Expression of a transmembrane protein in a multi-well plate format

Diploma thesis

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1 Introduction

1.1 The project

G-protein coupled receptors (GPCRs) are membrane proteins with seven transmembrane segments. They are the largest protein family known; they participate in a wide range of biological processes and are thus involved in many pathological conditions. They are target of 50 to 60 % modern drugs [1].

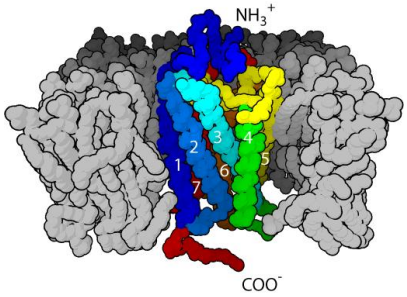

Figure 1: The seven transmembrane α-helical structure of a G protein-coupled receptor

Investigation of GPCRs is difficult, because their isolation and purification is arduous [2]. Instead of trying to isolate the functional receptor, we used the so-called *in vitro* approach: it was shown that it is possible to functionalize an artificial membrane by combining coding DNA with the protein synthesis machinery of a cell extract (TNT Promega). This way, the receptor can be synthesized into the artificial membrane (tBLM) in a functional form. The incorporation process is vectorial, meaning that the N-terminus is on the side of the membrane where the cell extract was added, whereas the C-terminus is located in the submembrane space [3].
This approach allows fast functionalization of the artificial membranes (approximately 90 min.). It is also easily possible to introduce another protein, only the corresponding coding DNA is needed (instead of having to develop a whole new purification and isolation protocol). Our approach is therefore suitable as a general method for the implementation of a biosensor.

The *in vitro* functionalization of an artificial membrane was already achieved in the flow-cell of a SPR setup. We would like to be able to express several proteins in parallel in a sort of "protein chip" approach. The 384 well plate was chosen because its small volume (ca. 100 μl) ensured minimal dilution. Also, standard sensing methods, as fluorescence, could easily be applied. This system would enable the simultaneous testing of different membrane proteins and/or of different ligands.

1.2 Biosensors

To detect receptors in their natural matrix, the phospholipid membrane, we need to combine the biological component with a physical detector system. For this, an intercessory component combining these two elements is used to obtain a biosensor.
The functional expression of one of these membrane based receptors, an olfactory receptor, has already been monitored by surfaces plasmon based techniques, like SPR. In this case, the biosensoric element consists of an artificial membrane that is attached to a gold surface. To analyze the sensor surface as well with a highly sensitive fluorescent component (in case of SPFS measurements) the sensoric platform is furthermore combined with a specific antibody sandwich system targeting the protein under investigation. Despite the fact that by the use of SPR/SPFS the general applicability of this biosensoric platform for membrane proteins could be proven, these techniques are time consuming and allow only for a "one dimensional" analysis: within a single run only one receptor species or one interacting ligand can be analyzed with this biosensoric setup.
We therefore want to implement a multidimensional biosensor [4] and have chosen a multi-well plate setup with fluorescence detection. In this case, a membrane is assembled in each well and expression of the membrane protein is carried out independently. A fluorescence detector then probes each well individually and quickly (residence time of 1-3 s). This technology would be very interesting for drug screening in the pharmaceutical industry. The main advantages of this setup would be speed, low cost and ability to assay several receptors and/or several ligands in parallel.

1.3 Olfactory Receptors

The olfactory gene family comprises approximately 1,000 different genes (three per cent of our genome) and gives rise to an equivalent number of

olfactory receptor types [5-7]. These receptors are located on the olfactory receptor neurons, which occupy a small area in the upper part of the nasal epithelium and detect the inhaled odorant molecules.
Studies in both insects and mammals suggest that each olfactory receptor cell possesses only one type of odorant receptor [8], and each receptor can detect a limited number of odorant substances. Our olfactory receptor cells are therefore highly specialized for a few odors. They send the information through their axons to the olfactory bulb, the first site for the processing of olfactory information. Most odors are composed of multiple odorant molecules, and each odorant molecule activates several odorant receptors. This leads to a combinatorial code forming an "odorant pattern" -This is the basis for our ability to recognize and form memories of approximately 10,000 different odors [9].
Olfactory receptors are G protein-coupled proteins with seven-transmembrane segments [10]. When an odorant receptor is activated by an odorous substance, an electric signal is triggered in the olfactory receptor cell and sent to the brain via neuronal processes. Each odorant receptor first activates a G protein, to which it is coupled. The G protein in turn stimulates the formation of cAMP (cyclic adenosine monophosphate). This messenger molecule activates ion channels, which are opened: a signal cascade is initiated, resulting in ion flux. The cell then sends small nerve impulses directly to distinct micro domains in the olfactory bulb, the so-called glomeruli. Olfactory cells carrying the same type of receptor send their nerve processes to the same glomerulus. From these micro domains in the olfactory bulb the information is relayed further to other parts of the brain, where the information from several olfactory receptors is combined, forming a pattern [11].

1.4 Tethered Bilayer Lipid Membrane

The lipid bilayer of cellular membranes represent the natural environment of membrane proteins like olfactory receptors. However, in order to be able to monitor specific receptor properties, the complexity of the biomembrane has to be reduced. This is achieved by Tethered bilayer lipid membranes (tBLMs) system [12, 13], which mimic the biological cytoplasma membrane. Just as natural membranes, they preserve the native structure and the functional integrity of integral membrane proteins. These tethered bilayers can be probed by Surface Plasmon Resonance (SPR), by Surface Plasmon Fluorescence Spectroscopy (SPFS), by resonant mirror and waveguide techniques as well as by electrochemistry [14].
Tethered bilayer lipid membranes (tBLMs) are a model membrane system

for the incorporation of proteins (see fig. 2).

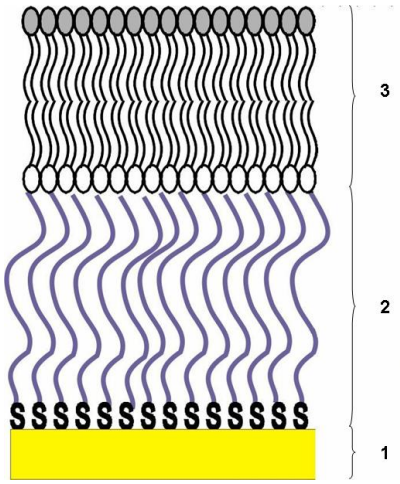

Figure 2: The tethered bilayer lipid membrane

They are constituted by (1) a solid substrate, (2) a tethering layer and (3) the lipid bilayer [15]. For these experiments, we used a gold film as substrate and a peptide, namely P19, as spacer between the surface and the membrane. The covalent attachment to a solid support ensures stability over a long period of time. The peptide spacer avoids interaction of the membrane with the surface; furthermore, it establishes a hydrophilic submembrane space and ensures sufficient space for protein insertion.
The tBLM was built up by a sequential layer-by-layer assembly of the constituents. The spacer peptide P19 has a N-terminal Cystein which attaches covalently to the gold surface. The C-terminal carboxy-group was activated by addition of N-ethyl-N'-(dimethylaminopropyl)carbodiimid (EDC) and N-hydroxysuccinimid (NHS, see fig. 3). Dimyristoylphosphatidylethanolamin (DMPE) was then added for the first lipid layer. By fusion of Phosphatidylcholin (PC) vesicles, the bilayer was completed.

