

PROJECT FINAL REPORT

Grant Agreement number: **CP-FP 214666-2**

Project acronym: **ASMENA**

Project title: **Functional assays for membrane protein on nanostructured supports**

Funding Scheme: **Small or Medium-scale focused Research Project**

Period covered: **from September 1, 2008 to August 31, 2011**

Name of the scientific representative of the project's co-ordinator¹, Title and Organisation:

Janos Vörös, Prof., ETH Zurich, Switzerland

Tel: +41 44 632 5903

Fax: +41 44 632 11 93

E-mail: janos.voros@biomed.ee.ethz.ch

Project website address: : **<http://www.asmena.ethz.ch>**

Final publishable summary report

1.1 An executive summary

The ASMENA project aims to create new tools and techniques for drug development and screening. Today, more than 50% of all drug targets are membrane proteins. The more that is known about membrane proteins, the easier it is to find out what effects certain substances may have in the body. If substances lacking the desired effects on the target proteins or having a harmful effect can be sorted out at an earlier stage, this considerably shortens the drug development process. In addition, this means less need for animal testing, and that there is likely less risk of harm to the lab animals as a result of the tests made. Once the substances have been developed into finished pharmaceutical drugs, the clinical trials (where drugs are tested on humans) will involve less risk, and the end result will be safer and more efficient pharmaceutical drugs.

The ASMENA project aims to develop the knowledge and methods needed to build label-free formats for membrane protein drug screening assays. By downscaling assays, costs can be reduced by reducing reagent and substance use, and also by allowing earlier detection of potential drug hits or toxicity effects. Combining several such assays on a chip will create a versatile platform where a great number of events can be measured at the same time at different spots on the chip, enabling functional screening of drug interactions with membranes and several classes of membrane proteins which are potential drug targets. ASMENA is a highly transdisciplinary, fascinating project with both scientific and technological aspects ranging from nanofabrication of materials and surfaces, polymer chemistry for the functionalization of biointerfaces, to physics and biochemistry of lipidic systems and membrane proteins, to device integration and quantitative biosensing and drug screening assays. It is a close partnership of seven academic and seven industrial partners that cover the necessary knowledge and expertise in the fields, and a student and postdoctoral researcher team fully committed to the overall goals and joint efforts.

Among the achievements of the project it is worth mentioning the successful and cost-effective fabrication of nanoporous membrane chips, development of dedicated macromolecules and assembly procedures for the surface functionalisation of the chips, improved or new protocols for the preparation and placement of liposomes and membranes on nanoporous chips by self-organization, and the incorporation of membrane proteins for electrochemical and optical readouts. In addition, several novel approaches have been invented and introduced during the project for the quantitative assessment of membrane transport processes and even a small library of drug leads have been screened resulting in interesting hits for aquaporins, an important class of membrane proteins.

Results have, during the whole duration of the project, been published in dedicated scientific journals and communicated at scientific conferences. ASMENA project background, concepts and achievements have also been communicated to a more general public through newsletters in the partner institutions, newspapers, and radio broadcasting. The ASMENA website provides downloadable information for both the scientific community and the general public.

Given the interdisciplinary character of the project, the dedication of each partner to collaborative efforts, open communication across all partners and specific student events, our project contributes greatly to student education and training, both in scientific, technological and social respect.

1.2 A summary description of project context and objectives

Current drug screening assays rely in the case of membrane proteins like ion channels on patch-clamp based techniques and in the case of ligand-binding assays, on fluorescent recording. Only the latter has found wide spread use in drug screening targeting membrane proteins, with the result that drug hits are scored for in vitro assays on the basis of binding or no binding. Labels are an unnatural attachment/component to the molecule to be investigated and may affect its biochemical behaviour. In the worst case this leads to false readouts. Thus, there is a strong desire to transfer fluorescence recording into label-free formats, such as surface plasmon resonance (SPR) or waveguide spectroscopy, which also allow routine measurement of affinity constants for ligand binding, and also to find an alternative to patch-clamp to expand in vitro assays for which ligand binding effect on protein function can be measured with a less labour intensive method. State-of-the-art approaches with respect to both patch clamp, which is one of the few label free techniques sensitive enough to probe single events, and label free affinity assays, which today requires millions of analytes for sufficient signals, both suffer from low throughput and in the latter case low information content. The lack of development in membrane protein assays is partially due to a lack of reliable procedures to immobilize membrane proteins onto sensor surfaces in a format suitable for label-free high-throughput screening of low molecular weight drug candidates. There is even higher demand to measure functions of membrane proteins which are highly diverse. In this proposal, we will develop methods to self-assemble membrane protein-containing lipid bilayers onto nanoporous substrates designed for both electrochemical recording of function and label-free nanoplasmonics compatible with an array format for membrane protein drug screening assays. The objective is to develop a versatile platform that can easily be scaled up from analytical profiling to functional screening of drug interaction with membranes and several classes of membrane protein which are potential drug targets. Techniques for drug screening or analytical profiling developed in this project include: (i) small label-free ligand binding affinity assays to membrane proteins using nanoplasmonic array sensor elements; (ii) electrochemical measurements on embedded membrane proteins over nanopore arrays using ion-selective electrodes, impedance spectroscopy or single-molecule voltage clamp measurements; (iii) ligand binding and induced conformational changes in proteolipid membrane functionalized waveguides.

The collaborating partners have researched various approaches to lipid membrane self-assembly,[1-8] membrane protein insertion and function investigation,[3, 9-12] sensor fabrication,[13-15] surface functionalization and patterning[2, 5, 16-18] both in parallel projects and in various collaborations. These platform skills will now be combined on a European level to realize a general platform for functional assays for membrane proteins. To ensure that valid model systems and platform assay formats are chosen a strong industrial component is present in both the development of measurement platform and in terms of end-users of drug screening tools, where industrial partners act as work package leaders.

Cell surface receptors, comprise a vast number of structurally diverse integral membrane proteins such as G-protein-coupled receptors, ion channels, transporters and aquaporins just to mention a few. Understanding cellular signalling mediated by cell surface receptors is in the focus of present biological research and central for drug development. Facing the rapidly growing number of (i) molecular membrane targets discovered by functional genomics and (ii) potential therapeutic compounds produced by combinatorial chemical synthesis, the need for downscaling assay formats is of utmost importance for accelerating functional screening, reducing costs by earlier detection of potential drug hits or toxicity effects, reduced reagent consumption, and open novel possibilities which cannot be reached by traditional techniques.

As mentioned above, all current platforms for screening of drugs towards membrane proteins suffer drawbacks, which have prevented their acceptance and scaling-up for industrial high-throughput applications for decades. The powerful patch-clamping method, which directly measures transmembrane signalling, is hampered by the need for highly trained specialists and its low throughput. Assays which require fluorescent dyes in vesicle solutions, risk altering the function through the labelling, do not provide quantitative results and generally require expensive sample preparations. Assay systems using black lipid membranes (free-standing over μm -apertures) are not long-term stable and traces of organic solvents left from lipid painting impair the functionality of membrane proteins.

Recently, it has been demonstrated by independent groups that the stability of free-standing bilayers in pores is significantly improved, if the pore diameter is in the nanometer range.[19] This observation, in conjunction with recent advances in the understanding of how to form planar (supported) lipid bilayers on substrates, opens the possibility for a new generation of free-standing membranes formed by self-assembly from proteoliposomes on nanostructures. Such lipid membranes, comprising membrane proteins relevant for drug screening on a sensor chip integrated with voltage-clamp recording and label-free array sensing, would be a revolutionizing technology for analytical profiling. A “voltage-clamp” current recording on the level of chip-based nanopore-supported Lipid Bilayers (npsLBs) is analogous to a measurement performed by the well-known electrophysiological “patch-clamp” technique.

1.2.1 References

1. Reimhult, E., F. Hook, and B. Kasemo, Intact vesicle adsorption and supported biomembrane formation from vesicles in solution: Influence of surface chemistry, vesicle size, temperature, and osmotic pressure. *Langmuir*, 2003. **19**(5): p. 1681-1691.
2. Stadler, B., et al., *Micro patterning of DNA-tagged vesicles*. *Langmuir*, 2004. **20**(26): p. 11348-11354.
3. Ataka, K., et al., Oriented attachment and membrane reconstitution of his-tagged cytochrome c oxidase to a gold electrode: In situ monitoring by surface-enhanced infrared absorption spectroscopy. *Journal of the American Chemical Society*, 2004. **126**(49): p. 16199-16206.
4. Naumann, R., et al., *Tethered lipid Bilayers on ultraflat gold surfaces*. *Langmuir*, 2003. **19**(13): p. 5435-5443.
5. Stadler, B., et al., Creation of a functional heterogeneous vesicle array via DNA controlled surface sorting onto a spotted microarray. *Biointerphases*, 2006. **1**(4): p. 142-145.
6. Reimhult, E., et al., *Novel surface architectures for biomimetic lipid membranes*. Technical Proceedings of the NSTI Nanotechnology Conference and Trade Show, 2007. **2**.
7. Reimhult, E., et al., A multitechnique study of liposome adsorption on Au and lipid bilayer formation on SiO₂. *Langmuir*, 2006. **22**(7): p. 3313-3319.
8. Richter, R.P., R. Berat, and A.R. Brisson, *Formation of solid-supported lipid bilayers: An integrated view*. *Langmuir*, 2006. **22**(8): p. 3497-3505.
9. Richter, R.P., et al., On the kinetics of adsorption and two-dimensional self-assembly of annexin A5 on supported lipid bilayers. *Biophysical Journal*, 2005. **89**(5): p. 3372-3385.
10. Giess, F., et al., The protein-tethered lipid bilayer: A novel mimic of the biological membrane. *Biophysical Journal*, 2004. **87**(5): p. 3213-3220.
11. Han, X., et al., Nanopore arrays for stable and functional free-standing lipid bilayers. *Advanced Materials*, 2007. **19**: p. 4466-4470.
12. Zheng, L., et al., *The mechanism of ammonia transport based on the crystal structure of AmtB of Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(49): p. 17090-17095.
13. Dahlin, A. and F. Hook, *Extinction spectroscopy for sensors based on localized surface plasmon resonance*. Abstracts of Papers of the American Chemical Society, 2006. **231**: p. 1.
14. Heyderman, L.J., et al., *High volume fabrication of customised nanopore membrane chips*. *Microelectronic Engineering*, 2003. **67-8**: p. 208-213.
15. Hook, F., et al., A comparative study of protein adsorption on titanium oxide surfaces using in situ ellipsometry, optical waveguide lightmode spectroscopy, and quartz crystal microbalance/dissipation. *Colloids and Surfaces B-Biointerphases*, 2002. **24**(2): p. 155-170.
16. Voros, J., T. Blattler, and M. Textor, Bioactive patterns at the 100-nm scale produced using multifunctional physisorbed monolayers. *Mrs Bulletin*, 2005. **30**(3): p. 202-206.
17. Benetti, E.M., S. Zapotoczny, and J. Vancso, Tunable thermoresponsive polymeric platforms on gold by "photoiniferter"-based surface grafting. *Advanced Materials*, 2007. **19**(2): p. 268-+.
18. Svedhem, S., et al., In situ peptide-modified supported lipid bilayers for controlled cell attachment. *Langmuir*, 2003. **19**(17): p. 6730-6736.

19. Janshoff, A. and C. Steinem, *Transport across artificial membranes - an analytical perspective*. Analytical and Bioanalytical Chemistry, 2006. **385**(3): p. 433-451.

1.2.2 Overall objectives of the ASMENA project

With the overall goal of creating new formats for drug lead profiling and screening versus membrane targets which close the gap between current analytical formats for measuring drug binding and complex assays for verifying drug induced functional changes, the main objectives can be summarized as generating the scientific and technological know-how to achieve:

- an electrochemical sensor platform for ion channel/transporter drug screening/analytical profiling
- a local surface plasmon resonance sensor platform for aquaporin drug screening/analytical profiling
- a membrane protein functionalized waveguide sensor platform for drug screening/analytical profiling based on self-assembled proteolipid membranes and label-free sensing

Realizing these objectives requires breakthroughs and pooling of knowledge from several areas of biointerface science. The strong knowledge base and skills within the consortium, manifested by a number of novel concepts for topographical and chemical nanopatterning, will be the starting point to develop sensor chips aimed to quantitatively measure membrane protein function. The key concepts of the platform are:

- nanoscale topographical features, specifically nanopores, to enhance stability of the bilayer and sensitivity of measurement integrated in a sensor chip format (WP1)
- nanoscale surface modifications allowing directed self-assembly of desired proteolipid structures on the chip (WP2)
- self-assembly of proteolipid membranes onto the nano-sized sensor structures from proteoliposomes to achieve a simple and robust preparation suitable for commercial applications. (WP3)
- affinity assays based on high-density membrane protein comprising lipid membrane-functionalized nanostructures on waveguides (WP4)
- insertion of membrane protein drug targets into the free-standing membranes for functional measurements (WP5)
- assays for quantitatively measuring membrane protein functionality for drug candidate screening and analytical profiling (WP6)

Each work package involves the concerted effort of several partners and although several of the work packages can be developed in parallel, the cross-fertilization of competences and results is substantial, as can be seen in Figure 1, where the inter-connectedness between essential objectives within WPs is schematically represented. Achieving this concerted effort of multiple competences is essential to realizing the objectives and will be accomplished based on our partners previously demonstrated ability to collaborate with each other.

