCl_2.6H_2O$)	7786-30-3	J.T.Baker	0162
Potassium Chloride (KCl)	7447-40-7	J.T.Baker	0208
S_A-protein	See protocol SEN-005		
Sodium Chloride (NaCl)	7647-14-5	J.T.Baker	0278
Sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$)	10028-24-7	J.T.Baker	0326
Sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$)	10028-24-7	J.T.Baker	0326
Sodium hydroxide (NaOH)	1310-73-2	J.T.Baker	0288
Tris Hydroxymethylaminoethane (Tris)	77-86-1	ProSciTech	C021
Tween 20	9005-64-5		
Ultra pure water	MilliQ of the Millipore system		

7.3.3 Solutions

a) 25 mM NaOH
   Prepare a solution of 1 g/L in MilliQ
b) Phosphate Buffered Saline, 10 times concentrated (PBS-buffer 10x)
   80.0 g/L NaCl, 2.0 g/L KCl, 18.05 g/L Na2HPO4·2H2O and 2,4 g KH2PO4 in MilliQ. (pH ≈ 6.8)
c) Phosphate Buffer Saline (PBS-buffer)
   Dilute "PBS-buffer 10x" 1:10 with MilliQ
d) 4M $MgCl_2$
   Prepare a solution of 813.2 g/L
e) 1M HCl
   Dilute 10 times 30% HCl
f) Tris-HCl buffer
   Prepare a solution of 12.1 g/L Tris, adjust the pH at 7.5 with 1M HCl
g) 2.5 mg/ml S_A-protein solution in 5M LiCl (See protocol SEN-005)

7.3.4 Procedure

a) Add 30 µL of a 2.5 mg/mL S_A-layer protein solution to an ultra filtration unit with a cut-off of maximum 30 KD.
b) Replace the 5M LiCl by Tris-HCl according to protocol SEN-006 (Ultra filtration)
c) Centrifuge the solution 10 min at 16,000 *g*
d) Prepare 250 µL of a 50 µg/mL S_A-layer protein solution in MilliQ
e) Add 100 µL S_A-layer protein solution to each well
f) Cover the wells with parafilm
g) Incubate overnight at room temperature
h) Remove the protein solution
i) Wash three times with MilliQ
j) Incubate two times with 4 M $MgCl_2$ for 1 minute
k) Wash one time with MilliQ
l) Incubate two times with 25 mM NaOH for 1 minute
m) Wash two times with MilliQ
n) Wash two times with PBS-buffer
o) Store the chip in PBS-buffer at 4°C until use

7.3.5 Remarks

a) The added amount of protein is subject to the exact concentration of the protein solution.
b) The S_A-layer protein has a molecular weight of 46,570 D. Buffer change can be performed with ultra filtration membranes with a lower cut-off
c) The centrifuge step after the ultra filtration step is to remove the aggregates formed during the ultra filtration step. As soon as the LiCl concentration decreases the self-assembly of the proteins will take place. The removal of the aggregates increases the stability and structure of the monolayer.
d) During the overnight incubation a multi layer of S_A-layer proteins will be formed on the sensor surface. This is due to van der Waals and hydrophobic interaction between the protein molecules. Because the sensor surface is highly hydrophobic the first mono layer of protein sticks with the hydrophobic part of the protein to the sensor surface. The second layer of proteins sticks by van der Waals interactions to the first protein layer. Van der Waals interactions will be disturbed by 4M $MgCl_2$, resulting in the removal of the multi layers on top of the first monolayer.
e) To keep the S_A-layer protein coating in shape it has to be stored in PBS-buffer. When the protein layer runs dry, it will crack and can not be used anymore for analyses.
f) At start of this protocol the sensor surface is highly hydrophobic. Contact angle of DCDMS is approximately 80 degrees or higher. After treatment with S_A-layer protein the sensor surface becomes hydrophilic. To control the treatment efficiency a drop of water will run over the surface when the surface is hydrophilic. A drop of water will not do this when the surface is still hydrophobic.

7.3.6 Literature

a) Sleytr U.B., Messner P., Pum D., Sára M., Crystalline Bacterial Cell Surface Proteins, Academic Press, ISBN 0-12-648470-8, January 1996
b) Smit E, Lactobacillus S-layer proteins Structure-function relationship and application potential, Posen & Looijen Wageningen, ISBN 90-9015872-3, June 2002
c) http://www.expasy.org/uniprot/P35829
d) http://www.lgcpromochem-atcc.com/common/catalog/bacteria/bacteriaIndex.cfm

7.4 SEN-005: Production and extraction of S_A-layer protein

7.4.1 Introduction

S_A-layer proteins are proteins from the outermost layer of the cell envelope of *Lactobacillus acidophilus*. These (glycol)proteins can be extracted from the cell envelope with 5M LiCl without destroying the cell envelope.