1.5 Surface Plasmon Resonance Spectroscopy

Surface plasmons are surface electromagnetic waves that propagate along the interface of two materials of different optical properties, e.g. at the boundary between a thin noble metal film (with a negative dielectric constant) and a glass slide (with a positive dielectric constant) [17]. Surface plasmons are bound to regions in the material where the optical properties reverse: they

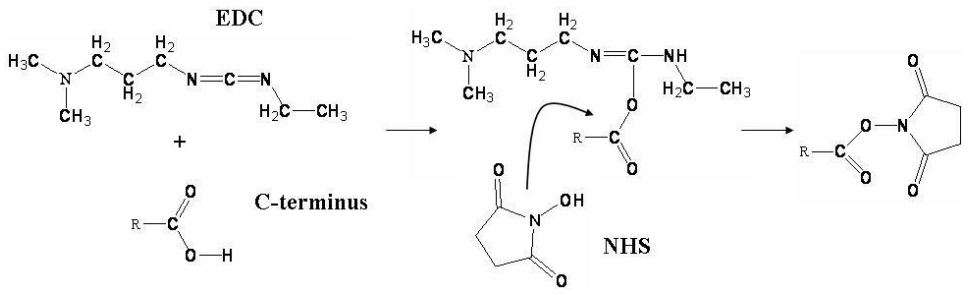

Figure 3: NHS-EDC Reaction [16]

decay exponentially into the media adjacent to the interface.
A plasmon is excited by light when it strikes the metal film at the resonance angle. At that angle, the free electrons on the surface absorb the energy of the photons and start to oscillate, producing a surface plasmon. The position and the width of this 'resonance point' are very sensitive to the properties of the surface and the media next to it. It makes it possible to use surface plasmon resonance techniques for chemical and biological sensing.
In the Kretschmann configuration (see fig. 4), the beam is reflected off the boundary between the optically dense medium (a prism) and the metallic layer. This enables us to monitor the plasmons.

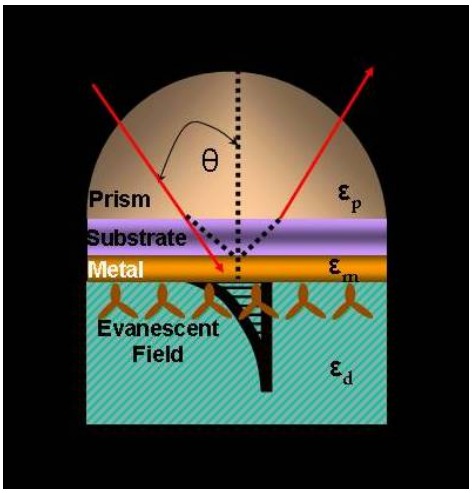

Figure 4: The Kretschmann configuration

1.6 Surface Plasmon Resonance Fluorescence Spectroscopy

In order to detect antibody binding, fluorescence measurement is coupled to SPR. When a surface plasmon is excited, the evanescent field on the surface is amplified. Dye molecules bound to this surface are therefore excited. The evanescent field decays exponentially perpendicular to the surface; thus, the excitation of dye molecules in the bulk solution is minimal. By detection of fluorescence emission the sensitivity of the system is improved by at least one order of magnitude.
The measurements were carried out in the experimental setup shown in fig. 5. The setup was the same as for SPR, with addition of the photomultiplier.

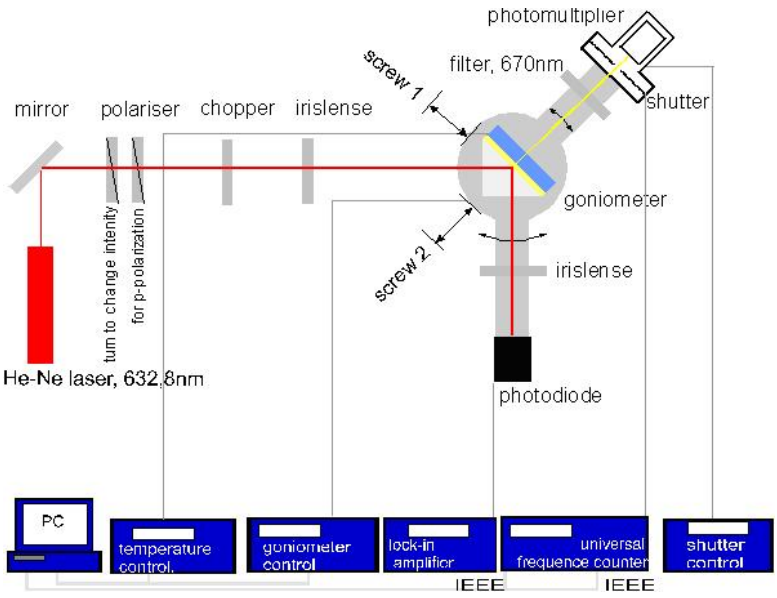

Figure 5: SPR / SPRF setup

2 Materials and Methods

2.1 *In vitro* Expression System

For the *in vitro* experiment, the final concentration range of the cDNA should be between 0.5 and 2 $\mu g/\mu l$, the ideal concentration being 1 $\mu g/\mu l$. I used

the plasmid pTNT-VSV-OR5 3-4 (3842 bp, Amp r, f1 orb) as a genetic information for the olfactory receptor OR5 from *rattus Norwegians*, with a N-terminal VS (Vesicular Stomatitis Virus) tag (see fig. 6).

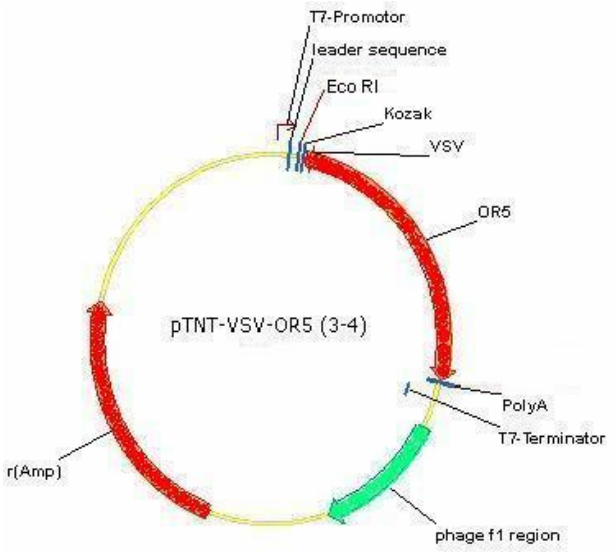

Figure 6: plasmid pTNT-VS-OR5 3-4

The TNT Quick Coupled Transcription/Translation Samples were prepared according to the procedure described in 2.2, then incubated 90 minutes at 32 °C. 5 μl of these solutions were taken to prepare the SDS samples (see 2.1.2) and loaded on the SODS-gel after 10 min incubation at 70 °C .
If the expression was done on a membrane, the well was washed extensively with PBS. It was the incubated with 50 $\mu g/\mu l$ of 1 ° antibody (mouse anti-VS) for 30 min. After washing, the background fluorescence measurement was performed. The 2 ° antibody (fluorescein labelled anti-mouse) was then added and incubated for 30 min. After washing, the final fluorescence measurement was performed [19].

TNT Quick Coupled Transcription/Translation System

TNT Quick Master Mix	$40\mu l$
Methionine, 1mM	1 μl
cDNA	2 μl
Nuclease-Free Water to a final volume of	50 μl

The TNT Quick Master Mix contains everything (RNA polymerase, nucleotides, salts etc.) necessary for *in vitro* transcription and translation [19].