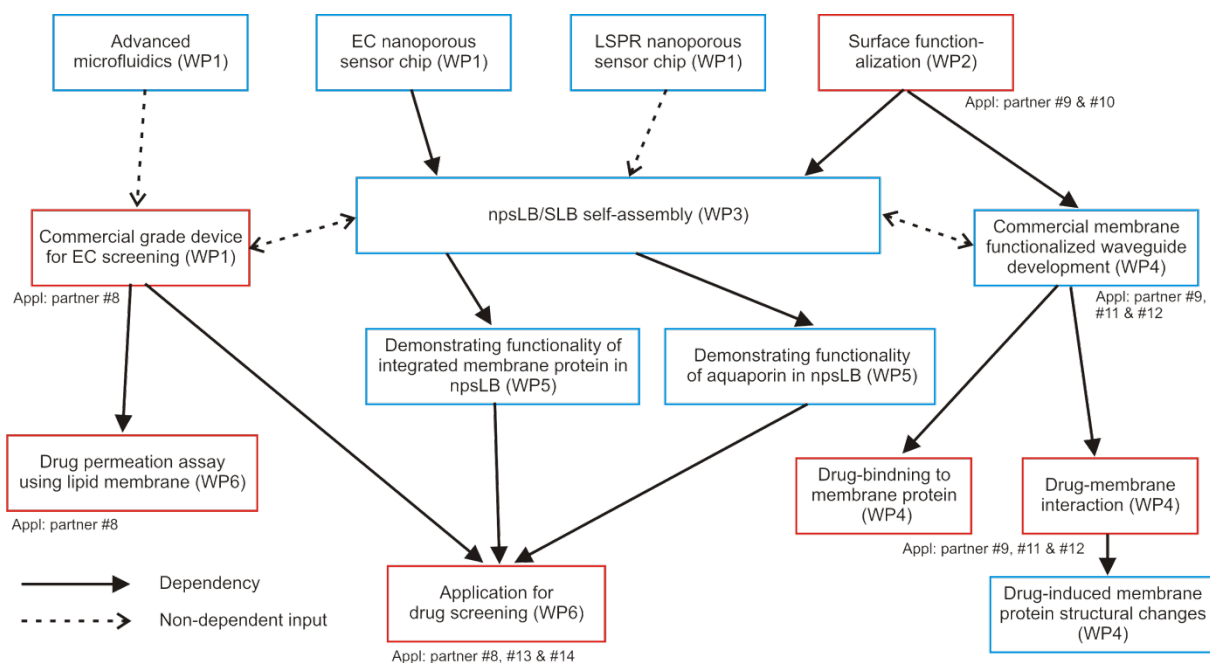


Figure 1. Overview of inter-connectedness scientific work package activities. Blue boxes mark fundamental exploratory activities and red boxes mark application oriented activities. Arrows mark dependencies and cross-fertilization of achieved results.

Central work packages for the work flow are WP1 where the sensor chips are developed, which are pre-requisite for subsequent development of nanotopological surface functionalisation and pore-spanning membrane assembly, and WP3 where integration and optimization of (proteo)lipid membranes with sensor platforms and nanostructures is achieved. WP2 addresses new concepts in surface functionalization that can be applied to facilitate control of membrane systems on biosensors and nanostructures. WP4-6 addresses various model systems and implementations of the generic platforms developed in previous work packages.

1.3 A description of the main S&T results/foregrounds

Here we describe WP by WP the main scientific and technological results following the order of the deliverables in the periodic reports.

1.3.1 WP 1: Fabrication of nanostructured supports, WP Leader: Marco Di Bernardino, Leister

In WP1 our task was to develop the necessary processes and platforms for the various nanopore sensor setups that were employed throughout the project. In three separate, but intertwined sub-projects we have developed platforms for nanopore electrochemical (EC) sensing, local surface plasmon resonance (LSPR) sensing, a combined EC and LSPR sensor platform and integrated microfluidics for advanced sample handling.

Summary of progress

In the first 18 months of the project we have demonstrated that the applied technology provides a means for manufacturing nanopore chips for research purposes. Nanostructuring of Si₃N₄-wafer by EBL followed by a post-processing step delivers reliable and reproducible chips for downstream analytics in the other work packages. Similarly, milling of well-defined pore arrays in Si₃N₄ diaphragms by FIB was shown to provide suitable supports for single channel recordings. The developed methods for particle lithography are cost-effective and provide, in contrast to the state-of-the-art technology, the possibility to control the spacing of nanostructured features. Particle lithography could thus represent a valuable alternative to EBL in applications that demand pores with diameters in the range of 80 to 500 nm. An additional, novel particle lithography fabrication scheme has been developed for sensor chips with short-range ordered nanoplasmonic pores. These supports provide a possible solution for combining electrochemical sensing and LSPR. In addition, with the achieved integration of microfluidics for rapid liquid exchange onto these LSPR chips, label-free studies of fast kinetic transport processes across membranes become accessible.

In the second half of this subproject efforts were focused on the development of a process suitable for manufacturing nanostructured chips for EC and LPCR sensing. Particle lithography was shown to provide a valuable solution, wherever nanopores are required for the specific measurements. However, classical photolithographic processes are alternatives that must be considered if EC measurements do not need structures at the nanoscale level (pores around 1 μ m) or demand precise layouts.

For controlled gradients and fast liquid exchange a first microfluidic device was developed and its proper operation for LPCR and fluorescent readouts shown. In addition, WP5 partner has developed in collaboration with a partner at a technical university a PDMS-based microfluidic system (MFS) consisting of two parts in which all standard-sized chips of any pore format can be integrated.

All WP objectives and deliverables of the reporting period have been fully reached.

Main significant results

We have successfully developed specific protocols for the reproducible fabrication of nanoporous chips using three different techniques: electron-beam lithography (EBL), focused ion beam (FIB) structuring, and particle (colloidal) lithography (PL). In particular, we have developed a novel version of particle lithography, which allows for unprecedented control of short- and medium-range order of colloid/nanopore arrays while still exploiting the cost advantages associated with self-assembly of particles and parallel fabrication of nanoporous substrates on large areas. Nanopore chips combining LSPR and electrochemistry capability have been manufactured as well as integrated with microfluidics to study fast kinetic transport processes.

Depending on the application requirements, we have now in place processes that allow for industrial mass production of micro- and nanostructured chips for electrochemical as well as for LPCR sensing. Microfluidic devices with the fastest ever reported switching times were developed on the one hand for rapid liquid exchange and optical readouts, and on the other hand also for automated lipid-bilayer formation on the micro- or nanostructured pores.

The PDMS-MFS with integrated nanopore array chips was tested for automated painting and formation of stable bilayers could be demonstrated as in macroscopic two-compartment systems. After extensive rinsing such bilayer are suitable for fusion of proteoliposomes. Alternatively, chips integrated in PDMS-MFS pre-treated with charged polymers allow solvent-free preparations of proteobilayers using the methods developed in WP3 and WP5. Thus, PDMS-MFS are an indispensable for reproducible sensor surface preparations. In the future, the microfluidic channels may be generated directly on chip surfaces.

Deviations from Annex I

All deliverables were fully achieved. Further WP 1 efforts in the reporting period reflected the need for additional chip platforms and properties: Protocols for the fabrication of (a) chips with microporous structures, and (b) low-capacitance chips were successfully and timely developed requiring an additional process step in the manufacturing process. In addition to the plan we also developed and tested a MFS for various chips allowing electrochemical detection (D.1.2.3). Such prototype devices are indispensable for reproducible proteobilayer formation and activity measurements. (See Figure 1.1 below.)

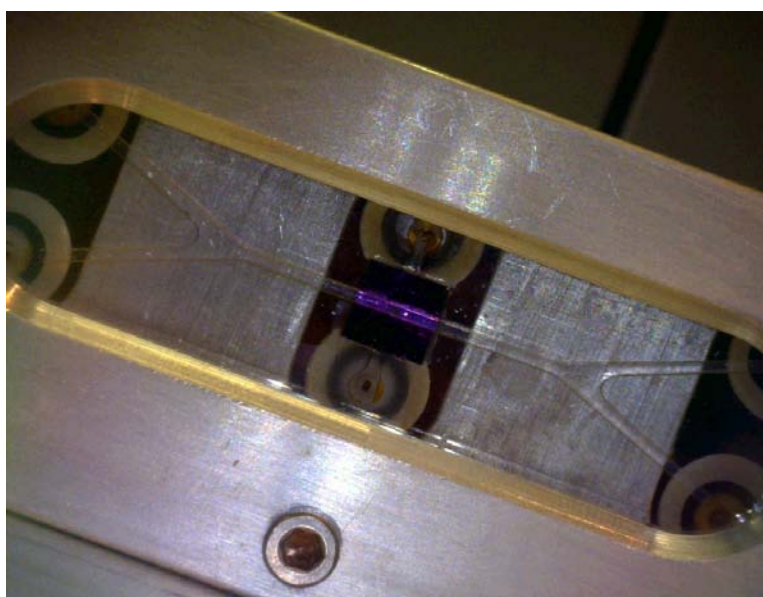


Figure 1.1: Picture of the microfluidic switching prototype allowing electrochemical measurements.

Achieved results and Deliverables

D1.1.1 Nanopore array chips by EBL (month 2)

Various types of nanopore (micropore) chips containing arrays, multiple or single pores were designed and manufactured for WP1, WP3 and WP5. In order to figure out the optimal geometry (nanopore size and density) for spanning the lipid bilayer over the pores, new wafer masks for variable pore size, number and distance were designed. Pores in the nanoscale range (< 200 nm) were obtained by E-beam lithography (partner PSI), particle lithography (partner Chalmers, ETH) or focused ion beam (partner MPG); larger micropores (> 800 nm) were fabricated by photolithography (partner Leister).

D1.1.2 Single-nanopore chips by FIB (month 2)

A process of milling well-defined arrays of through pores in Si_3N_4 diaphragms by the FIB has been successfully established. Pore openings were imaged by SEM on the pit (i.e. back) side and by AFM on the flat (i.e. front) side of the diaphragms. The most determinant parameters of the FIB patterning procedure on final pore specifications are nominal pore diameter and apparent milling depth, whereas parameters such as magnification at milling, pixel dwell time, or scan direction seem not to have a significant impact on pore characteristics. Arrays of 1-12 pores with controlled pore size (50-800 nm) and pore-to-pore distance (0.25-2 μm) have been produced. Substrates with the smallest pore diameter of ~ 50 nm (in 100-nm-thick

diaphragms) have also been fabricated reproducibly. Taper angle of $\sim 12^\circ$ towards the flat side has been estimated from high-resolution SEM images of 200-nm-wide pores. Conductance of unobstructed pores filled with electrolyte correlated well with theoretical predictions based on pore geometry.

D1.1.3 Nanopore array chips by particle lithography, random and spotted ordered assembly (month 8)

Nanopore chips with short-range ordered nanopores in the range 100-200 nm in diameter have been fabricated by defining the pattern using polystyrene nanoparticles adsorbed to the chip surface. Additionally, two new methods for particle assembly suitable as lithographic masks have been successfully developed. In the first method, particle suspensions were spotted onto a substrate in order to create 100-micron-sized spots with hexagonally ordered nanoscale circular features defined by nanoparticles assembled by capillary forces in the drying drop. Thus, this technique allows the production of nanopore sensing features in defined areas by a semi-parallel, cost-effective method. In the second method, particles were assembled at liquid-liquid interfaces and successfully transferred to the substrate material. This method has the great advantage over previously described methods for particle assembly at solid substrates, that patterns with short- and medium range order can be achieved (a) in a highly efficient parallel process, (b) over arbitrary large surfaces, and (c) with almost perfectly controlled nearest-neighbour spacing distances that can moreover be reproducibly varied over more than an order of magnitude. The two particle assembly processes have been combined with previously developed and now refined methods for etching nanopores in silicon nitride window chips, and could provide the basis for producing high quality, cost-effective nanoporous membranes needed in the industrial application.

D1.1.4 Process designed for industrial production of nanostructured (nanoporous) chips (month 30)

Partner 1 (ETHZ) has developed a new approach to particle lithography which allows nanoscale patterns to be created at low cost over large areas, with the nanoscale features spaced more than 10 diameters apart. The new method abbreviated SALI particle lithography makes use of assembly of nanoparticles at an oil-water interface and subsequent transfer of the particle mask to the substrate to be patterned. This process was optimized for features from 40-500 nm, scaled up to 2" wafers and integrated in a process flow for production of nanopore sensor chips for the ASMENA project with the company Porenix AB. Within ASMENA the process flow still includes the particle lithography step at the ETH partner, while the pre- and post-processing of the sensor chips is performed in Porenix fab. The entire process is cost effective on the level of current chips in the Porenix process portfolio. The chips are produced for and used within the other WPs of ASMENA, and the processing therefore also includes initially not foreseen steps aiding in the assembly of the molecular toolkit in WP2 as well as membranes in WP3 and WP5. This is further described in the reports for those work packages. The SALI process for nanoscale lithography including the application to nanopores has been published and features as method to produce nanopore substrates in one additional publication on nanopore-spanning lipid bilayers. The Porenix chips are also used in manuscripts on more advanced nanopore-spanning lipid bilayer preparations in manuscripts currently under preparation. [Particle lithography from colloidal self-assembly at liquid-liquid interfaces, L. Isa, K. Kumar, M. Müller, J. Grolig, M. Textor and E. Reimhult, ACS Nano 4,(2010):5665-5670], [Membrane biosensor platforms using nano- and microporous supports; E. Reimhult and K. Kumar, Trends in Biotechnology, 26,(2008):82-89]

Partner 3 (CHALMERS) has developed a particle lithography method for the fabrication of localized surface plasmon resonance (LSPR) active nanoscale apertures that span through optically transparent substrates with a thickness of between 200 and 500 nm. The process was developed in the cleanroom at MC2, Chalmers, and has been included in the Porenix AB process portfolio. [Locally Functionalized Short-Range Ordered Nanoplasmonic Pores for Bioanalytical Sensing; M.P. Jonsson, A.B. Dahlin, L. Feuz, S. Petronis; Analytical Chemistry, 82(5);(2010):2087-2094]

D1.2.1 Nanopore chip for combined LSPR and EC sensing (month 14)

A parallel fabrication scheme for the production of Localized Surface Plasmon Resonance (LSPR) nanopore chips has been successfully developed as a tool for future, combined LSPR and EC sensing. Sensor chips with short-range ordered nanoplasmonic pores were created. The method was optimized with respect to

yield and optical properties. A setup for microextinction measurement on the chips was developed and optimized. The sensor chips were successfully evaluated with respect to specific detection of protein binding to the pores in a flow-through format.

D1.2.2 LSPR imaging with the optimal resolution for practical applications (month 24)

Readout of LSPR active substrates with a spatial resolution limited by the diffraction limit have been explored, leading to the conclusion that optimal resolution is obtained at between $2\ \mu\text{m} \times 10\ \mu\text{m}$ and $10\ \mu\text{m} \times 50\ \mu\text{m}$ [REF: Dahlin AB, Chen S, Jonsson MP, Gunnarsson L, Kall M, Hook F: High-Resolution Microspectroscopy of Plasmonic Nanostructures for Miniaturized Biosensing. *Analytical Chemistry* 2009, 81:6572-6580.]. This is significantly larger than the diffraction limit, but not critical from the perspective applying the concept to the defined goals. The chips designed for combined LSPR and EC sensing have been designed by taking these criteria in consideration.