This protocol describes the production of *Lactobacillus acidophilus* and the extraction of S_A-layer protein

7.4.2 Reagents

	CAS number	supplier	product number
Lactobacillus acidophilus ATCC 4356	-	Oxoid	R4603007
Lithium Chloride monohydrate ($LiCl \cdot H_2O$)	7447-41-8	Merck	1.05677
MRS broth		Difco	
Ultra pure water	MilliQ of the Millipore system		

7.4.3 Solutions

a) 5 M LiCl solution
   prepare a solution of 302 g/L in MilliQ
b) 1 M LiCl solution
   Dilute the 5 M LiCl solution 5 times with MilliQ

7.4.4 Procedure

a) Add 10 ml MRS broth to 1 ampoule of freeze dried *Lactobacillus acidophilus*
b) Incubate over-night at 37°C under facultative anaerobic condition
c) Prepare 495 ml pre-warmed MRS-broth in a 500 ml sterile flask
d) Inoculate with 5 ml over-night culture of *Lactobacillus acidophilus* from step a)
e) Incubate over-night at 37°C under facultative anaerobic condition
f) Measure the optical density (OD) of the culture at 695 nm.
g) Harvest the bacterial cells when the OD_{695} is approximately 0.6
h) Centrifuge the bacterial culture at 10,000 *g* at 4°C for 20 min
i) Remove the supernatant
j) Add one volume chilled distillated water (w/v)
k) Centrifuge at 10,000 *g* at 4°C for 20 min
l) Add 0.1 volume 1M LiCl (w/v)
m) Incubate at room temperature for 30 min
n) Centrifuge at 10,000 *g* at 4°C for 20 min
o) Add 0.1 volume 5M LiCl (w/v)
p) Incubate at room temperature for 60 min
q) Centrifuge at 10,000 *g* at 4°C for 20 min
r) Collect the supernatant and store at 4°C

7.4.5 Remarks

a) In this protocol anaerobic condition can be described as follows:
   - Load the flask with MRS broth with the same volume of the flask
   - Close the cap of the flask. No airflow is allowed
   - Do not shake the flask during incubation

b) Use flasks of glass and not of polymers. The contact angle of polymer flasks are high. S_A-layer proteins will form a mono-layer at the wall of the flask. The contact angle of glass flasks is lower then 60 degrees so not adsorption will occur.

c) The bacterial cells are harvested when they are in their exponential growth phase. The measurement of the absorbance at 695 nm is to determine the exponential growth and has to be approximately 0.6.

d) The centrifugation steps are carried out at 4°C, to decrease the metabolism of the bacteria.

e) The 1M LiCl is used to remove all the molecules from the MRS broth that have an electrostatic interaction with the S_A-layer proteins on the cell envelope of the bacteria. This step is to increase the purity of the S_A-layer proteins collected.

f) The purity of the collected S_A-layer proteins is 95% or more.

g) The concentration of the collected S_A-layer proteins can be determined with the Lowry measurement (SEN-007) Generally, the concentration of S_A-layer protein is approximately 2.5 mg/ml.

7.4.6 Literature

a) Sleytr U.B., Messner P., Pum D., Sára M., Crystalline Bacterial Cell Surface Proteins, Academic Press, ISBN 0-12-648470-8, January 1996

b) Smit E, Lactobacillus S-layer proteins Structure-function relationship and application potential, Posen & Looijen Wageningen, ISBN 90-9015872-3, June 2002

c) http://www.expasy.org/uniprot/P35829

d) http://www.lgcpromochem-atcc.com/common/catalog/bacteria/bacteriaIndex.cfm

7.5 SEN-006: Ultra filtration of S_A-layer protein

7.5.1 Introduction

Ultra filtration has been used successfully for years and is an excellent and gentle method to purify and concentrate protein samples. Ultra filtration can be used to replace precipitation, evaporation, dialysis, lyophylization, and gel filtration to concentrate and desalt protein without significant protein loss

S_A-layer proteins are dissolved in 5M LiCl. For the assembly of S_A-layer proteins on the surface of a sensor the LiCl has to be replaced by a Tris-HCl solution. Ultra filtration is used for this replacement.

This protocol describes the replacement of LiCl by Tris-HCl by ultra filtration.