Components for membrane assembly

P19	CSRARKQAASIKVAVSADR Sigma, Cat.-No. C-6171
EDC	N-ethyl-N'-(dimethylaminopropyl)carbodiimid Fluka, Cat.-No. 03449
NHS	N-hydroxysuccinimid Aldrich, Cat.-No. 130672
DMPE	Dimyristoylphosphatidylethanolamin Sigma, Cat.-No. P-5693
PBS	Phosphate Buffered Saline PAA Laboratories GmbH,Cat.-No. H15-002
PC	Phosphatidylcholin Fluka, Cat.-No. 61757

2.2 SDS Polyacrylamide Gel Electrophoresis

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a commonly used technique to separate proteins according to their size. With the anionic detergent SDS, each protein is denatured and acquires a negative charge proportional to its size. The intrinsic charge some proteins might have is negligible compared to the large acquired charge. In an applied electric field, proteins travel towards the anode according to their molecular mass: the lighter the protein, the faster its migration towards the anode.
Two markers were added to the SDS gel: 7 μl of Seeblue Plus 2 (the stained bands allow to check the efficiency of the separation) and 3 μl of Magic Mark XP (which has recognition sites for antibodies of rabbit, goat and mouse and can be detected using the same chemiluminescent substrate as for the rest of the blot). Both markers contain a protein ladder and are therefore indicators of protein size. 500 μl Antioxidant were added into the anode chamber, before running the gel at 200 V for approximately 45 min.
A positive control was added to every gel.
NuPAGE 10 % Bis-Tris Gels were used. For the running buffer, 40μl NuPAGE MES SDS running buffer (20 x) was diluted in 760μl MilliQ water.

SDS Samples and Gel [20]

MilliQ Water	5.4 μl
LDS Buffer	4 μl
in vitro Expression Sample	5 μl
Sample Reducing Agent	1.6 μl

LDS Buffer contains SDS to denature the proteins. The Sample Reducing Agent contains mercaptoethanol to reduce disulfide bonds and ensure a regular running behavior.

2.3 Western Blot and chemiluminescence detection

Western Blotting is a commonly used method to transfer proteins from a polyacrylamide gel onto a membrane (in our case, a polyvinylidene difluoride (PVDF) membrane).This ensures immobilization of the proteins, which can be detected after incubation with the primary and secondary antibodies [21, 22].
The PVDF membrane was pre-wet for 30 seconds in methanol, then rinsed in MilliQ water and soaked in transfer buffer until use. The blotting pads were soaked in transfer buffer and air bubbles eliminated, because they could cause irregular transfer. The XCell II Blot Module was assembled (see fig. 7).

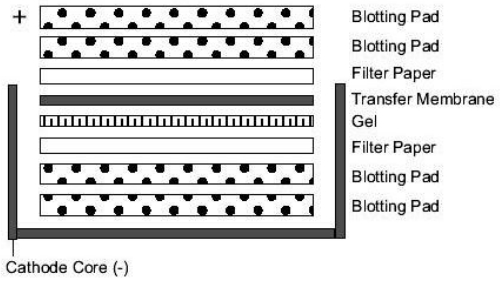

Figure 7: XCell II Blot Module

The transfer was performed for 1 hour at 30 V.
After the transfer, the membrane was washed twice in 20 ml MilliQ water, then placed on a shaker in 10 ml Blocking Solution (2.1.4), for 30 min to 1 hour. It was rinsed twice in 20 ml of water, for 5 min. After incubation of the membrane in 10 ml Primary Antibody Solution (2.1.5) for 1 hour, it was washed four times 5 min in Antibody Wash (2.1.6). The 30 min incubation in 10 ml Secondary Antibody Solution was followed by the same washing

steps. The membrane was then rinsed three times for 2 min in 20 ml water and placed on a sheet of transparency plastic. 2.5 ml of Chemiluminescent Substrate (2.1.7) was applied to the membrane. After developing the reaction for 5 min, the membrane was covered with another piece of transparency plastic and exposed to a high sensitivity color CCD. Images were taken in an interval of 3 min under an incrementation modus.

Western Blot Transfer Buffer

NuPage Transfer Buffer ($20x$)	50 ml
NuPage Antioxidant	1 ml
Methanol	100 ml
MilliQ Water	849 ml

Blocking Solution

MilliQ Water	5 ml
Blocker/Diluent (Part A)	2 ml
Blocker/Diluent (Part B)	3 ml

The Blocker/Diluent (Part A) is a concentrated buffered saline solution containing detergent. The Blocker/Diluent (Part B) is a concentrated Hammerstein casein solution.

Primary Antibody Solution

MilliQ Water	7 ml
Blocker/Diluent (Part A)	2 ml
Blocker/Diluent (Part B)	1 ml

Antibody Wash Solution

MilliQ Water	150 ml
Antibody Wash Solution ($16x$)	10 ml

The Antibody Wash Solution is a concentrated buffered saline solution containing detergent.

Chemiluminescent Substrate

The Chemiluminescent Substrate is a ready-to-use solution of CDP-Star chemiluminescent substrate for alkaline phosphatase.

2.4 Pipetting robot

In order for the experiments to be reproducible, a pipetting robot was built (see fig. 8).

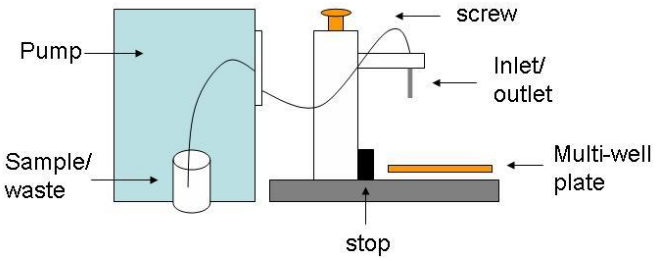

Figure 8: Side view of the robot and the pump.

The arm holding the tubes can be moved up and down by the screw on top. It holds two pump tubes (inner diameter: 0.76 mm), for sample and waste; both are connected to the same pump, the solution is therefore pumped in and out at the same speed. For the same reason, the surface of the liquid is exactly at the same height as the tip of the outlet. A 'stop' was fixed under the mobile arm so that it would stop at the height at which the outlet left about 50 μl in the well (see red arrow in fig. 9). This ensures that the membrane is covered at all time (contact with air would destroy it). Also, that way the bottom of the well cannot be touched by the inlet and the membrane cannot be damaged.

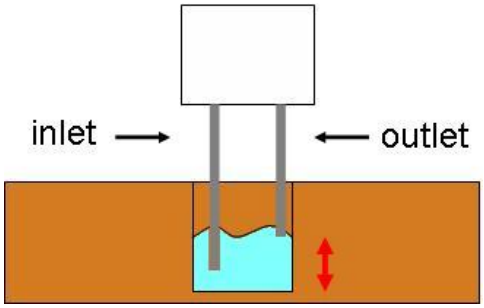

Figure 9: Front view of the robot and a well. The red arrow shows the height of the liquid when a volume of 50 μl is reached in the well.

2.5 Surface Plasmon Spectroscopy

2.5.1 Setup

Surface plasmons are surface electromagnetic fields and a direct access to the surface carrying the SP's is necessary. This can be achieved in the Kretschmann configuration (see fig. 4), in which the plasmons propagate in a thin metal film, evaporated on a high refraction index glass slide. This glass slide is coupled to a prism by immersion oil.

2.5.2 Scan measurement

In the Surface Plasmon Spectroscopy (SPS) setup, the reflectivity R is monitored as a function of the incident angle. At the resonance angle, light energy is transferred to the surface plasmons: the reflectivity drops almost to zero. When binding to the surface occurs, the refractive index of the surface changes; this shifts the plasmon curve. At the resonance angle, any change in the width of the surface or the index of refraction in the media or the surface can be sensitively monitored by the reflectivity (see fig. 10)
In short, SPS allows to detect the presence of molecules bound to a surface. If the refractive index of a layer is known, it is possible to calculate its thickness.

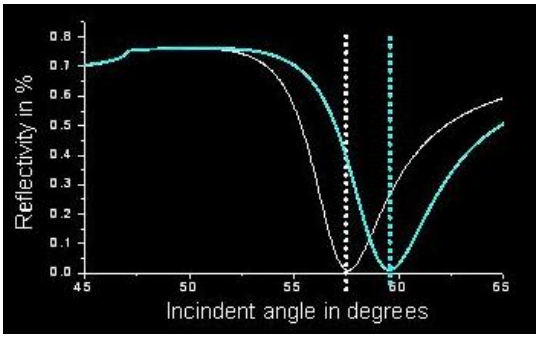

Figure 10: The white curve shows the reflectivity of a surface in a certain environment. For the blue curve, the refractive index of the media or the surface has changed, resulting in a shift of the total reflectivity angle.