D1.2.3 Optimized nanopore chip for combined array LSPR and EC sensing (month 36)

Nanoporeos chips compatible with combined LSPR and EC sensing have been fabricated [REF: Jonsson MP, Dahlin AB, Feuz L, Petronis S, Hook F: Locally Functionalized Short-Range Ordered Nanoplasmonic Pores for Bioanalytical Sensing. *Analytical Chemistry* 2010, 82:2087-2094.] and applied to investigate the limit of detection with respect to biomolecular binding. Using optimal readout conditions (see D1.2.2) a limit of detection comparable with conventional SPR was obtained. As outlined in D.1.2.6, a device for EC measurement using the standard-sized was developed and tested

D1.3.1 Microfluidic device for stationary gradients (month 18)

A microfluidic device was designed and successfully fabricated using PDMS moulding on a photolithographically defined master. Stationary concentration gradients of sample material in the channel as a function of time or spatial position could be achieved by controlled diffusion of molecules between parallel laminar flows. The flow rate of each of the parallel flows can be varied independently leading to switching of the sample volume over a well-defined detection area. Integration of the microfluidic system on a LSPR chip was successfully demonstrated, thus allowing for label-free sensing at rapid liquid exchange on a detection area of a few μm^2 compatible with the sensor setup. Currently, exchange rates are as low as <10 ms. Exchange times of 1 ms are within reach.

D1.3.2 Microfluidic device for transient chemistry – intermediate time scales (month 24)

A microfluidic device was fabricated using soft lithography. The device was constructed to allow controlled concentration gradients and rapid (< 10 ms) liquid exchange over sub $50\ \mu\text{m} \times 10\ \mu\text{m}$ measurement areas (see D1.2.1). Compatibility with LSPR and fluorescent readout was verified, and applied to probe passive and membrane-protein facilitated transport of uncharged solutes [REF: Olsson G, Tabaei S, Jonsson U, Kjellbom P, Tegenfelt J, Hook F: Sub 10 ms liquid exchange for permeation monitoring of single proteoliposomes. *Lab on Chip*, in writing]. The proteoliposomes were immobilized using kits provided by Layerlab AB (partner 9).

D1.3.3 Microfluidic device for transient chemistry – ultrashort timescales (month 36)

Mixing is induced by diffusion of the sheath flows through the sample flow as illustrated in for a fluorescent dye being mixed with pure water in Figure 2.2.2 (a). Due to the short diffusion distance in the narrow mixing region, the mixing occurs on a very short time scale. In the present system mixing times of 1-2 μs are observed see Figure 1.2 (b) below. By regulating the flow this can be decreased another order of magnitude (to the order of 100 ns or less).

Figure 1.2 (c) shows POPC liposomes (containing 40 μM calcein in 10 mM TRIS + 100 mM NaCl) that were fed at a flow rate of 1 nl/s through the central channel. The sheath flows contain 10 mM TRIS + 500 mM NaCl and were fed at the same flow rate (1 nl/s). When these mix with the sample in the mixing region an osmotic pressure will rapidly be applied on the liposomes causing water efflux, resulting in volume shrinkage and fluorescent quenching. Since the monitored intensity variation depends both on water efflux

and flow velocity, the trace of the former can be extracted after compensation of the effect of the latter. Figure 1.2 (d) shows the intensity times the flow velocity profile as a function of time for one single liposome, normalized to the initial value (from the blue square to the green circle, background subtracted). Before the mixing region this normalized intensity value is constant, while it decreases after the mixing. The curve can be fitted to a mono-exponential function, allowing the time constant for water transport to be calculated. The value is in agreement with literature data.

In the mixing region the velocity is ca 100 times higher than initially and the intensity is in the order of the noise level, which explains the spiky characteristics around that region.

Figure 1.2 (d) below has a time resolution of 100 μs in this specific case, which is faster than the 1 ms mixing time envisioned in D1.3.3.

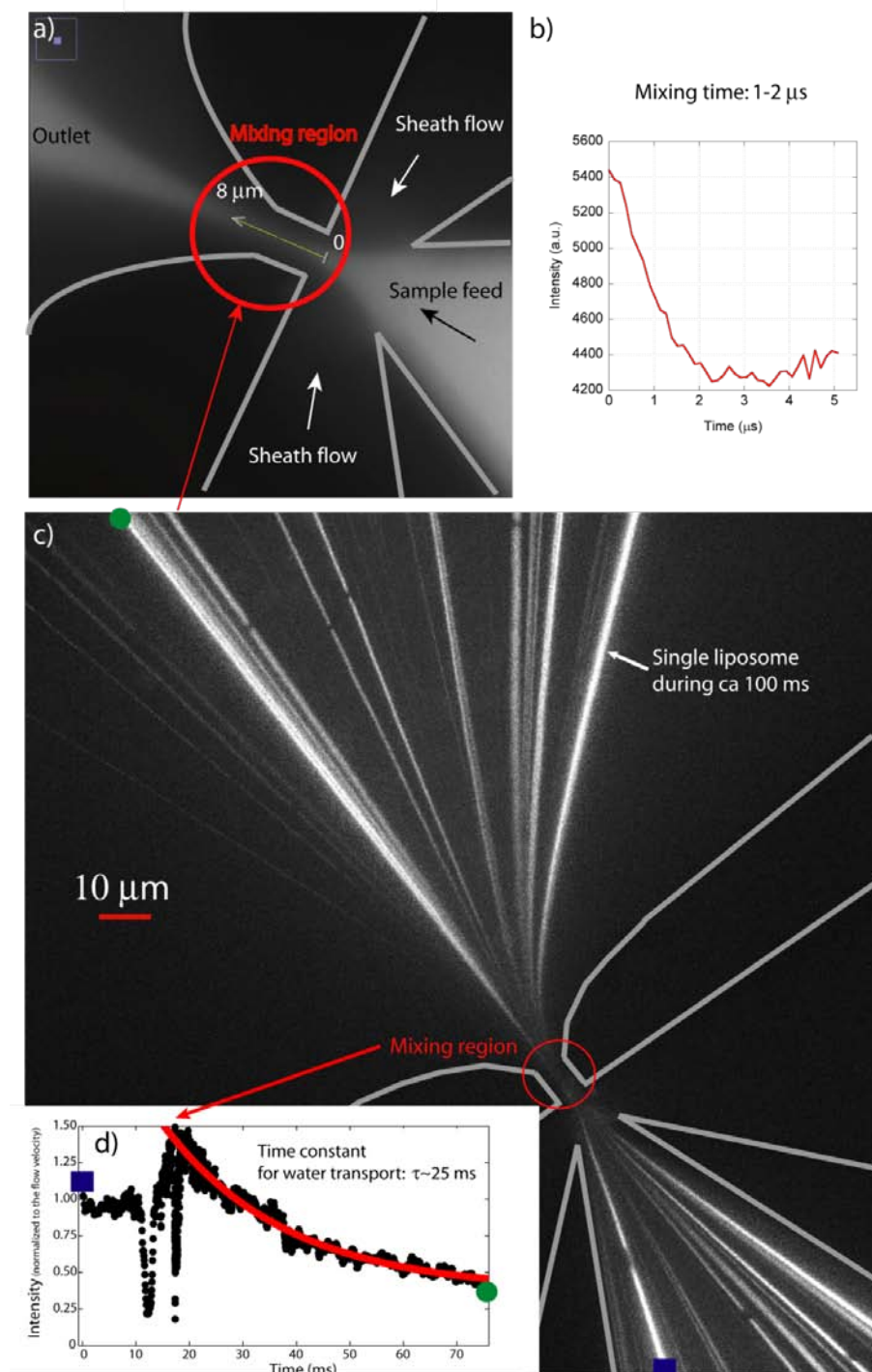


Figure 1.2: Proof of concept for ultrafast mixing in the context of monitoring transport events across lipid membranes.

1.3.2 WP 2: Chemical surface functionalisation of nanoscale features for guiding assembly and sensor integration of (proteo)lipid membranes by function

WP Leader: Erik Reimhult, ETHZ

In order to ensure that lipid membranes incorporating different membrane protein function can be assembled on selected spots on a sensor array, strategies for guided and triggered self-assembly on the nanostructures was developed. Chemical functionalisation of the surface was used also to ensure correct membrane protein orientation and position in the evanescent field, as well as no diffusion outside the sensing spot and high electrical resistance seal, for which new polymeric tethering strategies were developed.

Summary of progress

In the first 18 months of the project, new biomimetic anchor chemistry for assembling non-fouling, polymeric thin films (monolayers) and incorporation of sites for specific binding of liposomes has been successfully developed. These molecules were synthesized, fully characterized and the self-assembly conditions optimized for application with the specific materials used in the design of ASMENA chips. The key new development for objectives 2.1 and 2.2 is the use of nitrocatechol derivatives as anchors for poly(ethylene glycol) (PEG) brush layers on several relevant metal oxide, in particular TiO₂ surfaces. Nitrocatechols were found to be exceptionally strong and stable anchors for metal oxide surfaces in comparison to the state-of-the-art catechol (dopa) anchor systems. Further biofunctionalization of these polymers for conjugation to biological species include a range of (bio)chemically active moieties such as biotin, NTA and azides.

The nitrocatechol-PEG(-functional group) monolayers have been applied in an orthogonal fashion regarding substrate/material type, i.e. using surface modification methods that are specific to one or the other substrate chemical composition. Using our advanced toolbox of surface-active molecules and polymer, we successfully demonstrated, for example, the feasibility of site-specific formation of lipid bilayers on SiO₂ surfaces, non-fouling PEG films on Au and biofunctional groups on TiO₂ hole/pore structures. Additional linking of liposomes at sensing hot spots in these structures has been demonstrated using biotin-neutravidin linkage which is easily extended to addressed DNA linkage on an array chip.

Stimulus responsive polymer brushes grafted from substrate with tunable surface energy can be used to guide liposome adsorption and lipid membrane formation. Here, surface-initiated atom-transfer radical polymerization (SI-ATRP) has been used to synthesize polymer layers that respond to variations of pH on the ASMENA nanoporous platforms. Controlled radical polymerization methods provide a robust and reproducible tool for surface modification. Surface-grafted layers of poly(methacrylic acid) (PMAA) show a strong pH-dependent response, with a swelling/collapsing behaviour that can be triggered in a pH range near physiological conditions, where protein functionality is optimal. The protocol for functionalization of the nanoporous ASMENA substrates has been optimized by varying the aqueous medium of polymerization for optimal growth conditions at the nanopore level.

For the second half of the ASMENA project work continued in WP2 to adapt these deliverables to the specific requirements that materialized during work on deliverables in other work packages. There were also suggestions for new surface modification strategies that evolved during the work on ASMENA, which through decisions at ASMENA General Assemblies were added to the work portfolio of the WP2 partners. The partners in WP2 delivered molecular functionalization kits and functionalized chips to other consortium partners. Successful work also continued on further development of responsive polymer grafting in nanopores, which demonstrated that nanopores could be opened and closed by environmental stimuli.

A major task evolved through the realization that the originally proposed method for assembling nanopore-spanning lipid membranes by vesicle fusion onto nanopore chips which had unfunctionalized pore interiors intrinsically did not result in sufficient fraction of spanned pores (results published). We thus had to further develop the level of specific nanopore functionalization to control membrane assembly in, around and over the pores, which was originally just hypothesized as possible add-on work. This work included both a new platform additional to the original deliverables for molecular assembly developed by SuSoS and a nanoscale

molecular patterning technique developed additionally by ETH Zurich. Also Layerlab participated in this additional and successful research and development.

In Summary, all deliverables of WP2 were fulfilled and exceeded, as deemed necessary for successful accomplishment of deliverables in WP3 and WP5. A platform for surface functionalization of nanostructures in general and nanopores in particular which allows nanoscale control of the positioning of functional polymers, liposomes and proteins was developed and fully characterized. Additionally, response brushes with and without functionalization could also be selectively grown in and on the nanopore structures. These methods are applicable to the whole range of surface materials present in sensor platforms such as SiO₂, Si₃N₄, TiO₂ and Au. Annexin fusion proteins for use of sorting liposomes and membrane fragments into arrays in substrates were created and their function demonstrated.

Main significant results

We have developed catechol-derived anchors for assembling monolayers with a controlled biomolecular response using a grafting-to-technique. They have been shown to perform vastly superior to previously described methods in terms of both simplicity of use and performance demonstrated in several publications within and without ASMENA already published or in preparation. Optimization of the anchor and protocol has furthermore led to identification of low cost versions of the molecule suitable for biosensor applications. Efficient synthesis routes have been established and applied by industrial partner SuSoS, including a large variety of functional groups, which are available to ASMENA for specific binding/localization of membrane and membrane proteins. Furthermore, SI-ATRP has been shown applicable to synthesize responsive polymer films inside nanopores.

In the second half of ASMENA four main significant results were obtained. First, the development of a general grafting method built on the published PFPA linking strategy from SuSoS as a way to create both non-fouling PEG and functional PEG-biotin surfaces for coupling of liposomes to a non-fouling background. This method was adapted and proven for nanostructured surfaces as well. Second, a molecular lift-off strategy applicable to nanopores allowing selective functionalization of the pores to control their surface energy, adhesion properties and inserting specific binding sites was developed. Third, this method applied to patterning of initiators for radical polymerization allowed for growing responsive brush structure for local control of wetting of membranes on the nanopores. Fourth, a range of Annexin fusion proteins were created and their function demonstrated.

Deviations from Annex I

The objective of deliverable D2.3.3 was achieved and surpassed. However, this was done through a new nanoscale patterning method for molecular overlayers that was developed within ASMENA. The fulfilment of the deliverable with this method and the reasons why this method was chosen as superior is described in the summary of D2.3.3 below.

Achieved results and Deliverables

D2.1.1 Non-fouling PEG-based molecules with high affinity end groups for different oxides and expressing functional groups at the other terminus (month 6)

Catechol derivatives as anchor groups have been shown to provide high affinity for metal and metal oxide substrates. They were used as anchors on PEG-chains to serve as self-assembly systems on metal oxide substrates, providing stable polymeric surface films that exhibit very low non-specific adsorption of (bio)molecules. In total, 7 different surface-active molecules/polymers were synthesized, purified and characterized, two of which were end-functionalized with a biotin conjugation to biological molecules and/or liposomes.

All the active compounds described herein are available for the ASMENA participants and have already been used successfully by partners. Additional end-functionalities can now be synthesized on demand through established protocols. Additional end-functionalities can be synthesized on demand through established protocols.