7.5.2 Reagents/Materials

	CAS number	supplier	product number
Hydrochloric acid (HCl) (30%)	7647-01-0		
Nanosep 10K Omega	-	Pall	OD010C33
Tris Hydroxymethylaminoethane (Tris)	77-86-1	ProSciTech	C021
Ultra pure water	MilliQ of the Millipore system		

7.5.3 Solutions

a) 1M HCl
   Dilute 10 times 30% HCl
b) Tris-HCl buffer
   Prepare a solution of 12.1 g/L Tris, adjust the pH at 7.5 with 1M HCl

7.5.4 Procedure

a) Add 500 µl Tris-HCl to the Nanosep 10K Omega unit
b) Centrifuge at 12,000 *g* for approximately 12 min
c) Add sample to the Nanosep 10K Omega unit
d) Fill up to 500 µl with Tris-HCl buffer
e) Centrifuge at 12,000 *g* for approximately 12 min
f) Dilute the concentrate with Tris-HCl buffer to a volume of 500 µl
g) Centrifuge at 12,000 *g* for approximately 12 min
h) Repeat step f) and g) until a dilution factor for the LiCl concentration of minimal 1:1000 is reached
i) Collect the concentrate and store at 4°C
j) Wash the Nanosep unit three times with MilliQ
k) Fill the Nanosep unit with MilliQ
l) Store the Nanosep unit at 4°C until reuse

7.5.5 Remarks

a) The membrane of a new Nanosep unit is preserved with glycol. The glycol has to be removed before use. This is done by the ultra filtration of Tris-HCl buffer. The same step will be used to pre-wash the Nanosep unit before reuse.
b) Prepare an amount of S_A-layer proteins that will be processed within two days after buffer change. Tris-HCl is not strong enough to avoid aggregation forming. After two days the concentration of monomer S_A-layer protein becomes below the critical concentration needed for self-assembly.
c) For the buffer change of S_A-layer proteins also a Nanosep 30K Omega can be used. The centrifugation time will decrease as shown in table 1.

Table 1: Approximate Spin Times in Nanosep Devices

Nanosep Device MWCO	Color Code	RCF	Spin Time at 25 °C
3K	Gray	14,000 *g*	20 min
10K	Blue	14,000 *g*	15 min
30K	Red	14,000 *g*	8 min
100K	Clear	14,000 *g*	5 min
300K	Orange	14,000 *g*	3 min

Be aware that the spin times are guide numbers. Determine empirically the appropriate time by step wise increment of the centrifugation time.
d) The maximum centrifugation speed for Nanosep devices is 14,000 *g*. Do not exceed this limit because the membrane will crack resulting in dysfunction of the ultra filtration.
e) The dilution factor is set on a minimum of 1:1000. A lower dilution factor is not allowed because the remaining LiCl will disturb the self-assembly of the S_A-layer proteins on the sensor surface. An higher dilution factor is preferred.
f) Concentrate the S_A-layer protein solution to the same volume added at the beginning, to finish with the same S_A-layer protein concentration.
g) Take caution during the collection of the concentrate. Pipette points can crack the membrane resulting in dysfunction of the Nanosep ultra filtration unit.

7.5.6 Literature

a) http://www.pall.com/34696_40883.asp#40888

7.6 SEN-007: Lowry protein assay

7.6.1 Introduction

In order to build the S_A-layer on the DCDMS surface, the concentration of S_A-layer proteins in the initial solution has to be determined. A minimum concentration of S_A-layer proteins in solution is needed to build a proper homogeneous layer.

This protocol describes how to analyse the amount of protein in a solution. The protein measured is in this case the S_A-layer protein of *Lactobacillus acidophilus*.

7.6.2 Reagents

	CAS number	supplier	product number
Copper (II) Sulfate ($CuSO_4$)	7758-99-8	J.T.Baker	0104
Folin & Ciocalteu reagent (2N)	-	Fluka	F9252
Potassium Sodium Tartrate ($KNaC_4H_4O_6$)	304-59-6	Sigma	S2377
Sodium Carbonate ($NaCO_3$)	497-19-8	J.T.Baker	1400
Sodium dodecyl sulphate (SDS) ($NaC_{12}H_{25}O_4S$)	151-21-3	Serva	20760
Sodium Hydroxide (NaOH)	1310-73-2	J.T.Baker	0288
Ultra pure water	MilliQ of the Millipore system		

7.6.3 Solutions

a) $NaCO_3$ solution
   Dissolve 20 g $NaCO_3$ in 260 mL MilliQ
b) $CuSO_4$ solution
   Dissolve 0.4 g $CuSO_4$ in 20 mL MilliQ
c) $KNaC_4H_4O_6$ solution
   Dissolve 0.2 g $KNaC_4H_4O_6$ in 20 mL MilliQ
d) Copper reagent
   Mix the $NaCO_3$, $CuSO_4$ and $KNaC_4H_4O_6$ solution to prepare the copper reagent.
e) 1 % SDS solution
   Prepare a solution of 10 g/L in MilliQ
f) 1M NaOH solution
   Prepare a solution of 40 g/L in MilliQ
g) 2x Lowry concentrate
   Mix 3 parts copper reagent with 1 part SDS and 1 part NaOH.
h) 0,2 N Folin reagent solution
   Dilute a 2N Folin & Ciocalteu reagent solution 10 times with MilliQ