2.5.3 Kinetic measurement

In a kinetic measurement, the incident angle is held constant. An angle between the edge of total reflection and the minimum of reflectivity is chosen: in this area, any change in the layer thickness or refractive index can be monitored with a high sensitivity. This enables us for example to follow in real time the assembly of a bilayer membrane [23].

2.6 Artificial membrane set-up

The gold surface is incubated 30 min with the linker peptide P19. The N-terminal Cystein attaches covalently to the gold surface and establishes a monomolecular layer in a self-assembly process. Subsequent washing with MilliPore water avoids any double layer of P19. The C-terminal carboxy-group is then activated by incubation in an EDC/NHS mixture (see fig. 3) to yield a reactive succinimid ester. After 10 min, DMPE molecules are added and attached covalently to the activated carboxyl group with their free amino group.
Next, 300 to 400 ml of PC are extruded 21 times over a polycarbonate membrane to generate PC vesicles with a diameter of 50 nm. This solution is loaded into the cell and incubated for 90 min. The vesicles spread over the DMPE layer, generating a heterogeneous lipid bilayer. The membrane is then washed in PBS for 30 min.

3 Results

3.1 Membrane set-up

The first assembly of a membrane was continuously monitored by SPR. The left part of fig. 11 shows a scan measurement of a free gold surface. This allows us to check the quality of the gold surface and determine its thickness. The right side shows the same surface, covered with water. This was done as a reference measurement to determine the angle at which the reflectivity is 30 %. Note that the change in refractive index on the gold surface already shifts the minimum reflectivity by 31° !

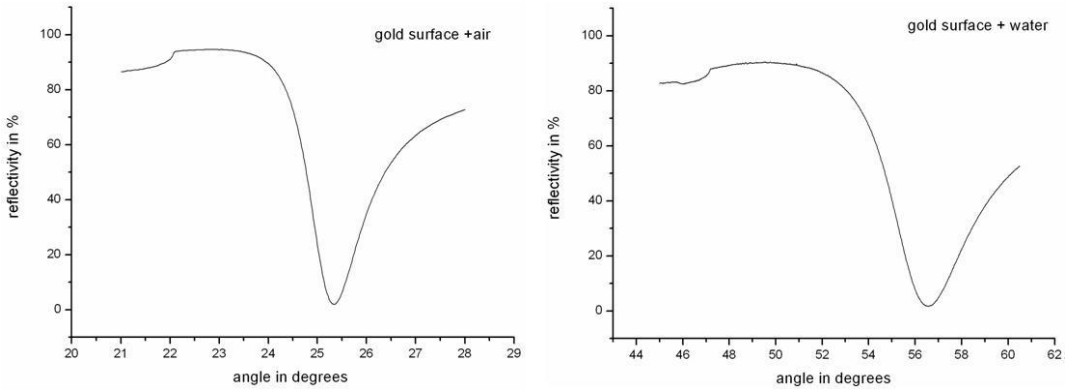

Figure 11: Scan measurement of the gold surface in air and covered with water

The monolayer was then built up and monitored by a kinetic measurement (see fig. 12). The surface was first incubated with P19, then rinsed at t=35 min. NHS and EDC were added at t=53 min. 10 min later, DMPE was added and the monolayer was built up. It was then rinsed at t=100 min.

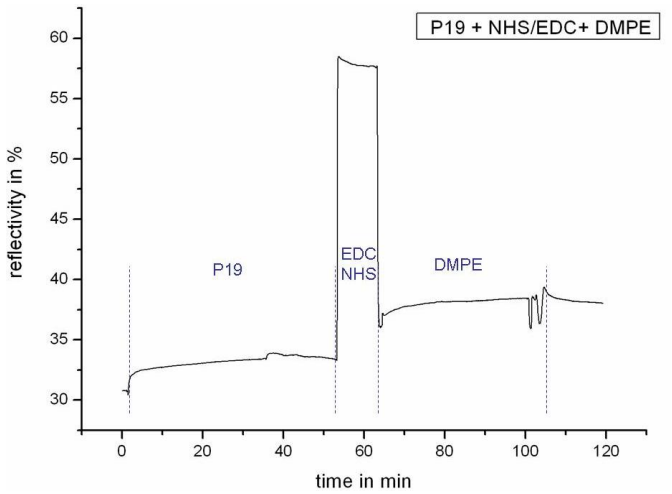

Figure 12: Assembly of the monolayer

The quality of this monolayer was then checked by a scan measurement (see fig. 13).

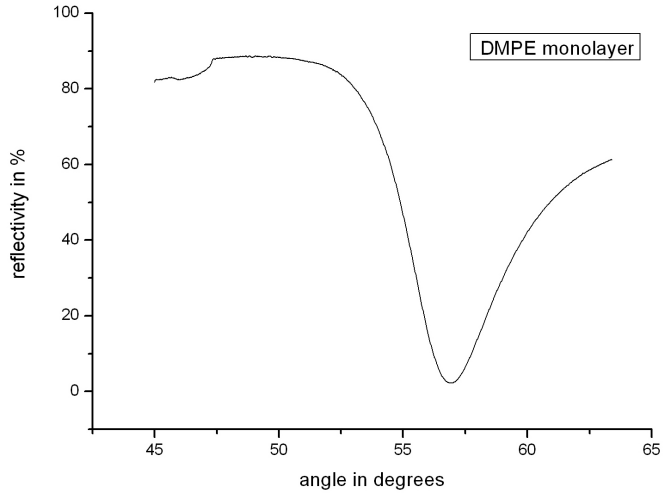

Figure 13: DMPE monolayer

Finally, the monolayer was incubated with PC vesicles (see fig. 14). The finished tBLM was rinsed with PBS (at t=35 min), which washes away the excess vesicles but retains the functional form of the bilayer.

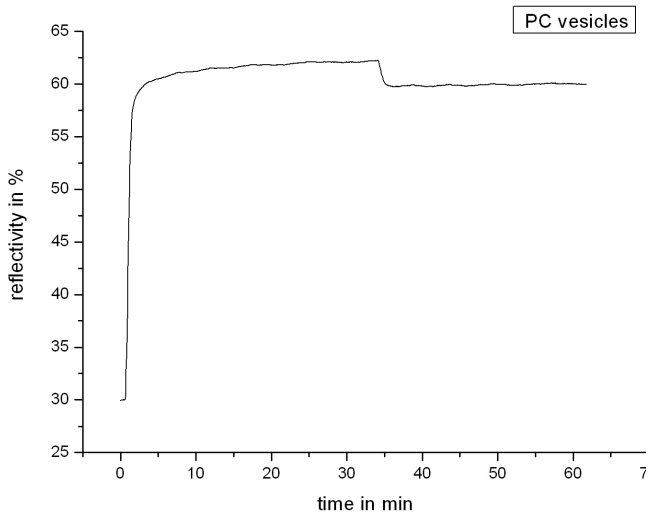

Figure 14: Spreading of the PC vesicles

3.2 Isolation of DNA

I used the PureYield Plasmid Midiprep System (Promega) to isolate the plasmid for our *in vitro* experiments.
A transformed E. coli cell culture (2* 50 ml) was grown overnight. The cells were pelleted by centrifugation (10 min. at 10000*g) and the supernatant discarded. The pellet was resuspended in 3 ml of Cell Resuspension Solution (containing EDTA to bind ions and RNase A to digest RNA), then the cells were lysed by addition of 3 ml Cell Lysis Solution (containing SDS). The mixture was incubated 3 min. at room temperature and then neutralized by addition of 5 ml Neutralization Solution. The lysate was allowed to sit until a white flocculent precipitate had formed: this contained insoluble cell components like membranes.
The lysate was poured into a clearing column (spanned by a cotton membrane retaining insoluble cell components) and centrifuged 5 min. at 1500*g. The lysate was filtered through and poured into a binding column. This binding column contains a polycarbonate membrane that bind DNA at a certain pH value. Vacuum was applied to the column until it was completely dry. The column was washed thoroughly with 5 ml Endotoxin Removal Wash (which elutes nucleases and endotoxins, i.e. small polypeptides) and with 20 ml Column Wash Solution (also containing Tris-HCl and EDTA), then dried by vacuum.
The column was placed onto a plastic tube and the DNA was eluted with 600 μl nuclease-free water. To concentrate the solution, an ethanol precipi-