D2.1.2 Protocol for SAM with controlled and selective protein and liposome interaction on nano-structured surfaces (month 12)

Thiol-poly(ethylene glycol) (thiol-PEG) and catechol derivative-PEG polymers were successfully investigated as self-assembly systems for the surface functionalization on gold (Au) and metal oxide surfaces, respectively. Thiol-PEG monolayers on Au exhibited close-to-zero non-specific adsorption of both proteins (<5 ng/cm² adsorbed mass) and liposomes (<0.1% surface coverage). Among all catechol derivatives studied, the novel nitrocatechols (nitrodopamine and nitroDOPA) anchors linked to PEG were found to bind exceptionally strong to metal oxide surfaces under cloud point assembly conditions, including to TiO₂, an important substrate material and surface coating for ASMENA chips.

A novel class of polycationic poly(L-lysine)-graft-PEG copolymers with a fraction of the free lysine groups covalently linked to catechol derivatives was found to provide stable, non-fouling surfaces on silica (SiO₂) surfaces when assembled from aqueous solution at 50-70°C.

D2.2.1 Protocol for patterning method for SAMs with different functional groups on the surface and in the nanopores (month 18)

Protocols for the formation of monolayers of PEG brushes developed in Deliverable 2.1.2 were tested for the orthogonal patterning of nanostructured substrates exhibiting a materials contrast of Au, TiO₂ and/or SiO₂. The developed protocols were shown to successfully allow the conversion of the heterogeneous inorganic surface into a biologically relevant contrast of (functionalized or non-functionalized) PEG brushes on one type of surfaces (e.g. PEG-catechols on TiO₂) and supported lipid bilayers on the other (e.g. on SiO₂). Specifically, we applied these protocols to site-specifically direct polymer adsorption into the nanopore structures as verified in Deliverable 3.1.1, and to the nanoplasmonic sensor platform.

D2.2.2 Protocol for lipid membrane sorting system based on Annexin fusion proteins (month 24)

Various types of Annexin fusion proteins have been produced for this purpose, which are made of an Annexin5 moiety coupled to a molecular recognition element. Coupling was achieved either at the cDNA level by molecular biology or by covalent chemistry between two reactive groups. The immobilization of liposomes and proteoliposomes on Annexin fusion proteins has been characterized by QCM-D. A standard protocol consists in 1) forming a supported lipid bilayer on a silica-coated sensor, 2) adsorbing a protein monolayer made of a dimer of Annexin5 obtained, 3) sorting liposomes containing phosphatidylserine, which is recognized specifically by Annexin5.

D2.3.1 Responsive polymer brush coatings (month 12)

Strategies for grafting of responsive polymer brushes were developed on planar silicon and gold surfaces. Using controlled surface-initiated atom transfer radical polymerization (SI-ATRP), pH-responsive polymer brushes of poly(acrylic acid) (PAA) or poly(methacrylic acid) (PMAA) were grown on silicon surfaces in aqueous medium at room temperature. The pH-induced swelling and collapse of the brushes were observed in situ by atomic force microscopy in liquid environment upon varying the pH of the buffer solution. The dissociation behaviour of the polymer layers were characterized by FTIR after incubating the polymer-grafted substrates in solutions with different pH values. To investigate the role of initiator surface coverage on the kinetics of growth, surface morphology, and nanoscale mechanics of the polymer brushes, PMAA layers were grafted from gold surfaces by controlled photopolymerization and the composition of the self-assembled monolayers (SAMs) of initiating molecules was varied systematically using a diluent agent.

PMAA layers on silicon surfaces showed a strong pH-dependent behaviour, with an effective pK_{abulk} of the polymer in the brush larger than pK_{asurf} due to ion confinement effects. The shifting of the effective pK_{abulk} of pH-responsive brushes is especially relevant for the screening platforms under development in the ASMENA project as swelling and collapsing of the polymer brush (that is pore switching), can be triggered in a pH range closer to physiological conditions, where protein functionality is optimal.

D2.3.2 Protocol for functionalization of nanopores with responsive polymer brush (month 18)

Protocols for functionalization of nanoporous substrates with the stimulus-responsive polymer brushes developed in Deliverable 2.3.1 were investigated. Surface-initiated atom-transfer radical polymerization (SI-ATRP) was conducted in mixtures of methanol and water to improve the wettability of the nanopores that are coated with hydrophobic initiator molecules. PMAA brushes that respond to variation of pH were grafted from substrates coated with a nanoporous layer of silicon nitride with pores 200 nm and 500 nm in diameter. The pH-induced opening and closing of these pores was investigated by atomic force microscopy. We have used surface-initiated atom-transfer radical polymerization ("grafting from" method) to create responsive polymeric structures on nanoporous substrates that are able to open and close pores in response to variations of pH. These results are useful for control of ion permeation through the nanoporous platforms under development in the ASMENA project.

D2.3.3 Chemically nanopatterned nanopore substrates (month 30)

In the development of the patterning of nanoporous chips a new method of performing molecular patterning of nanopores suitable for the guiding of membrane and protein assembly onto the pore sensor structures was discovered. The demonstrated method is broadly applicable to both physisorbed and chemisorbed polymer modifications specifically within or outside the pore, and uniquely fulfils the requirements of fabrication put forth in the ASMENA project. The method makes use of that a thin metal chrome mask is deposited after particle masking to define an etch mask for the high aspect ratio nanopores. After opening of the nanopores the chip is ready for polymer functionalization. Within this project we have developed a range of methods to immobilize liposomes and proteoliposomes on the nanopores using first poly(ethylene glycol) (PEG) brush passivation with some of the PEG carrying functional groups such as biotin. A pure PEG brush prohibits the adsorption of liposomes onto the structure or around the structure depending on where the brush is assembled. Through the functional group, e.g. biotin-streptavidin-DNA-DNA-cholesterol as in the Layerlab kit, liposomes can be guided to specifically adsorb onto the nanopore if this is desired.

Our new lift-off method for polymer patterning works by that the PEG (functional or non-functional) polymer coating is assembled onto the entire chip. By a mild acid etch which does not damage the polymer coating on the <10 min time-scale (demonstrated by ellipsometry, XPS and TInAS) the Cr layer that is covering the top surface is removed and the polymer coating on top is removed with it. This leaves a clean chip surface that can be used directly for supported lipid bilayer assembly or a second step of polymer functionalization. The new lift-off method, which is the first of its kind to produce nanoscale molecular patterns by self-assembly, has been tested to work for all the polymer functionalization protocols used and developed within ASMENA (e.g. physisorbed PLL-g-PEG, chemisorbed nitrocatechol-PEG and PLL-PFPA-PEG brushes) as well as on proteins attached to this structure retaining their native binding affinity.

We applied this nanoscale polymer patterning method in place of the originally proposed serial dip-pen lithography to modify the surface energy of the pores and control the assembly of nanopore-spanning lipid membranes and specific positioning of liposomes over nanopores, since the new method is parallel and can be performed on the wafer scale, while the, for these applications, inferior dip-pen lithography method in practice only allows functionalization of a few pores on a chip. It should also be noted that the new method can and was integrated directly in the process flow of the industrial chip manufacturing.

Additionally, the same novel patterning method was applied to create nanoporous films with pH-responsive polymer brushes in the vicinity of the pore opening. Initiator molecules used for grafting of polymer brushes via surface-initiated atom transfer radical polymerization (SI-ATRP) do not bind to the Cr layer, this strategy allowed to functionalize the inner walls of the pores and their vicinity with brushes of poly(methacrylic acid) (PMAA).

Besides nanopatterning of the nanopores, pH-responsive PMAA brushes were coupled with nitrilotriacetate (NTA) using EDC/NHS activation of the carboxylic acid groups. This approach can be used to guide the localization of His-tagged membrane proteins around the edge of the pores. Liposomes with His-tagged proteins can thus be guided to fuse around the edge of the functionalized nanopores. For this purpose nanoporous chips functionalized with PMAA-NTA were sent to PSI (partner #2) to perform experiments

involving spreading of proteoliposomes over the pores [G.W. de Groot, S. Demarche, M.G. Santonicola, L. Tiefenauer, G.J. Vancso, "Smart polymer brush structures for guiding the assembly of pore-spanning lipid membranes", manuscript in preparation].

1.3.3 WP 3: FORMATION OF PLANAR LIPID BILAYERS ON NANOSTRUCTURED SURFACES

WP Leader: Janos Vörös, ETHZ

WP3 focused on bringing together the technology platforms developed in WP1 and WP2 in order to achieve spontaneous formation of nanopore-spanning lipid bilayers (npsLBs) through induced fusion of vesicles, which was instrumental to investigations in WP5 and WP6. In addition, a combined experimental and theoretical approach was used to explore the parameters that determine the long-term stability and electrochemical seal of proteolipid membranes which are critical limiting factors for the industrial applications.

Summary of progress

During the first 18 months deliverables 3.1.1 and 3.4.1 were achieved on time according to the plan.

In the second half of the project WP3 was focusing on the formation of spontaneously formed, electrically sealing membranes over nanopores. This is required to fulfil the condition of easy manufacturability with high-throughput. In order to facilitate the spontaneous vesicle rupture process and to enhance the stability of the formed pore-spanning membranes the nanopores were filled with a polymer. Two different approaches were successfully demonstrated: the polyelectrolyte multilayer filling described under D3.2.1 and the stimulus responsive polymer filling described in D.3.3.1 and D3.3.2. In addition, planar lipid bilayers from proteoliposomes were also targeted using the giant unilamellar vesicles D3.2.2. Finally, these efforts were continuously supported by the theoretical partner #6 (ELTE) who provided predictive tools throughout the optimisation process. In summary, all the deliverables have been fulfilled as specifically described below.

Main significant results

Nanohole and nanopore topographical structures with substrate materials contrast were used to successfully demonstrate that orthogonal surface functionalization can be used to position liposomes over sensing structures with high accuracy, exploiting the selective surface modification schemes developed in WP 2.

The experimental strategies and work were complemented by theoretical work on energetic aspects of the interaction between lipid bilayers and solid surfaces, and its dependence on interfacial charge and charge redistribution.

The formation of proteoliposomes into planar membranes could be induced using spreading of lipid bilayers into proteoliposomes positioned into a nanopore. In addition, a new technique, the shear-flow driven motion of bilayers has been invented by partner 3 (Chalmers) and could be adapted to achieve the same goal. Polyelectrolyte filled nanopores provided an outstanding platform for achieving bilayers with Gigaseal and could be used to record the ionic currents through single peptide pores for over two weeks continuously. The stimulus responsive pore-function has also been successfully demonstrated providing unique opportunities for the development of controlled filtering mechanisms. The theoretical framework of induced bilayer formation has been developed, pointing out the importance of unexpected factors such as surface roughness and kinetic effects helping the work of the experimental partners.

Achieved results and Deliverables

D3.1.1 Protocol for positioning of labelled liposomes over predefined nanopores (month 12)

Nanohole and nanopore topological structures with materials contrast were used to successfully demonstrate that orthogonal surface functionalization can be used to position liposomes over sensing structures with high accuracy. In particular, a surface contrast of thiol-poly(ethylene glycol) (thiol-PEG) and nitrocatechol-PEG was exploited for the orthogonal patterning. To selectively bind liposomes at pores biotin-lipid containing vesicles docking through neutravidin bound to specifically adsorbed PEG-biotin was used. A steric constraint from liposome vs. pore size was observed for the liposome capture.

D3.1.2 Protocol for the induced rupture of proteoliposomes into a planar membrane over nanopore (month 24)

Partner 1 (ETHZ) in collaboration with Partner 2 (PSI) has developed several protocols during ASMENA which reached the objective of D3.1.2, using liposome fusion or the new technique of shear-driven membranes. First, it was shown that the spreading of a lipid membrane on a flat silicon oxide substrate can be used to rupture pre-placed proteoliposomes and even to further directionally move proteins within said supported lipid bilayer.

The easiest and most robust way to fuse liposomes into membranes specifically over a nanopore without filling the pore was based on the concept described in the following. Nanopores were first modified with the method described in WP2 to selectively graft PEG-brushes onto the walls of the nanopores, but leaving the top surface of the chip free. The addition of a dense PEG-brush to the nanopore wall makes it energetically unfavourable for a lipid membrane to form along the wall of the nanopore. Addition of liposomes fusing to the fusogenic top silicon oxide surface of the measurement chips or driving of membranes across the surface thus cannot result in membranes going into the nanopores. Without such functionalization the predominant occurrence is that the membrane enters the pores and follows the nanopore walls, as described in two publications on vesicle fusion and shear-driven membranes, respectively, on nanoporous substrates. [Formation of nanopore-spanning lipid bilayers through liposome fusion, K. Kumar, L. Isa, A. Egner, R. Schmidt, M. Textor and E. Reimhult, *Langmuir*, 27, (2011): 10920-10928]

As predicted in the theoretical work within ASMENA there is a critical pore size above which a membrane will circumvent a nanopore with membrane repulsive walls rather than form a spanning membrane across it if an unrolling membrane is approaching a nanopore. We showed that this can be reproducibly obtained for >100 nm nanopores, which is a suitable size for nanopore-spanning membranes. Thus, we could use this method both with shear-driven membranes and with fusion of liposomes smaller than the pore opening to form a completely surface-covering membrane, but which left all the pores on the substrate open. As the pores were uncovered after this preparation, high concentrations of proteoliposomes or proteoliposomes with larger diameters than the nanopore opening could then be added in a sequential step which fused the vesicles above the nanopores only. Nanopore spanning membranes over all pores could thus be formed and the localization of the new membrane material to the nanopore was demonstrated by complementary fluorescent labelling of surrounding lipid membrane and proteolipid membrane. The method, fully characterized and including the theoretical basis for the mechanism is being prepared for publication. [Membrane positioning and sealing on nanopore sensors; M. Pla Roca, S. Kresak, K. Fender and E. Reimhult, in preparation for *Angewandte Chemie*]

Alternative, but complicated, protocols make use of pre-placement of liposomes on the pores with residual liposomes also binding non-specifically to other parts of the surface and rupture of the proteoliposomes by the advance of a shear-driven membrane across the pores. Also in these cases the pores are first functionalized by repulsive PEG-brushes but including specific binding sites for the liposomes. Non-specific background adsorption of liposomes could be reduced by, e.g., playing with electrostatic repulsion and pH.

D3.2.1 Protocol for the spontaneous formation of sealing membranes over nanopores (month 36)

Although the direct formation of pore-spanning (as such also sealing) lipid bilayers over nanopores has also been demonstrated in the publications listed above, the real success for the electrical sealing membranes has been achieved using polyelectrolyte multilayer filled nanopores. A lipid bilayer with gigaohm resistance was fabricated over a single 800-nm pore in a Si₃N₄ chip of Partner 8 (Leister) from WP1 using 50-nm liposomes. The sample fabrication process and the electrical performance test of the system are illustrated in the schematic in Figure 3.1.