7.6.4 Procedure

a) Dilute samples to an estimated protein concentration of 0.025-0.25 mg/ml with a protein free buffer. If the concentration can not be estimated it is advised to prepare a range of 2-3 dilutions spanning one order of magnitude. Prepare 400 µL of each dilution. Duplicates or triplicates are recommended.
b) Prepare references by adding 40 – 400 µl of 0.25 mg/ml bovine serum albumin into 100 mm tubes. Add to each tube protein free buffer to a final volume of 400 µl/tube. Duplicates or triplicates are recommended.
c) Add 400 µL of 2x concentrated Lowry to each tube and mix thoroughly.
d) Incubate at room temperature for 10 minutes.
e) Add 200 µL 0.2 N Folin reagent very quickly, and vortex immediately. Complete mixing of the reagent must be accomplished quickly to avoid decomposition of the reagent before it reacts with protein.
f) Incubate for 30 minutes at room temperature.

g) Read the absorbances at 750 nm. If the absorbances are too high use 500 nm to measure the absorbances. Use glass or polystyrene cuvettes.

7.6.5 Remarks

a) If the 0.2 N Folin reagent is kept in an amber bottle, the solution is stable for several months.
b) It is preferred to prepare each time fresh 2x concentrated Lowry solution out of the three individual solutions. When storage is considered, the 2x concentrated Lowry solution can be used for 2-3 weeks after preparation. White or black precipitation can occur. Warm the solution to 37°C if a white precipitate is formed, and discard if there is a black precipitate.
c) The absorbance measurement has to be performed within 10 minutes after incubation.
d) As with most protein assays, the Lowry can be scaled up for larger volumes, however more protein will be consumed.
e) Proteins with an abnormal high or low percentage of tyrosine, tryptophan, or cysteine residues will give high or low errors, respectively.

7.6.6 Literature

a) Lowry O.H., Rosbrough N.J., Farr A.L. and Randall R.J., Protein measurement with the folin phenol reagent, J. Biol. Chem. 193, 265, 1951
b) http://www.ruf.rice.edu/~bioslabs/methods/protein/lowry.html
c) http://www.serva.de/

7.7 SEN-008: Biotinylation of antibodies with EZ-Link™ NHS-PEO_4-Biotin

7.7.1 Introduction

This protocol describes the biotin labelling of antibodies with EZ-Link™ NHS-PEO_4-Biotin. NHS-PEO_4-Biotin labeling reagent enables simple and efficient biotin labeling of antibodies, proteins and any other primary amine-containing macromolecule. The hydrophilic polyethylene oxide or polyethylene glycol (PEO or PEG) spacer arm of these biotinylation reagents imparts water solubility that is transferred to the biotinylated molecule, thus, reducing aggregation of labeled proteins stored in solution. The PEG spacer arm also gives these reagents a long and flexible connection to minimize steric hindrance involved with binding to large molecules.

7.7.2 Reagents

	CAS number	supplier	product number
Antibody			
NHS-PEO_4-Biotin	n/a	Pierce	21330
Potassium chloride (KCl)	7447-40-7	J.T.Baker	0208
Potassium hydrogen phosphate (KH_2PO_4)	778-77-0	J.T.Baker	0204
Sodium chloride (NaCl)	7647-14-5	J.T.Baker	0278
Sodium hydrogen phosphate dihydrate ($Na_2HPO_4{\cdot}2H_2O$)	10028-24-7	J.T.Baker	0326
Ultrapure water	MilliQ of the Millipore system		

7.7.3 Solutions

a) 20 mM NHS-PEO_4-Biotin solution
   Add 170 µL MilliQ to 2 mg of NHS-PEO_4-Biotin
b) Phosphate Buffered Saline, 10 times concentrated (PBS-buffer 10x)
   80.0 g/L NaCl, 2.0 g/L KCl, 18.05 g/L $Na_2HPO_4{\cdot}2H_2O$ and 2,4 g KH_2PO_4 in MilliQ.
   (pH ≈ 6.8)
c) Phosphate Buffer Saline (PBS-buffer)
   Dilute "PBS-buffer 10x" 1:10 with MilliQ (pH ≈ 7.4)
d) Antibody solution as determined by supplier

7.7.4 Procedure

In order to attach the analyte specific antibody on top of MαBiotine antibody, the analyte specific antibody has to be biotinylated. The biotinylation of the antibody is obtained according to the following procedure:

a) Add 500 µL of a 3.0 mg/mL antibody solution to an ultra filtration unit with a cut-off of maximum 30 KD.
b) Change buffer to PBS according to protocol SEN-006 (Ultra filtration)
c) Add 10 µL of 20 mM NHS-PEO_4-Biotin
d) Incubate at room temperature for 30 minutes
e) Change buffer to PBS according to protocol SEN-006 (Ultra filtration)
f) Store the antibody solution at 4˚C.