tation was performed, i.e. 1/10 volume 3M sodium acetate and 2.5 volumes 95% ethanol were added. The solution was placed 15 min. on ice, then centrifuged 10 min. at 14000*g.
5 μl of DNA solution was then digested with EcoRI (this is done to ensure a regular running behavior of the denatured DNA) and analyzed on an agarose gel. The bands (one for each 50ml E.coli culture, see fig. 15) are just under the 4000 bp marker band, as expected for OR5 (3842 bp).

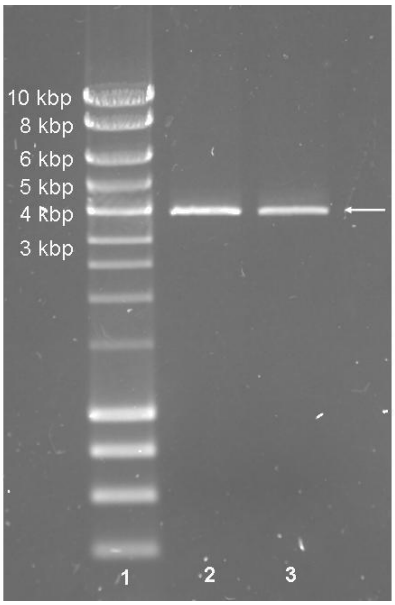

Figure 15: 1 % agarose gel from pTNT-VSV-OR5 3-4 Isolation; the arrow indicates at which height the OR5 band is expected

The resulting DNA solution had a concentration of 0.449 $\mu g/\mu l$, an A^{320} of 0.007 (this value gives a measure of the background turbidity of the solution) and an $A^{260/280}$ value of 1.52 (this gives a measure of fraction of RNA and protein, relative to the DNA).

3.3 Efficiency of expression for various DNA amounts

The protein expression was tested with different volumes (or amounts) of cDNA (see fig. 16). 1.5 μl DNA solution contains 0.673 μg plasmid, 2.0 μl contains 0.898 μg and 2.5 μl contains 1.122 μg .

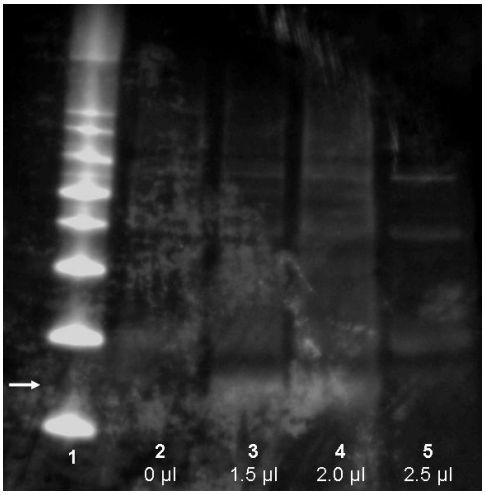

Figure 16: Expression at different DNA amounts.

3.4 Optimization of expression

As the first expressions in the 384 well plate yielded no results, the influence of the temperature, dilution and pipetting speed on the expression efficiency was checked.

3.4.1 Influence of temperature

The cell extract expresses badly under 30 °C, best results were obtained for incubation between 32 °C and 35 °C (data not shown).

3.4.2 Influence of dilution with PBS

The 50 μl TNT mix was diluted with $0, 10, 20, 30$ and 50 μl PBS. The incubation was done at 32 °C. The blot (see fig. 17) shows that even with 40 % dilution, the expression efficiency is still good enough to be detected.

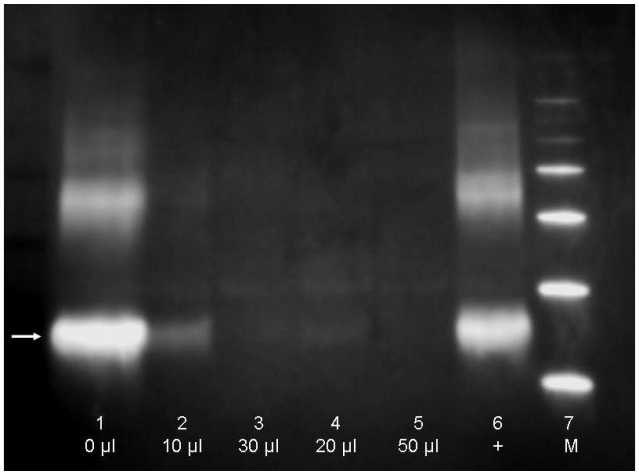

Figure 17: Blot after 45 min exposure. The amounts in μl refer to PBS. + stands for positive control, M for marker

3.4.3 Influence of pipetting speed

For the assembly of the membrane, a flow of 300 μl / min was used. I wanted to check if slower pipetting of the TNT mix on the PBS covered membrane would produce less dilution and therefore better results (see fig. 18).
The TNT mix was pipetted at 100 μl / min (lane 3), 200 μl / min (lane 4) and 300 μl / min (lane 5). The samples in lane 1 and 2 were incubated in wells in which no membrane had been assembled. Lane 6 contains a positive control.

3.5 Expression in the multi well plate

The membrane assembly and expression was attempted in a multi well plate, following the protocol described in §4.1 and 3.1. A commercially available 384-well plate had been previously coated with 50 nm gold. Because the coating was most uniform in the center of the multi-well plate, only the central wells were used. Several negative controls were performed:

1. for a general background check, an empty well was incubated with 1° and 2° antibody (well J19).

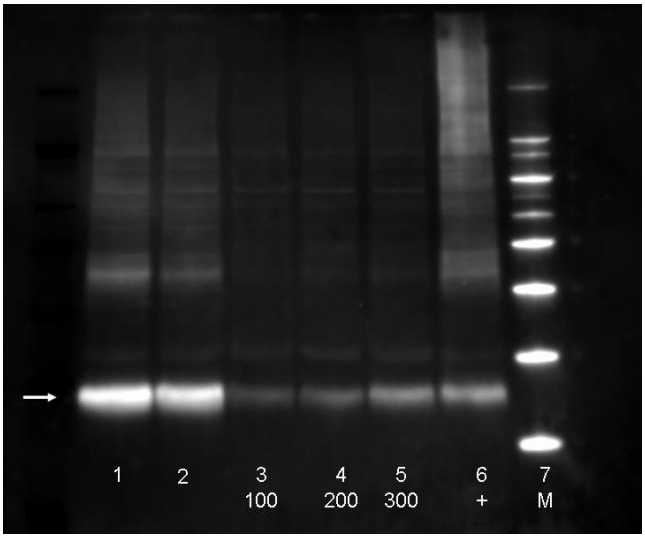

Figure 18: Blot after 27 min exposure. + stands for positive control, M for marker, the numbers refer to pipetting speed, in μl / min.

2. to check for unspecific binding on the membrane, a membrane was built up but no expression mix was added; the empty membrane was then incubated with 1° and 2° antibody (well G19).