Pore-forming peptide melittin was incorporated in the bilayer and single channel activities were monitored for a period of two and a half weeks. It is among the longest lifetime of pre-spanned lipid bilayer. The long lifetime of the system enabled the observation of the time-dependent stabilization effect of the melittin open state upon bias application. [K. Sugihara, J. Vörös and T. Zambelli, "A Gigaseal Obtained with a Self-Assembled Long-Lifetime Lipid Bilayer on a Single Polyelectrolyte Multilayer-Filled Nanopore", ACS Nano 4, 5047-5054 (2010).]

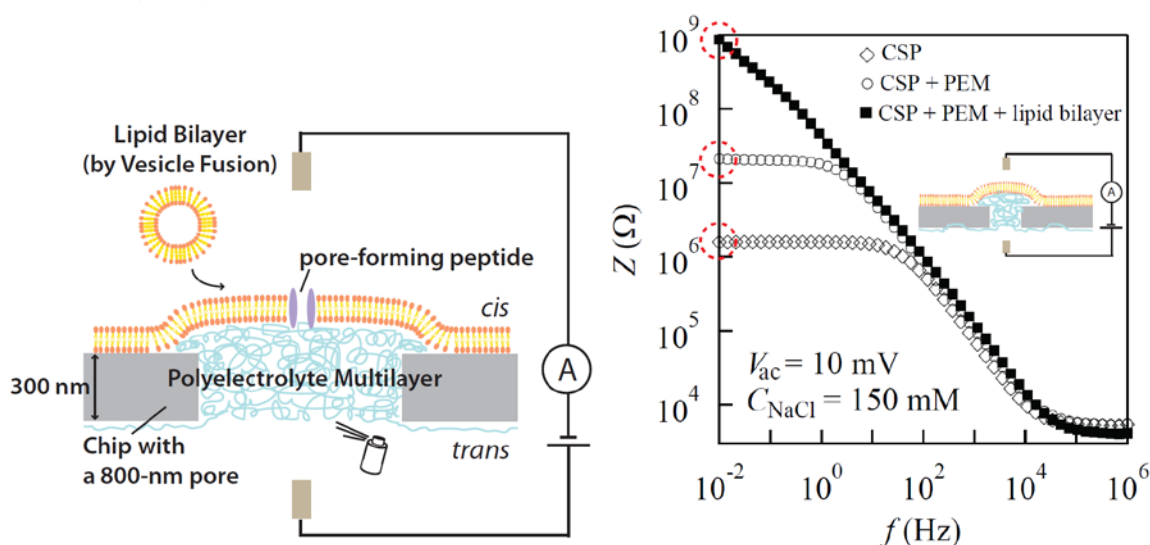


Figure 3.1. Schematics and impedance spectra of spontaneously formed nanopore spanning lipid bilayers.

D3.2.2 Protocol for the formation of protein containing bilayer over the nanopore using giant unilamellar vesicles (month 30)

Our strategy of formation of protein-containing npsLBs using giant liposomes (GLs) has focused on spanning the npsLBs from proteo-GLs. The main objective has thus become to obtain *floating* unilamellar GLs with membrane channels functionally reconstituted into their envelope. Floating is emphasized as many protocols have been reported to yield proteo-GLs, yet most of these remain adhered to a supporting surface, whereas detached mobile proteo-GLs are required for our purpose. Proteo-GLs have been targeted by three different methods: 1) partial dehydration of small proteoliposomes on conductive surfaces and formation of proteo-GLs at rehydration induced by electric-field, 2) partial dehydration of small proteoliposomes and spontaneous swelling thereof into proteo-GLs at rehydration, and 3) fusion of small proteoliposomes to pure lipid GLs. Method (1) has failed in general, as electroformation of GLs is generally incompatible with physiological media, which, on the contrary, are crucial to preserve the functional state of the membrane protein. In the rare instances where (1) works, the resulting proteo-GLs remain adhered to the electrodes. (2) has been found to yield reasonable amounts of (often multilamellar) floating GLs and other large membrane structures at physiological conditions. (3) has generated mostly unilamellar floating GLs exhibiting significant envelope fluorescence after incubation with small proteoliposomes with fluorescently-labelled lipids, again in physiological media. Electrophysiological detection of membrane channels in GLs yielded by methods (2) and (3) has been done by glass micropipette patch clamp. Until recently, the progress has been delayed by the unavailability of proteoliposome preparations of a relevant membrane channel that would be free of unspecific channel

contaminants and where the specific functionality would verifiably comply with published data. Shortly before the end of the project the KvAP bacterial voltage-gated potassium channel has been engineered, produced and reconstituted into classical BLMs in our laboratory. Specific labeling of a cysteine residue in the sequence of the channel by a fluorescent dye allowed us to quantify the extent of channel reconstitution into GLs by methods (2) and (3). The protocol of proteo-GL formation has been optimized to yield maximum channel density in the liposomal envelope. Proteo-GLs generated by this protocol were allowed to unfold over and seal single pores in Si₃N₄ diaphragms. Characteristic KvAP currents were observed in electrophysiological voltage clamp experiments upon potential steps that are known to activate the channel in classical BLMs. (Ruta et al., 2003, Nature, doi:10.1038/nature01473)

D3.3.1 Polymer brush supported pore-spanning membranes for enhanced membrane stability and storage capability (month 24)

Planar surfaces functionalized with zwitterionic sulfobetaine-based polymers were investigated as possible platform for support of functional lipid membranes. Specifically, we used surface-initiated atom-transfer radical polymerization (ATRP) to graft well-defined and robust polymer layers where DOPC membranes could self-assemble from fusion and spreading of liposomes. By controlling the polymer architecture using ATRP, we show that formation of a stable neutral phospholipid membrane can be achieved on a charge-balanced zwitterionic polymer cushion covalently attached to a surface. We demonstrate that our approach allows one to obtain a tuneable hydrophilic polymer interface to facilitate the assembly of neutral lipid bilayers with high mechanical stability and reproducibility on various synthetic materials.

The kinetics of lipid vesicle adsorption and membrane formation was investigated on polymer-modified gold surfaces under different ATRP conditions by surface plasmon resonance spectroscopy. Figure 2.2.4 shows a comparison of liposome adsorption on poly(SBMA)-modified gold surfaces, with polymerization times of 15 and 30 min using phosphate buffers where the ionic strength was varied by addition of 150 mM KCl. When poly(SBMA) surfaces synthesized for 15 min (water contact angles of $31^\circ \pm 2^\circ$) were incubated in a DOPC vesicle solution, an adsorption and subsequent rearrangement of the vesicles on surface can be observed by SPR in both phosphate buffer solutions. The self-assembled lipid membranes were very stable upon rinsing with buffer, and samples could be used for imaging by atomic force and fluorescence microscopy for several days. The polymer-grafted surfaces could be regenerated by rinsing with a mild surfactant solution (DDM, 0.1% by weight) in the same buffer used for the lipid membrane formation (Figure 3.2a).

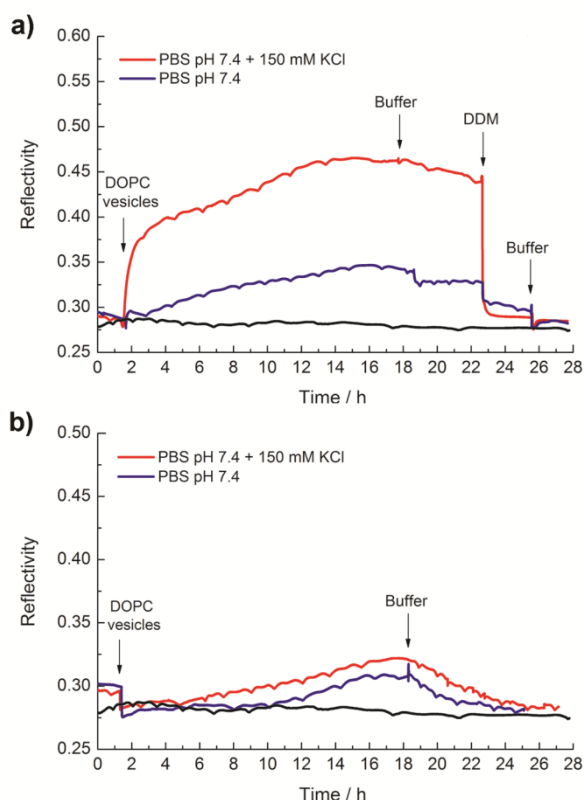


Figure 3.2 Left: Kinetics of DOPC vesicle adsorption and membrane formation on poly(SBMA) layers in phosphate solutions with different ionic strengths (by addition of KCl) followed by SPR spectroscopy. Polymer layers grafted from initiator-coupled gold surfaces using ATRP ($[SBMA] = 0.5\text{ M}$, $[CuI]/[CuII] = 3$, methanol/water 4:1 v/v) for 15 min (a) and 30 min (b). Right: Scheme for grafting of zwitterionic poly(SBMA) from initiator-coupled surfaces using atom-transfer radical polymerization (ATRP).

These results indicate that stable phospholipid membranes do not assemble on highly hydrophilic surfaces, such as zwitterionic poly(SBMA) layers with swollen thickness values of approximately 35 nm, whereas they form on the thinner poly(SBMA) layers. This behaviour can be related to the lower density of the zwitterionic groups in poly(SBMA) layers grafted for short times, as indicated by the larger values of water contact angle measured on these layers with respect to the thicker ones.

Grafted polymer layers and supported lipid membranes were characterized in fluid environment by atomic force microscopy (AFM) operated in Peak Force Tapping™ mode. Recently, the enhanced force control accuracy of Peak Force Tapping mode™ has made imaging at low interaction forces and without lateral friction possible, thus allowing non-destructive imaging of very soft samples such as polymer-supported lipid bilayers in liquid environment. In addition, this mode allows for high-resolution quantitative mapping of mechanical data, among which deformation and adhesion, with conventional AFM imaging speed. Figure 3.3 shows high-resolution height and deformation maps, and typical force curves by Peak Force Tapping mode™ for the poly(SBMA) layer and the corresponding supported DOPC membrane in phosphate buffer (50 mM phosphate, pH 7.4) with 150 mM KCl. As expected, we observed larger deformations (Figures 3.3b and e) in the supported DOPC membrane than in the underlying polymer layer.

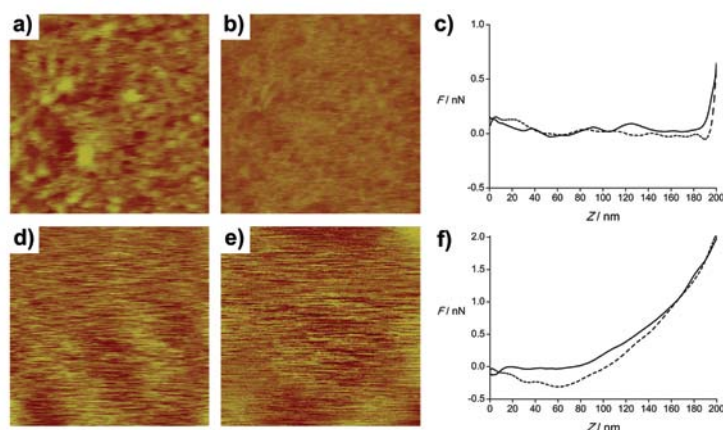


Figure 3.3 Topography images (a, d), deformation maps (b,e), and typical force curves (c, f) from AFM measurements in Peak Force Tapping™ mode for a poly(SBMA) layer in phosphate buffer (top) and for a DOPC membrane supported on the same poly(SBMA)-grafted surface (bottom).

Finally, we investigated the fluidity of the DOPC membrane self-assembled on the zwitterionic poly(SBMA) layer using fluorescence recovery after photobleaching (FRAP). Figure 3.4 shows FRAP results of a DOPC membrane doped with NBD-PC (2 mol%) formed on a poly(SBMA)-grafted glass coverslip using ATRP for 15 min. The diffusion coefficient was determined by fitting the normalized fluorescence recovery data following procedures reported in the literature and found to be $1.16 \pm 0.06 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$. No formation of supported DOPC membrane was observed on initiator-coated glass surfaces, whereas lipid membranes formed on glass surfaces at the same conditions were not homogeneous and did not show recovery within 1 h from photobleaching (M.G. Santonicola, M. Memesa, A. Meszyńska, Y. Ma, G.J. Vancso, “Surface-Grafted Zwitterionic Polymers as Platforms for Functional Supported Phospholipid Membranes”, submitted to Soft Matter).

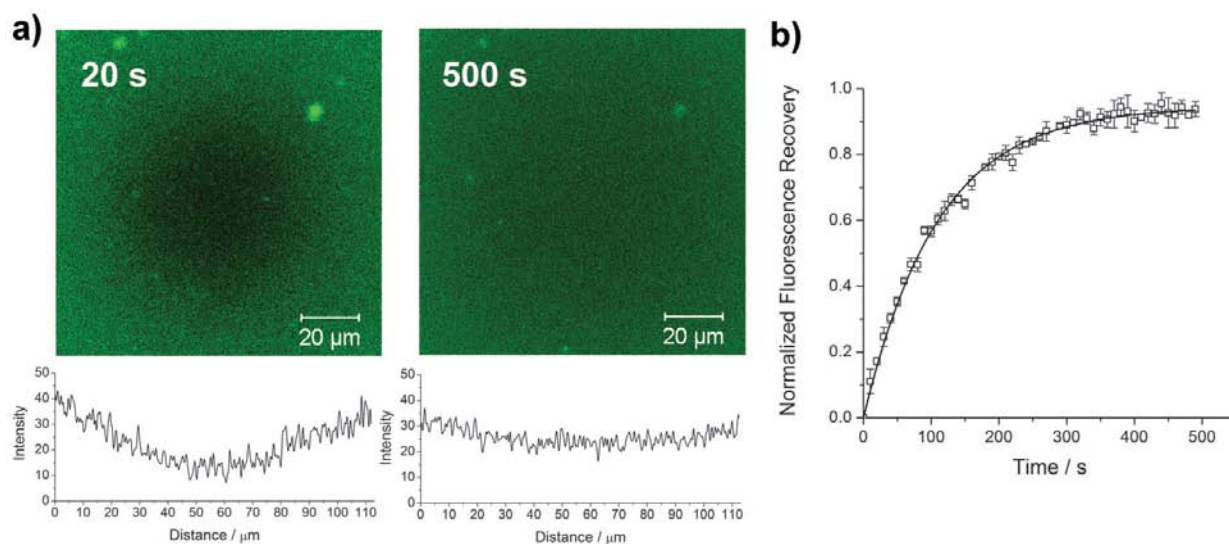


Figure 3.4:(a) FRAP characterization of a poly(SBMA) supported DOPC membrane after 20 s and 500 s from photobleaching in phosphate buffer pH 7.4 with 150 mM KCl. (b) Normalized recovered fluorescence intensities and fit to the data for the evaluation of the lipid diffusion coefficient following the procedure by Axelrod and co-workers.