7.7.5 Remarks

- NHS-PEO_n-Biotins are moisture sensitive. Store product in original bottle at 4°C with desiccant.
- To avoid moisture condensation onto the product, vial must be equilibrated to room temperature before opening.
- No-Weigh Microtubes: Immediately before use, puncture the microtube foil with a pipette tip, add water and mix by pipetting up and down.
- Avoid buffers containing primary amines (e.g. Tris or glycine) as they compete with the biotin labelling reaction.
- Prepare NHS-PEO_n-Biotins immediately before use. The NHS ester moiety readily hydrolyzes and becomes non-reactive; therefore stock solutions cannot be prepared for storage. Discard any unused reconstituted reagent.

7.7.6 Literature

a) http://en.wikipedia.org/wiki/Phosphate_buffered_saline
b) http://www.piercenet.com/Objects/View.cfm?type=ProductFamily&ID=01030914
c) http://www.piercenet.com/files/1299dh5.pdf
d) Altin, J.G. and Pagler, E.B., A one-step procedure for biotinylation and chemical crosslinking of lymphocyte surface and intracellular membrane-associated molecules. Anal. Biochem. 224, Issue 1, 382-389, 1995
e) Gretch, D.R., Suter M. and Stinski M. F., The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpes virus hydrophobic proteins or glycoproteins. Anal. Biochem. 163, 270-277, 1987
f) Manning, J., Pellegrini M., Davidson N., A method for gene enrichment based on the avidin-biotin interaction. Application to the Drosophila ribosomal RNA genes. Biochemistry. 16, 1364-70, 1977
g) Updyke, T.V. and Nicolson, G.L., Immunoaffinity isolation of membrane antigens with biotinylated monoclonal antibodies and immobilized streptavidin matrices. J. Immunol. Meth. 73, 83-95, 1984

7.8 SEN-008 supp: Biotinylation of antibodies with EZ-Link™ NHS-PEO_4-Biotin, supplement

7.8.1 Structure of the PEG-spacer containing biotin labeling reagents, NHS-PEO_4-Biotin.

NHS-PEO_4-Biotin labeling reagent enables simple and efficient biotin labeling of antibodies, proteins and any other primary amine-containing macromolecule. The hydrophilic polyethylene oxide or polyethylene glycol (PEO or PEG) spacer arm of this biotinylation reagent imparts water solubility that is transferred to the biotinylated molecule, thus, reducing aggregation of labeled proteins stored in solution. The PEG spacer arm also gives these reagents a long and flexible connection to minimize steric hindrance involved with binding to avidin molecules.

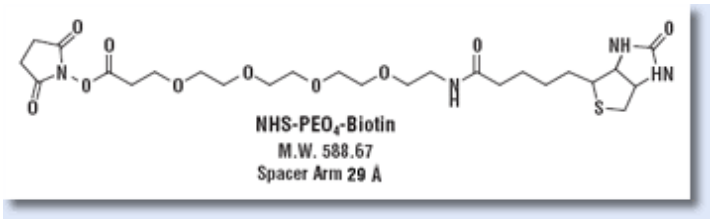

Figure 29: Chemical structure of NHS-PEO_4-Biotin

Cell surface biotinylation has emerged as an important tool for studying the expression and regulation of receptors and transporters, differentiation of plasma membrane proteins from those localized to organelle membranes, and distribution of membrane proteins in polarized epithelial cells. Because these PEG-containing molecules dissolve readily in polar solutions, they do not permeate the cell membrane. As long as the cell remains intact, only primary amines exposed on the surface will be biotinylated with PEG-Biotin reagents.

7.8.2 Reaction of NHS-PEG_n-Biotins with a primary amine-containing protein.

The *N*-hydroxysuccinimide (NHS) ester group on this reagent reacts with the ε-amine of lysine residues and the α-amine at the N-terminus of each peptide to produce a stable product. NHS esters react with deprotonated primary amines, therefore, the reaction requires neutral to basic pH values to proceed. Primary amines react with NHS esters by nucleophilic attack and NHS is released as a byproduct. Hydrolysis of the NHS-ester competes with the reaction in aqueous solution and increases with increasing pH.