3. as a blank, an empty expression mix (containing no cDNA) was added on a membrane, which was then incubated with 1° and 2° antibody (well H19).

The influence of different pipetting speeds was checked again. In two wells, the TNT mix was added with 200 μl / min (well I19 and I20) and in two other, the mix was added with 300 μl / min (well H20 and G20). See fig. 19. After the incubation with the expression mix, 5 μl of the supernatant was taken from each well. A SDS electrophoresis and Western Blot were done, as described in §3.2 (see fig. 20). The sample from well H19 (blank, NC 3) was applied onto lane 1, from well H20 onto lane 2, from well G20 onto lane 3, from well I19 onto lane 4 and from well I20 onto lane 5.

3.6 Optimizing

As no expression into the membrane was observed, I wanted to check if it was due to the low sensitivity of the detection methods or to the absence of expression. Therefore, the whole process (membrane assembly and *in vitro*

	Well number	Background	Experiment	Difference
NC 1	J19	1393	1376	-17
NC 2	G19	1400	1390	-10
NC 3	H19	1403	1388	-15
200 µl/min	I19	1394	1385	-9
200 µl/min	I20	1378	1359	-19
300 µl/min	G20	1391	1390	-1
300 µl/min	H20	1390	1375	-15

Figure 19: Comparison between fluorescence after incubation with the 1° antibody (background) and after incubation with the 2° antibody (experiment). NC stands for negative control.

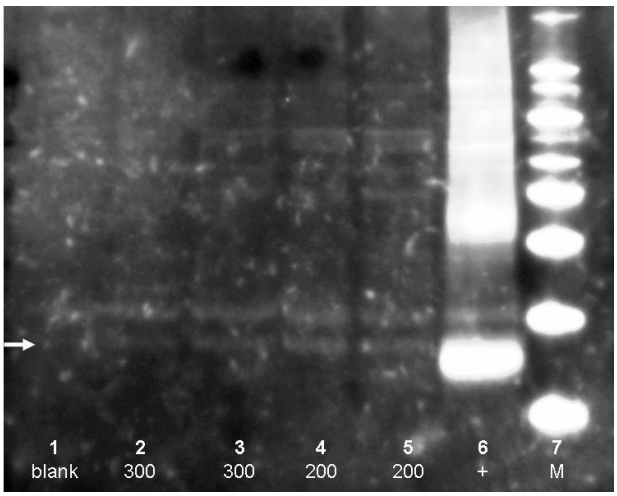

Figure 20: Blot after 60 min exposure. + stands for positive control, M for marker, the numbers refer to pipetting speed.

expression) was done in ten wells and I tried to concentrate the proteins by putting together all the wells after the expression. On the one hand (exp. A), the supernatant were put together. On the other hand (exp. B), the membranes were dissolved from the gold layer by reusing the same detergent (therefore hoping to have the membrane proteins of all the 10 wells in this detergent solution).

Exp. A: Putting together all the supernatants yielded 500 μl solution which was supposed to contain enough OR5 to be detected (besides lots of other proteins, ribosomes, etc...). The proteins were precipitated by heat precipi-

tation: 10 min incubation at 90 °C, then 10 min. centrifuging at 13000 rpm. The supernatant was kept at −20 °C, while the pellet was resuspended in 400 μl LDS (a SDS containing detergent). Both the resuspended pellet (lane 4) and the supernatant (lane 2) were run on the gel (see fig. 21).

Exp. B: To dissolve the membranes from the gold layer, 15 μl LDS buffer were pipetted into the first well and incubated for 2 min. The solution was then taken up and pipetted into the second well. The same procedure was followed through all 10 wells. It was then tried to precipitate the proteins by heat precipitation but no pellet was obtained (note that the proteins were dissolved in the same detergent usually used to resuspend pellets). This solution was then also run on the gel (lane 3).

In another well, 50 μl TNT mix were incubated at the same time, but without membrane (lane 1). This allows to check the efficiency under conditions that already were found to be suitable. Lane 5 contains the positive control and lane 6 the MagicMark XP marker.

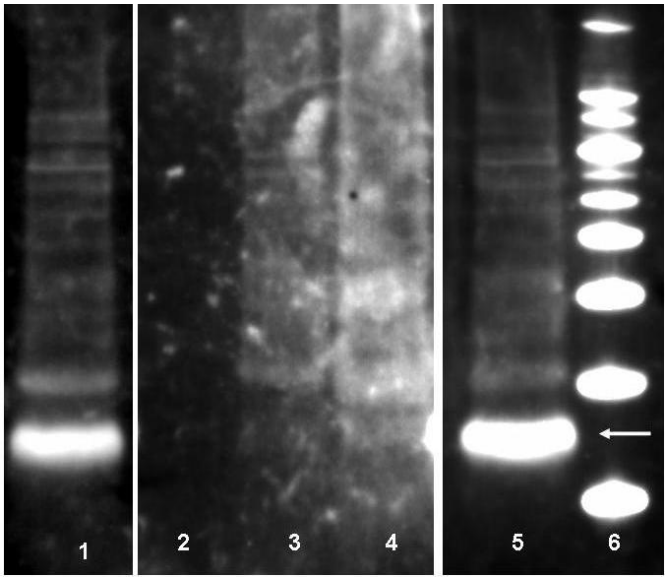

Figure 21: lanes 1, 5 and 6 are represented after 78 min exposure, lanes 2, 3 and 4 after 123 min exposure . Lane 1 contains the expression sample without membrane, lane 2 the supernatant of the expression mix pellet (expA), lane 3 the sample of the expression into the membrane (expB), lane 4 the resuspended pellet of the expression mix (expA), lane 5 the positive control and lane 6 the marker.

3.7 SPFS measurement

The first steps are similar to SPR measurements, except a control SPFS measurement performed on the gold surface covered with water (see fig. 22): the fluorescence of the bare surface is very low.

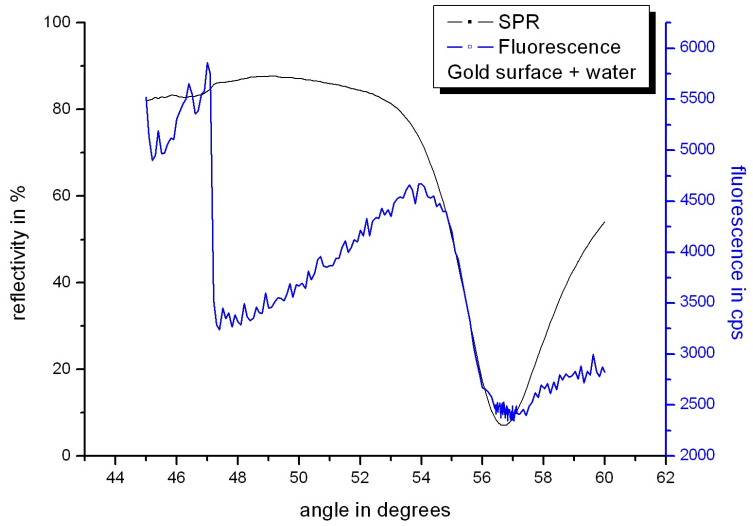

Figure 22: SPFS measurement of the gold membrane covered with water.

A membrane was then assembled in the flow-cell, following the same procedure as explained in 4.1. The completed membrane was incubated with the TNT mix, which was monitored by kinetic measurement (see fig. 23).