D3.3.2 Stimulus responsive pore function for spanning membranes (month 36)

Nanoporous chips modified with pH-responsive polymer brush were switched between the closed and open states by varying the pH of the surrounding phosphate buffer solution. Stimulus responsive pore function was followed by electrochemistry measurements and permeation measurements. The electrochemistry measurements were performed in the lab of partner #1 (ETHZ).

Nanoporous silicon nitride chips with pore diameters of 200 and 400 nm were obtained from Leister, Switzerland (partner #8). These nanoporous chips were functionalized with pH-responsive poly(methacrylic acid) (PMAA) as described in the protocol in deliverable D2.3.2. Current-voltage measurements were performed in solutions of various pH values (Figure 3.5) in collaboration with ETHZ (partner #1). Measurements show that the current increased by 3-fold between phosphate buffer of pH 4 (non-charged state of polymer) and phosphate buffer of pH 8 (charged state of polymer). This can also be displayed by plotting electrical resistance against pH value (Figure 3.5). These measurements indicate that ion permeation in PMAA-coated nanopores can be controlled by varying the pH of the solution. Permeation experiments in a cross-flow module were also performed and they show a ten-fold decrease in permeation between the open state (phosphate solution with pH 4) and the closed state of the nanopore (phosphate solution with pH 8) (G.W. de Groot, K. Sugihara, M.G. Santonicola, T. Zambelli, J. Vörös, G.J. Vancso, "Switching transport through nanopores with pH-responsive polymer brushes for control of ion permeation", *manuscript in preparation*).

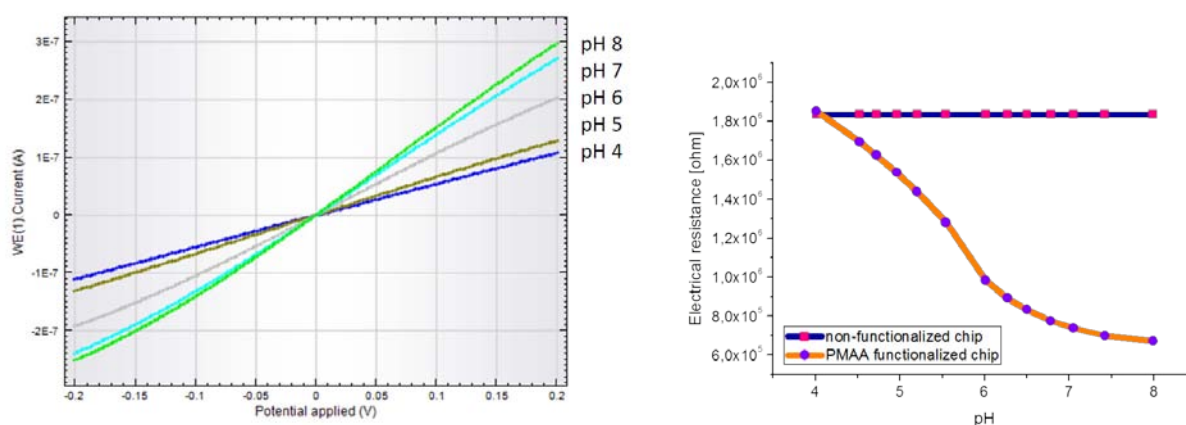


Figure 3.5 Left: Current-voltage measurement of PMAA functionalized nanoporous chip (4 x pore diameter 400 nm) at varied pH values. Right: Electrical resistance of PMAA brush functionalized nanopore chip at varied pH values. Control shows that response is not influenced by variation in pH of the phosphate buffer solution, so only by the pH-responsive polymer brush.

Table: Permeation measurements with PMAA functionalized nanoporous chip (512 x pore diameter 200 nm) performed with phosphate solutions of pH 4 and pH 8.

pH 4	52 mg/s
pH 8	6.2 mg/s

D3.4.1 Report on theoretical framework for understanding the rupture of lipid vesicles and the formation of supported lipid bilayers (month 18)

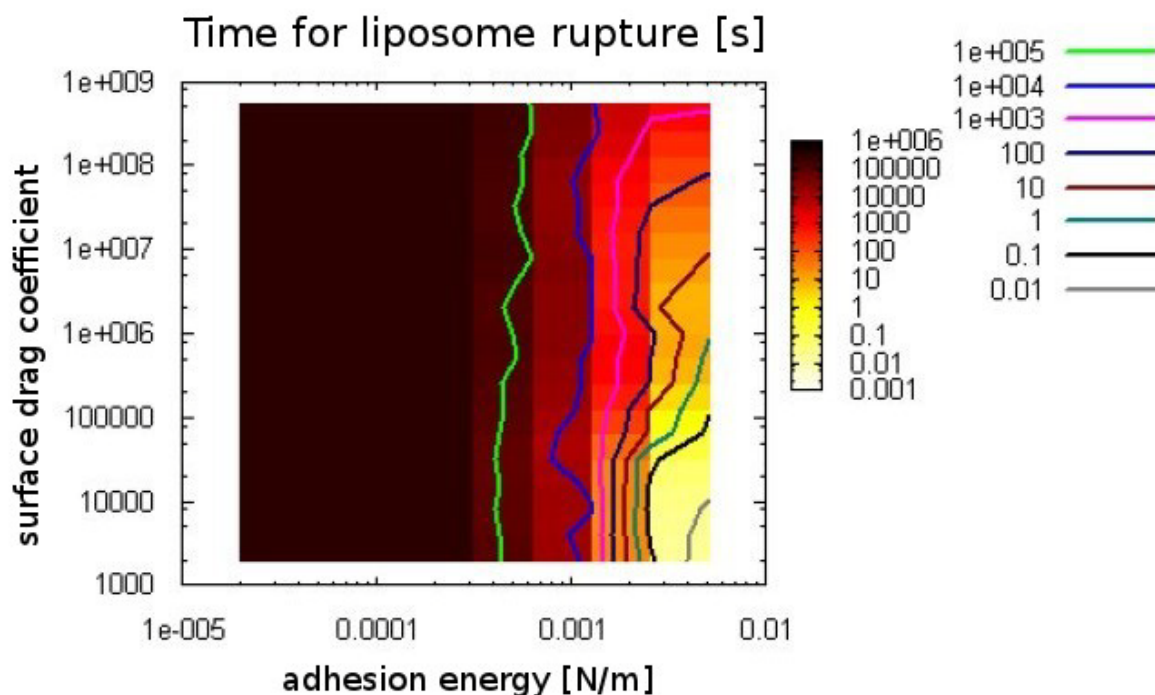
The interaction between lipid bilayers and solid surfaces is a theoretically poorly understood phenomenon. We have investigated how the redistribution of charged lipids near charged surfaces affects this interaction addressing the questions what the energetic aspects of membrane pore formation at the contact lines between vesicles and surfaces are; under what conditions the membranes tend to enter the nanopores of the surface; and what the energetic aspects of this latter process are in the context of different geometries. The obtained results will aid in choosing and designing the optimal strategy for liposome rupture and formation of nanopore spanning membranes in WP 3-5.

D3.4.2 Report on predictive theoretical tools for optimising liposome adsorption and supported lipid bilayer formation (month 36)

Combining elastic membrane theories and fluid dynamics we developed a detailed understanding of the process during which liposomes first adhere to solid surfaces, then rupture (or go through a series of transient ruptures) due to the mechanical tension induced by the adhesion, and finally spread along the surface forming a supported lipid bilayer. In our theoretical description we simultaneously considered the coupled dynamics of (i) spontaneous pore opening and closing; (ii) volume loss of the liposome via leakage through the pores; (iii) and the advancement of the adhesion front.

Extensive numerical simulations of the dynamical equations and analytical calculations revealed that the rupture process of liposomes consists of three well distinguishable phases: a fast initial volume loss; followed by a slow volume loss; ending with a final burst and surface spreading. The slow second phase can be skipped and, consequently, a fast bilayer formation can be achieved, if either the first phase advances far enough or the third phase sets in early enough. This strongly depends on the parameters of the system. Our simulations showed that the smaller the vesicle, the further the first phase can advance. The third phase can start earlier if either the surface is smooth enough (i.e., the surface drag against the propagation of the adhesion front is small enough), or the adhesion energy is large enough, or the line tension of a free membrane edge is small enough. When the second phase is not skipped the time needed for the rupture process can take very long (well over the experimental time scales) with a large variance. One of the most important results of this study is that we can now predict the range of parameters (including liposome size, adhesion energy, line tension, bending rigidity, drag coefficients, etc.) when fast bilayer formation is expected to occur.

As a demonstration, the figure below displays the rupture time of a vesicle of initial radius of 2.8 microns as a function of the surface adhesion energy and the surface drag coefficient (other parameters include: line tension = 2×10^{-11} N, membrane bending rigidity = 10^{-19} J). Notice the sudden drop of the rupture time around the value of 0.001 N/m of the adhesion energy. [The rupture of membrane vesicles near solid surfaces, A. Nyeste and I. Derenyi, Physical Review Letters, in preparation]



1.3.4 WP 4: INTEGRATION OF TRANSMEMBRANE PROTEIN ASSAYS WITH WAVEGUIDE BIOSENSORS

WP LEADER: MARCUS SWANN

In this work package we focused on adapting protocols developed in earlier WPs to functionalise waveguide sensor systems by industrial partners Farfield and Microvacuum for various screening assays. WP4 also had a high content of theoretical analysis and simulation to understand and develop new sensitive assays for deducing changes in membrane and membrane protein conformation in addition to pure affinity assays.

Summary of progress

This work package focused on applying methods developed in other workpackages for forming proteolipid layers on waveguide sensors, understanding the structural signals from the responses and applying this to drug binding assays.

A method for nanostructuring waveguides has been implemented based on colloidal lithography. However, even at relatively low pore densities the scattering effect was found to be appreciable. Whilst this is being mitigated by using a more complex waveguide multilayer structure, which should reduce this effect, it has not yet been shown to produce the anticipated advantages. An alternative methodology to fulfil the objective has been developed, involving a polymer coated waveguide.

A large number of protocols for assembly of supported lipid bilayers from liposomes have been described and verified on both waveguide platforms within ASMENA: optical waveguide lightmode spectroscopy (OWLS, Microvacuum) and dual polarisation interferometry (DPI, Farfield). The waveguide response to the supported lipid bilayers has been modelled and an appropriate analysis method has been determined, which has been incorporated as a standard tool in the commercial software of Farfield. In an extension of this analysis, significant progress has also been made to demonstrate the detection of structural changes in lipid bilayer modified waveguides. This provides the platform for further analysis of binding events to incorporated membrane proteins.

A methodology has been developed for the formation of tethered liposome mono- and multi-layers on waveguide surfaces. A number of different methods have been used to probe functional changes in the

liposomes to determine differences in porosity, mass and dimensions as a function of model interactions. This is now already being commercially utilised and experiments are currently being extended to cover a wider, more generic range of lipid systems and interactions.

A variety of methods have been trialled on the partners' waveguide sensor devices for the immobilisation of proteolipid layers, enabling objective 4.2 to be reached in several different ways. Methods tested were immobilisation of lipid layers on PEG-cholesterol functionalised surfaces, tethering liposomes via DNA-tags and bilayers via high density DNA tags. The DNA-tethered proteo-liposomes also fulfilled objective 4.3 and is also elaborated upon in WP5 for drug binding assays using proteoliposomes from partner 13. Tethered proteolipid bilayers have been formed via coordination of 6xHis tagged trans membrane protein to NTA-Ni functionalised surfaces. This has enabled significant progress towards objective 4.5, though this has not been fully met as confirming that the observed response was due to structural changes in the protein has not yet been possible. The potential for forming proteolipid layers by electrostatic interaction on charged polymer and brush surfaces have also been demonstrated by partner 1 for polymer coated OWLS waveguides and partner 11 for metal ion functionalised, PEG layer coated DPI waveguides. [Simultaneous OWLS and EIS monitoring of supported lipid bilayers with the pore forming peptide melittin, K. Sugihara, M. Delai, I. Szendro, O. Guillaume-Gentil, J. Vörös, and T. Zambelli, in press]

Theoretical modelling of anticipated changes, in conjunction with experimental measurements has enabled us to clearly identify structural changes in lipid bilayers (objective 4.4) due to a variety of factors. This has shown that the different structural effects of thermal phase transitions of the lipids, their solvent composition, as well as drug partitioning can be clearly identified. It is also possible to distinguish the various disrupting and pore forming effects of lipid binding anti-microbial peptides on the lipid bilayer structure.

A completely different approach has also been tested for the use of a waveguide device as a trans-membrane channel protein activity assay platform. In this instance the proteolipid membrane is formed sufficiently above the surface of waveguide, so that transport through the membrane can be probed by measuring changes in the bulk Refractive Index at the waveguide surface.

All deliverables due by month 36 have been met.

Main significant results

Bilayer formation protocols have been applied and optimised for a range of lipid systems on OWLS and DPI sensing platforms. The DPI approach to analysis, providing both Birefringence and Mass values for the bilayer has been demonstrated and integrated into the "Farfield Explorer" analysis software for the DPI instrument. The approach has also been published. Furthermore, a method for producing polymer supported bilayers has been established and patented.

DLPC liposome mono and multilayers have been formed using DNA-tag coupling kits provided by partner Layerlab. These have been used to monitor the interaction of liposomes with melittin. The interaction has been shown to produce changes in liposome size, which can be related to the interaction mechanism. The successful demonstration of the combination of the two technologies has led to joint marketing.

Applying the analysis method for lipid bilayer mass and anisotropy data, obtained with DPI in the first portion of the project, to probing structural effects in the bilayers caused by a variety of factors such as temperature, solvent (DMSO, Ethanol) or drug partitioning has proved to be very successful. This has enabled these effects to be followed and rationalised in terms of their impact on the bilayer structure. This has resulted in new measurements of membrane conformational changes as a function of drug molecule physico-chemical structure.

Extension of this method to lipid peptide interactions has been even more successful. Modelling of the interaction mechanisms alongside the experimental data has allowed mechanistic details of the interaction mechanisms to be elucidated and has prompted a promising uptake of the method in the wider scientific community.