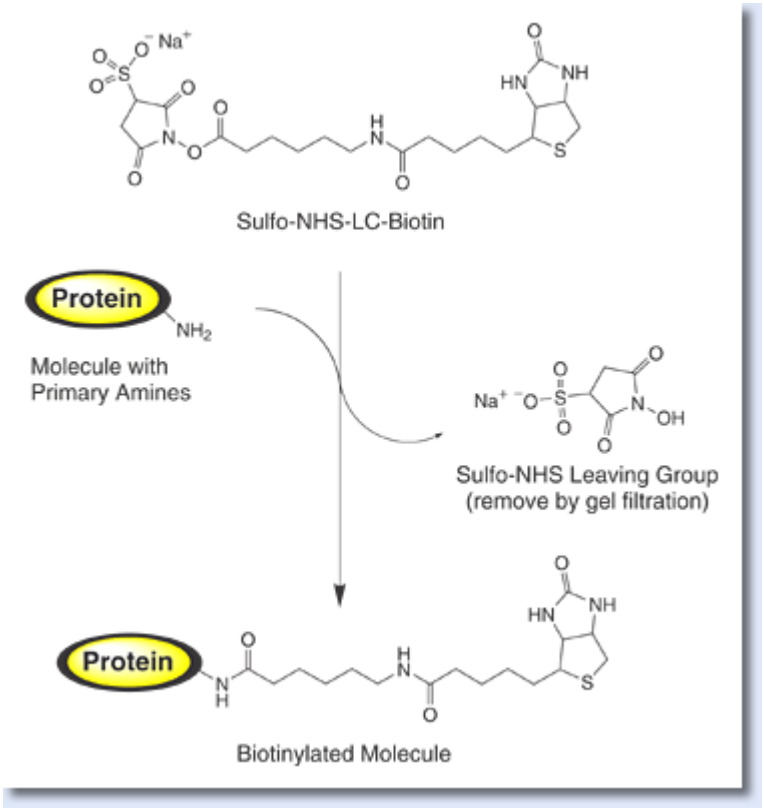

Figure 30: Reaction scheme of a similar Sulfo-NHS-LC-Biotin

There is considerable flexibility in conditions used for conjugating NHS-PEO_n-Biotins to a protein or peptide. NHS ester reactions can proceed at temperatures of 4-37°C, reaction mixture pH values of 7-9, and at incubation times from a few minutes to overnight. A particular set of conditions will result in different degrees of biotinylation optimal for a given application. Because of protein variability, a particular set of conjugation conditions that is optimal for one protein may not yield optimal results when applied to a different protein.

7.8.3 NHS-PEO_n-Biotin Reagent Specifications

Pierce manufactures biotinylation agents to the following specifications

Table 4: Properties of NHS-PEO_4-Biotin

Properties of NHS-PEO_4-Biotin Labeling Reagent	
Alternate names	NHS-PEG_4-Biotin, NHS-dPEG_4-Biotin
Molecular formula	$C_{25}H_{40}O_{10}N_4S$
Molecular weight	588.67
Spacer arm length	29 Å
Mass added to target	474.59 daltons
Storage conditions	4°C with desiccant, protect from moisture, use only fresh solutions
Reactive groups	NHS ester, reacts with primary amines at pH 7.0-9.0
Spacer arm composition	polyethyleneoxide (PEO), 4 ethyleneoxide subunits

7.8.4 Literature

a) http://en.wikipedia.org/wiki/Phosphate_buffered_saline
b) http://www.piercenet.com/Products/Browse.cfm?fldID=02030212
c) Altin, J.G. and Pagler, E.B., A one-step procedure for biotinylation and chemical crosslinking of lymphocyte surface and intracellular membrane-associated molecules. Anal. Biochem. 224, Issue 1, 382-389, 1995
d) Gretch, D.R., Suter M. and Stinski M. F., The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpes virus hydrophobic proteins or glycoproteins. Anal. Biochem. 163, 270-277, 1987
e) Luo Y., Vassilev P.M., Li X., Kawanabe Y. and Zhou J., Native Polycystin 2 Functions as a Plasma Membrane Ca2+-Permeable Cation Channel in Renal Epithelia, Molecular and Cellular Biology, Vol. 23, No. 7, 2600-2607,April 2003
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7.9 SEN-010: Antibody immobilisation on S_A-protein monolayers with EDC-NHS

7.9.1 Introduction

This protocol describes the antibody immobilization on S_A-protein monolayers with N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS).
NHS esters react with primary amines at pH 7-9 to form stable amide bonds, along with release of the N-hydroxysulfosuccinimide leaving group. Proteins, including antibodies, generally have several primary amines in the side chain of lysine (K) residues and the N-terminus of each polypeptide that are available as targets for NHS-ester cross-linking reagents.

7.9.2 Reagents

	CAS number	supplier	product number
2-(4-Morpholino)ethanesulfonic acid monohydrate (MES·H_2O)	145224-94-8	Fluka	69892
6-Aminohexanoic acid	60-32-2	Fluka	07260
Antibody			
Ethanolamine hydrochloride	2002-24-6	Sigma	E6133
N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)	25952-53-8	Sigma	E7750
N-Hydroxysuccinimide (NHS)	6066-82-6	Aldrich	130672
Potassium Chloride (KCl)	7447-40-7	J.T. Baker	0208
Sodium Azide (NaN_3)	26628-22-8	J.T. Baker	9099
Sodium Chloride (NaCl)	7647-14-5	J.T. Baker	0278
Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	10028-24-7	J.T. Baker	0326
Sodium hydroxide (NaOH)	1310-73-2	J.T.Baker	0288
Sodium phosphatemonobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$)	13472-35-0	Acros	271750010
Tween 20	9005-64-5	Merck	8.22184
Ultra pure water	MilliQ of the Millipore system		