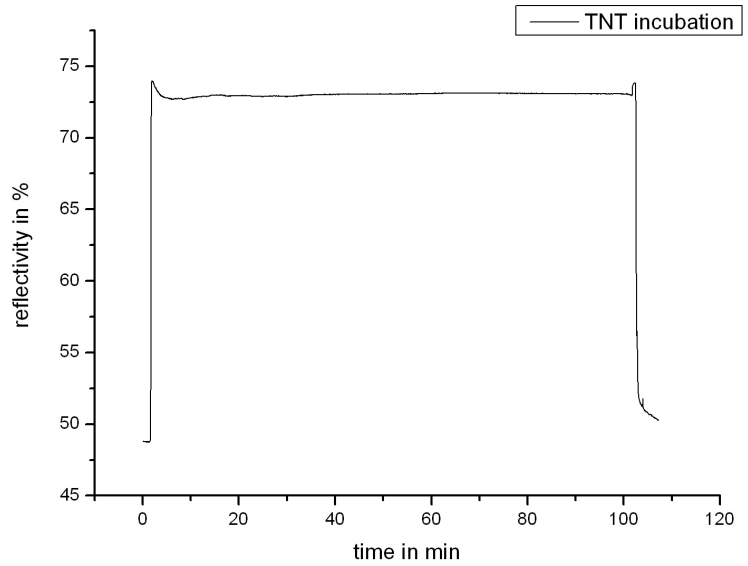

Figure 23: Kinetic measurement of TNT mix incubation.

A scan measurement was then performed (see fig. 24) to determine the angle at which the reflectivity is 30 %.

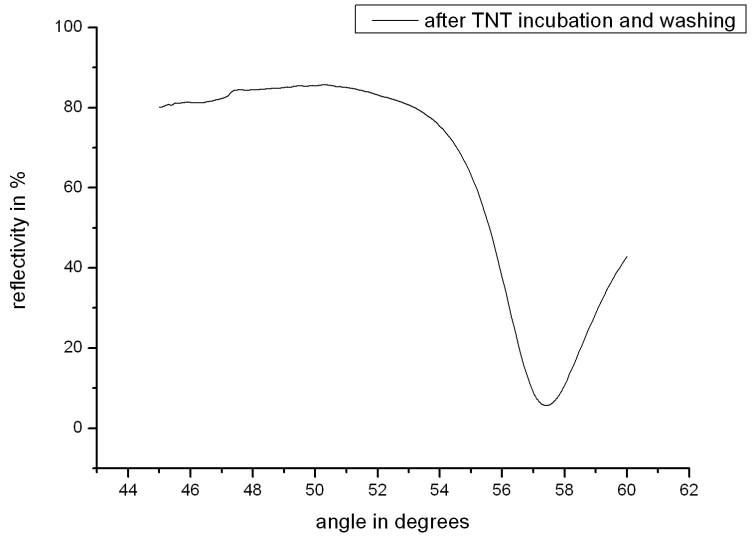

Figure 24: Scan measurement after TNT mix incubation and washing.

After extensive washing with PBS, the 1° antibody was added (see fig. 25).

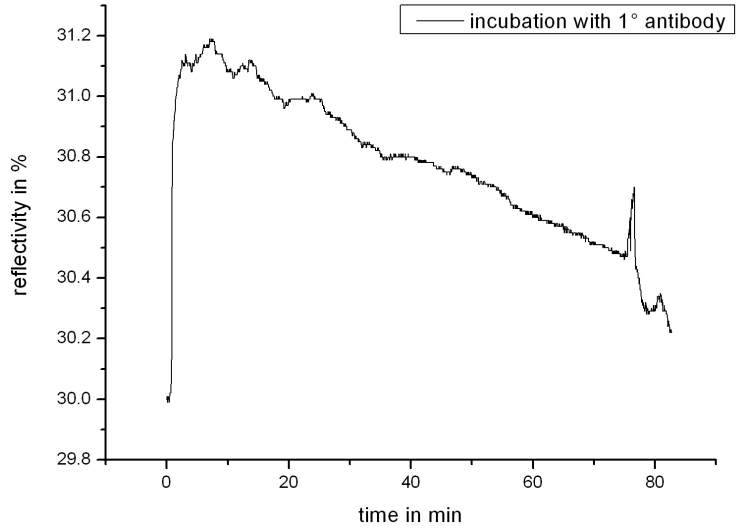

Figure 25: Kinetic measurement of 1° antibody incubation.

Another scan measurement was performed to check for unspecific fluorescence (see fig. 26).

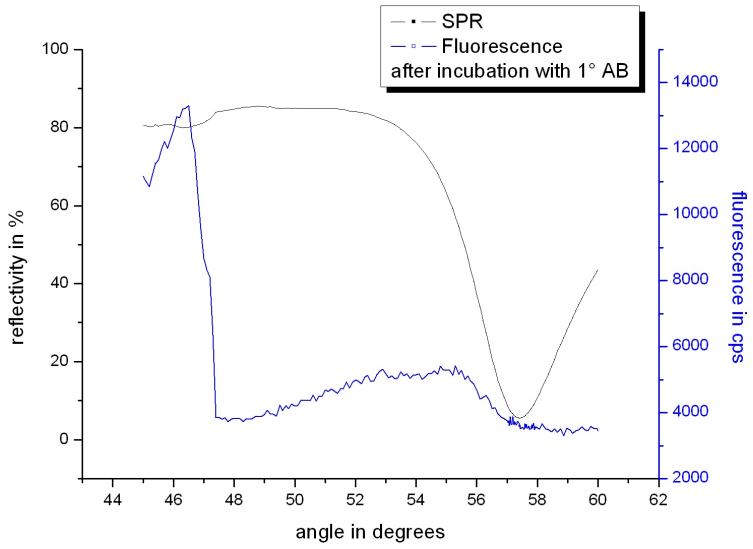

Figure 26: Scan measurement of the rinsed surface after 1° antibody incubation.

The next kinetic measurement (see fig. 27), to monitor the incubation of the 2°, Cy5-labelled antibody, was started at 20 % reflectivity because at this level, the fluorescence is most sensitive. Washing with PBS afterwards left behind only the 2° antibody that was well attached.

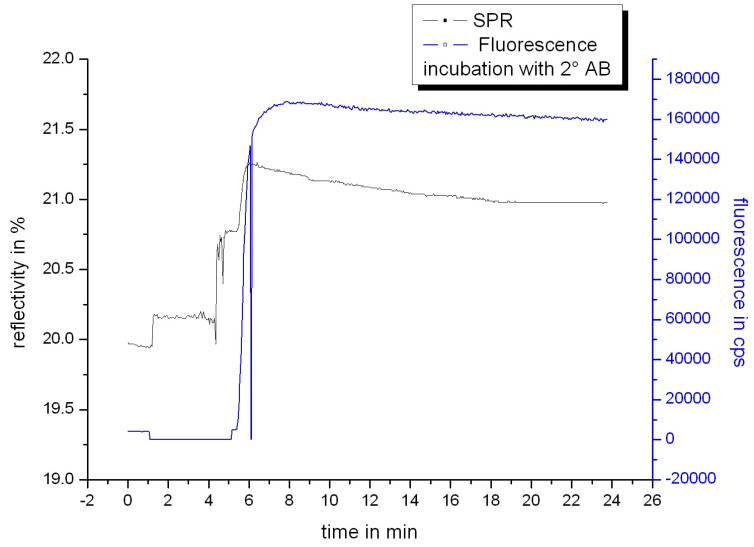

Figure 27: Kinetic measurement of 2° antibody incubation.

4 Discussion

As a first step, the plasmid for the VS tagged OR5 receptor was isolated from bacteria. A pure DNA preparation should produce a 260/280 purity value of 1.8, however, contamination with protein or RNA should not influence the expression efficiency of the plasmid. This was proven by the subsequent experiments. Nucleic acids do not absorb at 320 nm at all, i.e. the A^{320} should be as low as possible. The value measured for our DNA solution indicate a high purity (see §3.2).