A new method has also been developed and demonstrated for measuring ion channel function using a membrane-modified OWLS waveguide sensor.

Deviations from Annex I

Additionally to the work envisaged in Annex 1, Partner 12 has implemented the alternative assay format for probing membrane protein activity and inhibition detailed above. i.e. by forming proteolipid layers significantly above the waveguide surface of OWLS chips, which has enabled transport through the membranes to be probed by measuring changes in the bulk RI proximal to the waveguide surface.

Achieved results and Deliverables

D4.1.1 Report on production of nanoporous waveguides (month 12)

Particle lithography has been applied to etch nanopores in the grating part of grating-coupler waveguides supplied by Microvacuum. Confinement of the pores to this section of the waveguide reduced the undesirable scattering by the pores to an extent that allowed sensing with the same sensitivity as obtained with a non-porous waveguide of the same type.

In view of the substantial complexity and potential drawbacks of this approach, a second, alternative approach was investigated where instead of a porous waveguide, a standard waveguide coated with a polyelectrolyte multilayer is used. Polyelectrolyte multilayers (PEMs) are highly hydrated and allow diffusion of, for example, ions thanks to their open molecular structure. Based on a novel protocol, supported lipid bilayers (SLB) could be successfully formed on top of the polyelectrolyte multilayer while the underlying polymer cushion allowed for waveguide sensing of transport through the SLB. Since this method is highly sensitive to translocation of ions across the membrane, e.g. through ion channels, this approach is considered to be more attractive than the original porous waveguide method, and will be further pursued as part of this deliverable by implementation of an optimal waveguide structure for sensing the PEM-SLB overlayer. A provisional European patent application has been filed for the device.

D4.2.1 Protocol for lipid membrane functionalised waveguides (month 12)

Optimized protocols for the formation of supported lipid bilayers (SLBs) from liposomes have been applied to grating coupler chips for in situ waveguide sensing. SLBs can now routinely be achieved on unmodified and modified waveguide sensor surfaces from both industrial partners Farfield and Microvacuum. The characteristic signature response of SLB formation through liposome fusion has been recorded for a number of different lipid compositions. Furthermore, we have created an analysis model for SLBs that extracts from experimental data structural information such as molecular alignment in lipid membranes and membrane phase behaviour through analysis of the optical anisotropy. This model has been published and implemented in the analysis software of industrial partner Farfield.

D4.2.2 Protocol for incorporation of transmembrane protein into waveguide-supported lipid membrane (month 24)

The protocol delivered by partner 2 (PSI) for the formation of planar lipid bilayers containing trans-membrane proteins consists of immobilising the surfactant stabilised protein via a 6xhis tag (inserted on expression and used for purification of the protein) and then diluting and rinsing the surfactant out in the presence of lipids. During this process the lipid bilayer is formed around the protein above the chip surface. The method tested here utilises PLL-PEG-NTA(Ni) as the 6xhis tag coupling surface, though would also apply to other Ni or for example Cu functionalised surfaces. The PLL-PEG-NTA(Ni) adsorption, protein immobilisation and subsequent lipid addition was verified by QCM-D, OWLS and DPI. The lipid layer formed was also probed by FRAP and showed complete recovery of fluorescent intensity and a high level of mobility, consistent with a lipid bilayer being present on the surface.

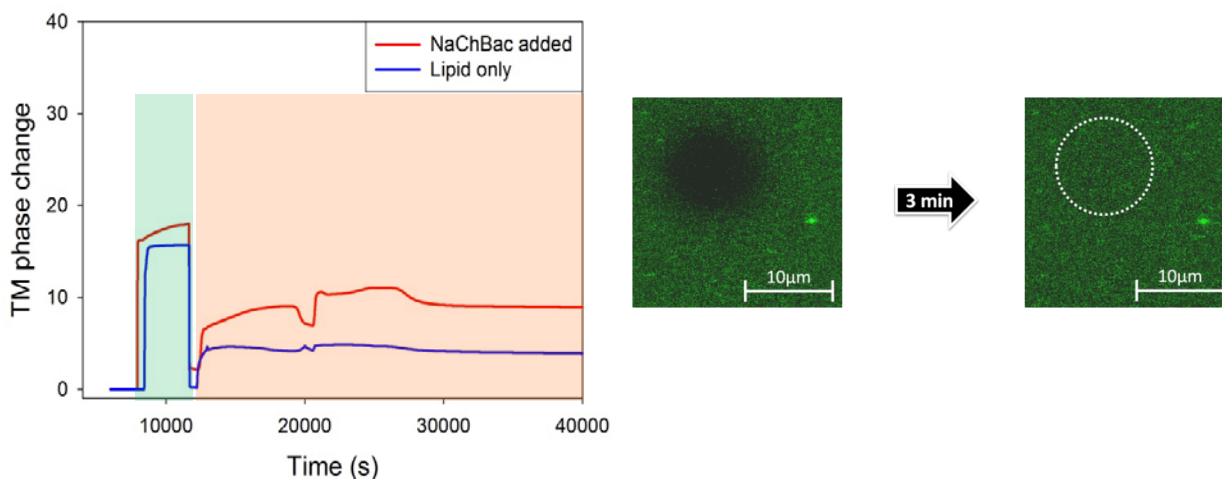


Figure 4.1. Left: DPI measurement showing addition of 1 mg ml⁻¹ liposomes in RB and 0.1 % DDM with a washout to RB alone, followed by injection of liposomes in RB and 0.03 % DDM with a washout followed by injection of liposomes alone (Fresh NaChBac on C1 and just PLL-PEG-NTA on C3) Right: FRAP analysis of a proteobilayer formed by in-situ reconstitution. The liposomes used in this experiments are composed of 47%POPC, 3%NBD-PC and 50%POPS

D4.3.1 Protocol for assembly of transmembrane protein-containing liposomes or rafts into multilayer structures on waveguides (month 18)

Large unilamellar liposomes have been physisorbed directly to optical waveguide sensor chips of partner Microvacuum. Changes in bulk refractive index response due to permeabilisation with gramicidin could be successfully recorded. Physisorption can be very dependent on lipid composition, so based on the Layerlab methodology for immobilizing a multilayer of liposomes onto sensor surfaces, the deliverable is to adapt and optimize this methodology, to produce a protocol and proof-of-concept for use of the methodology on waveguide bioanalytical sensor platforms.

A protocol has been produced for use on Fairfield's DPI-sensor platform and a manuscript, for publication in a Scientific journal, has been written, which describes a study of the action of the lytic protein (a small peptide), melittin on liposomes attached to the DPI waveguide surface using the Layerlab methodology. Structural changes in the liposomes measured in this way provide more information than mass changes would alone. Fairfield has reached a commercial arrangement with Layer Labs and has launched the Layerlab kit as a product for use with DPI and promoted through its marketing channels.

D4.4.1 Report on standard methods for analysing lipid membrane response and conformational changes due to drug interactions and environmental changes using waveguide spectroscopy (month 24)

Waveguide measurements using DPI are made in Transverse Magnetic (TM) and Transverse Electric (TE) polarisations. These two data sets are insufficient to completely characterise a bilayer structure which in the simplest case requires three parameters of thickness, Refractive Index (RI) and Birefringence. The RI being a function of bilayer density and the birefringence of lipid alignment. The analysis of DPI data has been developed (by partner 11) so that assuming a fixed value for one of these parameters, the other two can be calculated. Fixing RI for example gives a thickness value which is in effect proportional to mass and a birefringence value which is a function of the alignment of the lipid bilayer, i.e. a structural parameter. For characterising bilayers, fixing thickness to a known value may make most sense, however for the partitioning of drug compounds, or peptides fixing the RI has been shown to provide the most robust and generic analysis.

Structural changes in lipid bilayers have been measured and analysed using this method for a number of different interactions and environmental effects. The phase change of DMPC from the fluid to gel phase has been measured, and the structural transition observed as a change in the birefringence and lipid mass

per unit area as a function of temperature. The plot of Birefringence against mass has been shown to be a generic method for viewing and interpreting such data.

The effect of solvents on lipid bilayers has also been assessed for DMSO and ethanol. The former is used in the industry for the solubilisation and storage of drug libraries and so the stability of lipid bilayers in the presence of DMSO is critical. The swelling effect and slight disruption of the solvents on lipid bilayers could be observed, though at commonly used concentrations lipid bilayers can be formed and probed with drug compounds. This has been demonstrated as an automated assay process.

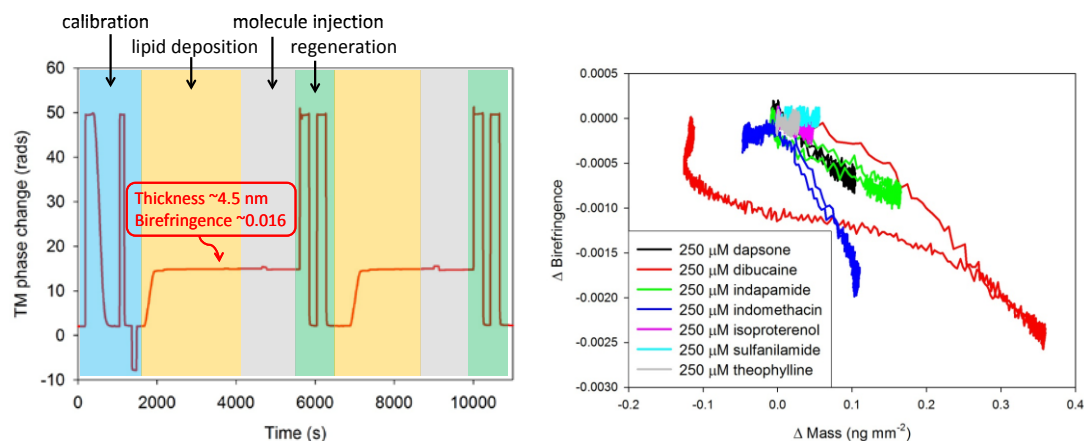


Figure 4.2. Left: Formation and regeneration of DOPC bilayers in an automated drug partitioning assay. Right: Birefringence: Mass plots for a range of drug compounds showing different partitioning behaviour

Partitioning of small molecule drug compounds into lipid bilayers and their structural effect have been measured. As well as varying affinities, different structural responses can be clearly observed depending on the drugs physico-chemical nature and where in the bilayer it resides. The effect of a model peptide, melittin, has also been measured. This compound is well known to form trans-membrane pores in the lipid bilayers and the different stages of the interaction mechanism can be distinguished from the different mass and structural changes. A range of other compounds have also been measured, namely peptides such as melittin, aurein, a-synuclein, alamethicin and HPA. For these compounds the different interaction mechanisms possible have been modelled and correlated against the observed wide variety of responses [e.g. PH-PLCδ1 Interaction with PIP2 Containing Supported Lipid Bilayers monitored in situ with Dual Polarization Interferometry, M. Baumann, M. Swann, M. Textor, E. Reimhult, Analytical Chemistry 2011. Effect of acyl chain structure and bilayer phase state on the binding and insertion of HPA3 onto a supported lipid bilayer, Daniel J Hirst, Tzong-Hsien Lee, Marcus J Swann, Sharon Unabia, Yoonkyung Park, Kyung-Soo Hahm and Marie Isabel Aguilar, European Biophysical Journal, 2011].

D4.5.1 Report on studying the effect on transmembrane protein embedded in planar lipid membrane subject to ligand binding event using waveguide spectroscopy (month 36)

The formation of a lipid bilayer containing the trans-membrane protein NaChBac has been studied using DPI by partners 2 and 11, following the immobilisation protocol delivered in D4.4.1. This proteolipid system was challenged with a selection of specific and non-specific compounds. The protein was successfully immobilised at high density, which is a pre-requisite to be able to determine a specific response. The control channel was coated with a lipid bilayer without the protein. The structural and mass binding response from the compound partitioning into the lipid layer is significant however, so whilst a net difference in structural response between the two channels was determined it has not yet been possible to confirm that this is due to a structural response of the protein rather than the replacement of a small amount of lipid by protein.

An alternative method for forming a drug binding assay platform on a commercial waveguide sensor was also tested by partner 12 using their OWLS device. This involved immobilising lipid membranes on a

nanoporous carrier membrane above the waveguide surface. In this way, transport through the membrane could be quantified by following bulk RI changes in the solution immediately proximal to the waveguide surface. The principle of the technique was demonstrated with transmembrane pores formed by the insertion of gramicidin. Pore activity was discriminated by comparison of Sodium ion with various iminium ions.

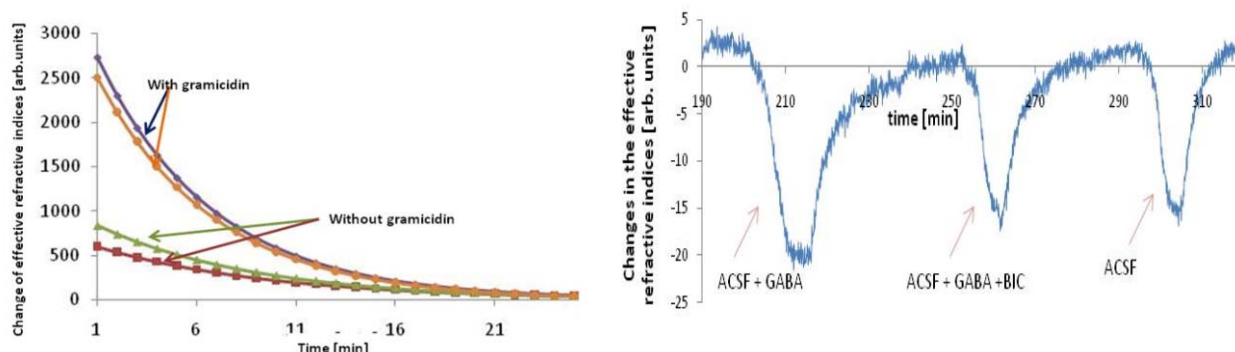


Figure 4.3. Left: Change of effective refractive index as a result of the formation of gramicidin pores. Right: Changes in Cl⁻ ion permeability of GABAA receptors in the presence of GABA and the channel blocker bicuculline

The technique was further demonstrated using cellular membrane fractions which were isolated from HEK293 cells expressing human GABAA receptors with $\alpha 5$ and $\beta 2$ subunit composition. The OWLS signals indicated a significantly increased Cl⁻ permeation in the presence of GABA, a natural ligand of the Cl⁻-channel. On addition of the GABA-channel blocker bicuculline, the GABA-induced increase of Cl⁻ permeation was prevented. The data demonstrated that GABA channels can function in the membrane-sandwich model, and the system might provide a tool to measure lipid-embedded, multi-subunit channel functions. A publication on this topic can be found in the Appendix of this report [OWLS assay techniques for investigating membrane-bound ion-channel activities; Inna Székács, Katalin Erdélyi, István Szendrő, Nóra Kaszás, Pál Gróf, Emilia Madarász, in preparation].