7.9.3 Solutions

a) 1 M NaOH solution
   Prepare a solution of 40 g/L in MilliQ
b) MES-buffer pH = 4.5
   Prepare a 10 mM solution of MES·H_2O in MilliQ. Adjust pH to 4.5 with 1 M NaOH.
c) 400 mM EDC solution
   Prepare a solution of 77 g/L in MES-buffer pH=4.5
d) 100 mM NHS solution
   Prepare a solution of 11.5 g/L in MES-buffer pH=4.5
e) EDC-NHS solution
   Add 200 µl EDC solution to 100 µl NHS solution
f) MES-buffer pH = 6.5
   Prepare a 10 mM solution of MES·H_2O in MilliQ. Adjust pH to 6.5 with 1 M NaOH.
g) 6-aminohexanoic acid solution
   Prepare a solution of 50 g/L in MES buffer pH = 6.5
h) Antibody solution
   prepare a solution of 50 µg/mL antibody solution in MES buffer pH=6.5
i) 1 M ethanolamine hydrochloride solution
   prepare a solution of 97.5 g/L in MilliQ
j) Phosphate Buffered Saline with Tween 20, 10 times concentrated (PBS-buffer Tween10x)
   80 g/L NaCl, 2.0 g/L KCl, 18.05 g/L $Na_2HPO_4 \cdot 2H_2O$, 2.75 g/L $NaH_2PO_4 \cdot 2H_2O$ and 0.05% V/V Tween 20 in MilliQ.

k) Phosphate Buffered Saline with Tween 20 (PBS Tween buffer)
   Dilute "PBS-buffer Tween 10x" 1:10 with MilliQ
l) Phosphate Buffered Saline with Tween 20 and Sodium Azide (PBS Tween Azide buffer)
   Dilute "PBS-buffer Tween 10x" 1:10 with MilliQ, while adding 0,05% M/V NaN_3.

7.9.4 Procedure

When a S_A-protein monolayer has been formed on a surface (see protocol SEN-003) the immobilisation of the antibody is obtained according to the following procedure:

a) Wash 3 times with 10 mM MES-buffer pH = 4.5
b) Incubate for 10 minutes with an EDC-NHS solution at room temperature.
c) Remove the EDC-NHS solution
d) Incubate for 10 minutes with 50 g/L 6-aminohexanoic acid solution
e) Remove the 6-aminohexanoic acid solution
f) Wash 3 times with 10 mM MES-buffer pH = 4.5
g) Incubate for 10 minutes with an EDC-NHS solution at room temperature.
h) Remove the EDC-NHS solution
i) Incubate for 10 minutes with of 50 µg/mL antibody solution
j) Remove the antibody solution
k) Incubate with 1 M ethanolamine hydrochloride solution for 10 minutes at room temperature
l) Remove the ethanolamine hydrochloride solution
m) Wash three times with MES buffer pH = 6.5
n) Observe signal of MES buffer pH = 6.5 versus antibody solution
o) Wash three times with PBS Tween buffer
p) Store with PBS Tween Azide buffer

7.9.5 Remarks

- Mixture of EDC and NHS has to be freshly prepared.
  EDC is easily hydrolysed by water effecting in a reduction of the reactivity of EDC. After thawing or preparation of the solution add immediately to the cuvet.
- Stock solutions of EDC or NHS can be prepared beforehand and have to be stored in the freezer at -20 °C.
- Immobilise each well, one at the time, to observe the change in signal due to changes on the sensor surface.

7.9.6 Literature

a) Stigter E.C.A., Jong G.J. de, Bennekom W.P. van, An improved coating for the isolation and quantitation of interferon-γ in spiked plasma using surface plasmon resonance (SPR). Biosensors and Bioelectronics 21, 474-482, 2005

7.10 SEN-015: Atrazine measurement on MZI

7.10.1 Introduction

This protocol describes the measurement sequences for the direct detection of atrazine with a Mach Zehnder Interferometer (MZI) based optochemical sensor. This protocol is a guideline describing which chemical solutions can be used and which sequential steps are needed to perform the atrazine analyses. The exact order of steps will be determined by the researcher and will be based on the results obtained from previous experiments.