The efficiency of the expression for various DNA amounts had to be checked first (see §3.3). The protocol predicted good efficiency between 0.5 and 1.5 μg plasmid (for a total of 50 μl expression mix) with an optimum at 1 μg. The concentration of our DNA solution being 0.449 $\mu g/\mu l$, the optimum should be at approximately 2 μl.
The efficiency was therefore tested for 1.5 μl, 2.0 μl, 2.5 μl(i.e. 0.673 μg

,0.898 μg and 1.122 μg plasmid respectively) and 0.0 μl as negative control. The blot (fig. 16) shows that expression was successful for 1.5 μl and 2 μl, while too high concentration suppressed it in the third case (2.5 μl): this can be explained by substrate inhibition. However, the drop in efficiency is very sudden. As expected, no OR5 band is visible in the negative control.

The temperature range found in §3.4.1 corresponds well to the activity curve of the polymerases and other enzymes of the TNT mix: they are inactive at too low temperature ($< 30\,^{\circ}\mathrm{C}$), and inefficient at too high temperature ($> 37\,^{\circ}\mathrm{C}$). Subsequent experiments were therefore done by placing the 384-well plate in a $32\,^{\circ}\mathrm{C}$ or $35\,^{\circ}\mathrm{C}$ incubator during PC-vesicles fusion (the material of the plate is not very conductive, it was therefore put to heat up 90 min in advance) and the expression.

In the multi-well plate, when the expression was done on a membrane, a certain dilution with PBS cannot be avoided. At any time, the membrane has to be covered to avoid contact with air (the membrane would collapse if it was left dry). Before pumping in the expression mix, the membrane is covered by approximately 50 μl of PBS. The TNT mix is then added close to the membrane, and sinks to the bottom (it is heavier than PBS because it contains glycerol). At the same time, the PBS is pipetted out close to the surface (see fig. 9).
Two experiments were performed with respect to this problem: exp. 3.4.2 checked the critical dilution up to which the expression was still working; exp. 3.4.3 checked if the speed at which the TNT mix was added onto the membrane influences the dilution. Fig. 17 shows that even with 20 μl PBS dilution, the expression is still efficient enough to be detected. The influence of the pipetting speed seems to be considerable (see fig. 18). The best results were obtained for 300 $\mu l/min$, probably because the TNT mix is injected so quickly that the dilution by the overlaying PBS is minimal, while almost pure PBS is pipetted out. Subsequent experiments were therefore done by pipetting in the TNT mix at that speed, except exp. 3.5 where I hoped to see expression into the membrane (while in exp. 3.4.3, only the efficiency of the expression in the supernatant could be verified).

The first part of exp. 3.5 consisted of several negative controls: unspecific binding of $1\,^{\circ}$ or $2\,^{\circ}$ antibody to the gold surface (well J19, NC 1) and to the membrane (well G19, NC 2) was checked; as a general blank measurement, the whole experiment was done with an "'empty"' expression mix, i.e. containing no DNA (well H19, NC 3). I can conclude that no unspecific binding takes place (see fig. 19); also, 3 min washing at 300 $\mu l/min$ is efficient,

otherwise unbound 2° antibody would have been seen in the Experiment measurement. Fig. 19 also shows that no fluorescence was observed in the wells that were incubated with complete TNT mix. So, either the expression did not occur or it is too weak to be measured by the applied fluorescence method.
I could check if expression occurred in the supernatant by casting the 5 μl TNT mix I had taken after the incubation. The blot shows that expression occurred in all four wells (see fig. 20). In contrast to exp. 3.4.3 (see fig. 18), the expression is more efficient in the wells where the TNT mix was added with $200\mu l/min$. On one hand, the lower dilution favors addition at $300\mu l/min$; on the other hand, addition at $200\mu l/min$ strains less the membrane architecture, i.e. if the membrane is slightly damaged it could perhaps withstand the $200\mu l/min$ but not any higher flow.

So, exp. 3.5 showed that expression in the well plate in presence of a membrane is detectable. I wanted to check if expression also occurred into the membrane. As it was not possible to view it by fluorescence, I tried to put together 10 membranes, hoping that the signal of the receptor present in these membranes would add up.
Exp A serves as a positive control as it shows that expression in presence of the membrane took place (see fig. 21). In Exp B, LDS was used to detach the membrane from the gold surface; the same 15 μl were used 10 times, so it should contain all the proteins (and membrane constituents) of those 10 membranes. I tried to precipitate the protein by heat precipitation, which was unsuccessful as they were dissolved in too strong a detergent. So 5 μl of the solution was directly used as IV-sample and prepared following § 2.2.
For this sample (lane 3, fig. 21), no line is clearly visible. In a subsequent assembly of a membrane in the SPR setup, I noticed that the PC vesicles did not spread at all. The same solution had been used for exp 3.6, therefore it is probable that the bilayer was not completed which would lead to a very hydrophobic surface on which the ribosomes and other components of the TNT mix would attach and denature. This explains why no clear band can be seen in lane 3, but also why the one in lane 4 (which should contain protein from the supernatant of 10 wells) is so faint.

For exp 3.7, fig. 22 and 26 show a low background fluorescence, i.e. the gold surface and the membrane/receptor have no fluorescence on their own (at the monitored wavelength). Fig. 25 and 27 show a sigmoidal start of the curve, which is characteristic of affinity binding kinetics. Unspecific binding would show a steep slope, similar to the one in fig. 23. The fluorescence in fig. 27 drops because of bleaching of the fluorescent dye.

Fig. 27 shows steps in the reflectivity measurement: the first and the last can be explained by switching the pump on and off. The second one must be due to a perturbance in the flow-cell, for example an air bubble. Note that after injection of the antibodies, the reflectivity rises approximately 1.2 % for both antibodies (see fig. 25 and 27), but, while it drops quite low for the 1° antibody, in the case of the 2° antibody, the signal stabilizes after a loss of 0.2 %; this can be explained by the fact that only one 1° antibody attaches per receptor, whereas several 2° antibodies attach per 1° antibody. The signal in fig. 27 has to be compared to a fluorescence measurement done without receptor insertion [3]. In this case, the fluorescence is about 80 000. So, our signal is approximately double.

The difficulties encountered for the expression in the multi-well plate are probably due to the high dilution and the difficulty to control the environment (like temperature). Even in the flow-cell of the SPR setup, lower expression efficiency is observed in the presence of a membrane, compared to expression in an Eppendorf tube. Indeed, the membrane is amphiphilic, perhaps the ribosome or another essential component attaches to the membrane and is therefore dysfunctional. In any case, free lipids are extremely harmful because of their high hydrophobicity. Also, the TNT expression mix was developed for cytoplasmic proteins and its efficiency for the transmembrane synthesis is only documented for small integral proteins.
However, it was shown that membrane assembly in a commercially available 384 wells plate is possible and that expression of protein under these condition is detectable. I was not able to prove clearly that expression occurred into the membrane. In order to do that, optimization of the expression system and the detection method is necessary.

5 Outlook

The impossibility to check the quality of the membrane before performing the expression is very negative. This could be done remedied in a system developed by Biacore, where 4 lanes on a chip constitute something similar to a very big SPR flow-cell. Another possibility is a system by Iongate: a 384 well-plate in which each individual plate is connected to an electrode. By electrochemistry, the assembly of the membrane can be monitored in real time and, eventually, improved.
Changing the architecture of the gold layer might also improve results. By

evaporating gold only on the bottom of the wells, the membrane would not be forced to perform a 'turn' to cover the well sides. Also, the membrane would never be in contact with the surface of the liquid or air.
The optimization of the system would be much easier if a protein with a higher *in vitro*expression efficiency was used. This would be the case for the claudins (important components of the tight junction; four transmembrane domains) whose expression *in vitro* is one or two orders of magnitude better than that of OR5.
In order to reduce the background, one could use luminescence instead of fluorescence. This means using a 2 ° antibody that is not fluorescein labelled but has a peroxidase attached; the signal is then generated by adding a substrate, for example Luminol, that is converted by the peroxidase. This signal might have a lower background and therefore a higher difference between background and experiment.

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