7.10.2 Reagents

	CAS number	supplier	product number
Acetonitrile	75-05-8	Boom	61009017.2500
Ethylene glycol, anhydrous, 99.8%	107-21-1	Sigma	324558
Glycine	56-40-6	Sigma	G7126
Hydrochloric acid (HCl) (30%)	7647-01-0	Merck	1.00313
Magnesium Chloride ($MgCl_2.6H_2O$)	7786-30-3	J.T.Baker	0162
Methanol	67-56-1	Boom	61008402.0001
Potassium Chloride (KCl)	7447-40-7	J.T. Baker	0208
Sodium Azide (NaN_3)	26628-22-8	J.T. Baker	9099
Sodium Chloride (NaCl)	7647-14-5	J.T. Baker	0278
Sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	10028-24-7	J.T. Baker	0326
Sodium hydroxide (NaOH)	1310-73-2	J.T.Baker	0288
Sodium phosphatemonobasic dihydrate ($NaH_2PO_4 \cdot 2H_2O$)	13472-35-0	Acros	271750010
Tween 20	9005-64-5	Merck	8.22184
Ultra pure water	MilliQ of the Millipore system		

7.10.3 Solutions

a) Phosphate Buffered Saline with Tween 20, 10 times concentrated (PBS-buffer Tween10x)
   80 g/L NaCl, 2.0 g/L KCl, 18.05 g/L $Na_2HPO_4 \cdot 2H_2O$, 2,75 g/L $NaH_2PO_4 \cdot 2H_2O$ and 0,05% V/V Tween 20 in MilliQ.
b) Phosphate Buffered Saline with Tween 20 (PBS Tween buffer)
   Dilute "PBS-buffer Tween 10x" 1:10 with MilliQ
c) Phosphate Buffered Saline with Tween 20 and Sodium Azide (PBS Tween Azide buffer)
   Dilute "PBS-buffer Tween 10x" 1:10 with MilliQ, while adding 0,05% m/v NaN_3.
d) 4M $MgCl_2$
   Prepare a solution of 813.2 g/L
e) 10 – 1000 mM glycine, pH 1.5
   Prepare a solution of 75.07 g/L and adjust the pH with HCl (1M). Dilute this solution x times with MQ or buffer solution to obtain the concentration needed
f) 0.1 – 10% v/v Acetonitrile
   Dilute acetonitrile 10 to 1000 times with MQ or buffer solution
g) 0.1 – 10% v/v Methanol
   Dilute methanol 10 to 1000 times with MQ or buffer solution
h) 1 M NaOH solution
   Prepare a solution of 40 g/L in MilliQ
i) 0.1 – 50% v/v ethylene glycol
   Dilute ethylene glycol 2 to 1000 times with MQ or buffer solution
j) 1M HCl
   Dilute 10 times 30% HCl

7.10.4 Procedure

When an antibody has been immobilised on the sensor surface (See protocol SEN-004) the analyses of atrazine is following the next procedure:

a) Fill the fluidic system with running buffer (PBS)
b) Start data acquisition software
c) Wait until the baseline of the sensorgram is stabilised
d) Fill the sample loop with ShαAtr (optional)
e) Inject the ShαAtr (optional)
f) Incubate the ShαAtr for a fixed time period (if necessary, adjust duration based on previous results) (optional)
g) Stop injection (optional)
h) Wait for a fixed time period (if necessary, adjust duration based on previous results) (optional)
i) Fill the sample loop with sample
j) Inject the sample
k) Incubate the sample for a fixed time period (if necessary, adjust duration based on previous results)
l) Stop injection
m) Wait for a fixed time period (if necessary, adjust duration based on previous results)
n) Fill the sample loop with regeneration buffer
o) Inject the regeneration buffer
p) Incubate the regeneration buffer for a fixed time period (if necessary, adjust duration based on previous results)
q) Stop injection
r) Wait for a fixed time period (if necessary, adjust duration based on previous results)
s) Stop data acquisition software
t) Sequentially repeat steps "b until s" for sequential samples

7.10.5 Remarks

- The biochemistry of the sensor coating need some time to stabilise before measurements can start. Wait until baseline is stable in angle (drifting) and noise.
- The steps "d" until "h" are optional for in the case a two-step-rocket principle is used for the immobilisation of the antibody against atrazine
- Make use of an injection valve with sample loop to define the volume of the sample. This increase the repeatability of the experiments.
- The waiting times from "c", "h", "m" and "r" are necessary to determine the differences in signal levels. Signal changes only can be determined when in the fluidic system the same running buffer is in place. The refractive index difference in these cases is only caused by the bound mass to the sensor surface.
- The composition of the running buffer can be adjusted to the needs of the biochemical system tested. Adjustments can be made in salt type and concentration and pH.
- The composition of the regeneration buffer can be adjusted to the needs of the biochemical system tested. The antibody – antigen interaction differs from case to case. Therefore there is a need for different regeneration buffers. Adjustments can be made in salt type and concentration, pH and mixtures of salts and organic solvents. Be aware of the fact that the compatibility with organic solvent is not always good. "High" concentrations of organic solvent may give rise of inactivation of the antibody recognition.

7.10.6 Literature

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