1.3.5 WP 5: FUNCTIONAL ASSAYS OF MEMBRANE PROTEINS

WP Leader: Louis Tiefenauer, PSI

WP5 explored the npsLB platform for activity measurements on different model systems. The outcome of this work package was directly correlated with the achievements in the other work packages. The nanoporous chips suitable to generate stable lipid bilayers were used to integrate membrane proteins therein and to measure their activities.

Summary of progress

Within the first half of the ASMENA-project we could demonstrate that peptides (melittin, alamethicin) and proteins (hemolysin, a Na⁺-channel) can be integrated in free standing lipid bilayers and that the suspended proteolipid bilayers are sufficiently stable during of the required duration of a measurement. We have established three different highly sensitive electrochemical detection methods. The results demonstrate that these methods are sensitive for single channel measurements.

Formation of long-term stable lipid bilayer in nanopores and electrochemical functionality testing of integrating peptidic channels has been demonstrated in the first part of the ASMENA project. However, the critical step as identified already in the proposal is the integration of membrane proteins in stable lipid bilayers. A general applicable method (a) has been developed applying a two-step procedure: first generation of a stable bilayer by painting and second fusion in of proteoliposomes. The resulting planar proteo-lipid bilayer exhibit a GigaOhm sealing in which individual sodium channel ion channels could be measured. Thus, the main two objectives (5.1 & 5.2) of this WP5 have been achieved.

However, this first successful method has some severe limitations: (i) The pore diameter should not be smaller than about 6 micrometres and the high stability of free-standing bilayers as demonstrated for

nanometer diameters can't be achieved; (ii) the lipid composition of the proteoliposomes is more or less fixed; (iii) the number of inserted protein molecules is rather low and (iv), very important, this method is not organic solvent-free. We therefore evaluated further methods which may be applicable for all families of membrane proteins, especially also eukaryotic origin. In a first phase several stimuli were investigated in order to induce a direct fusion of a proteoliposome to a nanopore of similar diameter. Based on reported effects from the literature the following stimuli have been tested separately and in combinations: pH-changes, elevated temperature, suppression, high-voltage application and Ca^{++} addition. Some of these stimuli showed small effects, but none of them did result in a lipid bilayer of a sufficiently high sealing.

Based on the achievement of WP3 we (b) first filled a nanopore layer-by-layer with polymers. If the last polymer layer is positively charged, added negatively charged PS-liposomes or proteoliposomes fused on this polymer at higher temperature (45 to 55 °C) and form confluent lipid bilayers as confirmed by electrochemical impedance spectroscopy (EIS) and fluorescence FRAP-analysis. In order to prevent the exposure of the protein to such temperature (as in b), a further two-step procedure (c) has been developed: first formation of a negatively charged bilayer at 55 °C followed by fusion of a positively charged proteobilayers into this first bilayer at 37 °C. The resulting lipid bilayer of mixed lipids has a reasonably high sealing in the range of 100 MΩ up to 500 MΩ and ion channel activities could be detected. This method (c) is solvent-free and over 50 % of the lipids of the proteoliposomes can be the uncharged POPC which is a common lipid in all eukaryotic cells. Thus, this solvent free-method is applicable for many reconstituted membrane protein of interest. There is room for optimization aimed at improving the sealing of the bilayer by this method. The preparation of proteobilayers by this two-steps procedure may be performed using an automated microfluidic system.

The third proteo-lipid-bilayer preparation method (d) can circumvent the limitation concerning the lipid composition as for methods (a), (b) and (c) outlined above. The nanopores are first filled by a polymer carrying NTA-residues which form Ni^{++} complexes with histidine-tags (His-tag) frequently used in recombinant proteins. The immobilization of the detergent-solubilized protein in nanopore(s) offers two advantages: (i) the density of proteins can easily be defined and (ii) all protein molecules are oriented in the same direction. The second step of method (d) is formation of a lipid bilayer by replacing detergent molecules with lipid molecules using detergent-adsorbing Biobeads or by dialysis. This method (d) was developed using first unstructured surfaces and quartz crystal microbalance (QCM) and FRAP techniques, followed by EIS experiments using chips with one nanopore.

Methods for electrochemical measurements of channel activities in nanopore suspended lipid bilayers has been successfully established and open, closing and inhibition of the NaChBac could be demonstrated.

Looking back it turned out that not the use of different membrane proteins - as planned - is the critical factor for developing functional methods, rather the evaluation of different procedures to form stable lipid bilayers of high sealing with integrated membrane proteins.

Main significant results

The ASMENA-project aimed at exploring technologies for functional assays of reconstituted membrane proteins using nanoporous supports. Due to the close collaboration between many partners of a different background i.e. in material science, surface chemistry, membrane protein expression, purification and reconstitution, electrophysiology, instrument manufacturing and application for drug discovery, the interdisciplinary research activities received a new quality. The close interaction between all involved researchers facilitated the exchange of ideas, materials and know-how and stimulated the motivation of all involved persons. Thus, the scientific outcome as judged from the list of publications is wide. It should be kept in mind that many experimental attempts as mentioned above were not successful and this information could not be published. Nevertheless, also this knowledge of not successful strategies is also important. In summary, the following main results have been achieved:

Nanoporous supporting materials: Many different types of chips as outlined in WP1 have been produced and used to evaluate various methods for generating free-standing lipid bilayers. It turned out that the tiny

silicon nitride membrane (300 nm) leads indeed to the intended low aspect ratio, but also to an enhanced electrical capacitance. The perforated thin membrane covers a relative large area on the chip (0.25 mm²) and typically a capacitance of about 800 pF was found. For current measurement at a constant applied voltage and for optical measurements this inherent high capacitance of the chip is acceptable. However, in voltage-clamp measurement such a high capacitance can't be compensated. By addition of a SU-8 polymeric layer to the chip surface outside the pore area, the capacitance could be reduced to about 10 % which is acceptable. Alternatively, the silicon nitride membrane area could be reduced to about 0.0225 mm² (0.15 mm x 0.15 mm). However, the dimension of the backside cavity is then also reduced and as a consequence the wettability of the cavity surface has to be enhanced by a hydrophilic silanization to avoid air bubble entrapment therein. The number of pores needed depend on the membrane protein of interest. For ion channels a pore of 800 nm diameter is sufficient, whereas for transporters pore arrays are needed. The necessary fabrication techniques have been all developed and evaluated in WP1.

Membrane protein expression, purification and reconstitution: In the proposal both, recombinant (AmtB, aquaporin) and extracted membrane proteins (AChR, CoC) were considered to be investigated. Recombinant proteins usually have a His-tag which is used for transiently immobilization to NTA-nickel column in the purification procedure. Since the transmembrane part of membrane proteins is hydrophobic, pure membrane proteins would aggregate and would be immediately inactivated. Thus, detergents are needed to keep them in a functional state. Different detergents are available. Thus, considerable efforts are required to find the appropriate conditions and procedures to keep a given membrane protein functional until it is integrated in a functional lipid bilayer. We have identified two ways to use these membrane proteins: (i) the detergent-stabilized (=solubilised) His-tag membrane proteins are first bound to a NTA-polymers present in the nanopores. In this way membrane proteins are oriented which is an advantage since ion translocation and transport are usually directed either from outside to inside (importer) or in the opposite direction (exporter). (ii) From solubilised membrane proteins proteoliposomes were made by replacing the detergents by lipids either using Biobeads or by dialysis. The diameter of the resulting proteobilayer was adjusted by extrusion across filters of defined nanopore diameters.

Prokaryotic membrane protein: Instead of the proposed AmtB partner 2 and 4 first used a bacterial sodium channel (NaChBac) as a model protein. This protein with a His-tag is relatively robust and can be produced in sufficient amounts (some mg). These factors are important in the development of a method. In addition a viral ion channels KvAP has been expressed (partner 4).

Eukaryotic protein: As mentioned expression, purification and solubilisation of multi-domain eukaryotic protein is a big challenge. The final aim is to have hERG channels in bilayers which are highly relevant for early identification of putative side-effects of drug candidates. However, this membrane protein is very delicate and not yet available. Therefore partner 14 has produced a eukaryotic analogous ion channel which is acid sensitive (ASIC). The protein has been purified, solubilized and frozen. The activity of the solubilized protein can't be checked so it is not clear if it is in active form. Nevertheless the His-tag variant has been used in the reconstitution experiments.

In situ reconstitution: The solubilised and immobilized His-proteins can be integrated into a self-assembling lipid bilayer, when the detergent molecules are removed. This procedure comprising removal of detergents and concomitant replacing with lipid molecules needs time and has been monitored by QCM. The EIS investigation as well as the FRAP-investigations confirmed that an intact lipid bilayer is formed. The diffusion rates of dyed lipids after photo-bleaching were in the same order of magnitude as free-standing bilayers, but always somewhat lower probably due to interactions of the bilayer with the supporting polymer. However, the diffusion of ions across the supporting polymer is still good. We could demonstrate the formation of NaChBac-lipid-bilayer in single pores. The moderate sealing quality can be caused by open ion channels or imperfect sealing.

For future investigations also pH-sensitive polymer covalently grafted within the pores are available (from partner 7). Upon pH-changes the observed barrier effect can probably be removed after the formation of the bilayer. Most important for a good sealing is a good contact of the formed bilayer to the surface. In order to further improve this sealing, several surface modifications can be carried out: (i) hydrophobic silanization to enhance the lipid contact to the surface as previously demonstrated for the painting method (a). (ii) Protection of the surface from deposition of polymers which disturb later the contact with the lipid molecules in layer-by-layer preparation techniques.

Fusion of proteoliposomes: Membrane proteins in proteoliposomes are much more stable than in solubilised form and can be frozen. Thus, from a practical point of view the use of proteobilayers is very attractive and can also be used in automated microfluidic systems. Despite many attempts direct fusion of proteoliposomes to nanopores could not be observed. However, at specific condition negatively charged liposomes fuse to positively charged polymers as demonstrated by partner 1. Fusion of positively charged proteoliposomes with at least 50 % POPE lipids to 100 % PS bilayers formed on PEI-polymers has been confirmed by FRAP-investigation and in EIS-experiments. Thus, the feasibility of this fusion method could be demonstrated. It remains open which membrane proteins can tolerate this restriction of lipid composition.

Electrochemical measurement: One of the most sensitive detection techniques are current measurements. A prerequisite for single ion channel measurement is a sealing in the GigaOhm range. In other words a good sealing has a direct influence on the sensitivity of detection. We could demonstrate that one voltage-gated ion channel NaChBac in physiological ion concentration leads to a current of a few pAs. By increasing the ion strength and applied voltage the signal can be enhanced. For most ion channels a voltage-pulse is applied.

Microfluidic systems (MFS): Supplementary to the original proposal partner 2 spent about 6 months for developing a microfluidic system in which all different types of nanoporous chips can easily be integrated. In collaboration with an external partner (New Technical University Buchs (NTB), Switzerland) partner 2 has developed a MFS based on PDMS slabs, which are hold together by PMMA-plates to which PEEK-tubes are connected. Fluidic transport is achieved by syringe pumps connected to the MFS-device through the tubes. The transport of microliter volumes to both sides of the chip surface is controlled by the NEMESYS software. This versatile system also allows automated transport of organic and aqueous solutions and thus the generation of painted lipid bilayers. Small volumes are also a prerequisite for solvent-free preparations methods (c and d) using polymer-filled nanopores and undiluted proteoliposomes to enhance contact to preformed bilayers. Finally, a MFS is indispensable for sequential transport of effector (inhibitor) compounds and for washing steps as frequently required in preparation and analysis procedures. In summary, this MFS is indispensable part of assay systems to prepare lipid bilayers with integrated membrane proteins and to investigate their activity. This was not fully recognized at the definition of the proposal.

Deviations from Annex I

In the original proposal functional investigations of channels (AChR), transporters (AmtB, aquaporin) and redox membrane proteins of different origin were proposed in order to enhance the chance of success to develop a method to assess function of membrane protein. As discussed during the general assembly meeting in Bordeaux, the major challenge is not the diversity of the proposed membrane proteins and their measurement, rather their complexity. Whereas prokaryotic proteins consist of a single domain and are relatively simple, eukaryotic ion channels are multi-domain structures and much more difficult to produce in a functional form and to integrate into free-standing lipid membranes. Therefore, we decided to use both, a prokaryotic ion channels (NaChBac, from partner 2) and the eukaryotic ASIC channel (from partner 14) as a model proteins to develop solvent-free assays.

Furthermore, we obtained the AChR channel protein (from partner 5) as a membrane fraction. Since we found that the direct fusion of proteoliposomes with neutral lipids to nanopores is unlikely even with

stimulation, we proposed to exchange in the future a portion of the natural lipids of the AChR-fraction by positively charged lipids and apply the fusion method (c) to preformed PS-bilayers in nanopores.

The electrochemical method to quantify diffusion of NH_4^+ across free-standing lipid bilayers and hemolysin pores has been reported (Ref. Studer et al. Colloids & Interfaces B, 2009). In the proposal we intended to further investigate the transporting mechanism of AmtB. In the meantime this mechanism has almost completely been elucidated and from a scientific point of view such investigations are not interesting any more. Since the unit activity rates (i.e. the number of molecules transported per membrane protein per second) of transporters is about thousand times lower than that of ion channels, concentration and sensitivity of detection of non-electro-genic (uncharged) species are critical factors. Thus, a small volume of the trans-compartment is a prerequisite for the investigation of transporter activities. Beside the mentioned need of pore arrays, a MFS with minimized trans-compartment volumes of some microliters is an absolute prerequisite to investigate this class of membrane proteins. Thus, partner 2 concentrated on the development of the assay method and additionally on the development of a microfluidic system (MFS) as outlined above. Based on these experiences, the original list of deliveries of later deadlines has been discussed in the meetings (Wildhaus, Texel and Bordeaux) and continuously adapted (see below).

Achieved results and status of Deliverables

D5.1.1 Report on EIS on nanopore-spanning lipid bilayers on nanopore chips (month 8)

The first crucial deliverable of the ASMENA-project is to demonstrate that free-standing lipid bilayers are present in nano-scaled pores. We could successfully demonstrate that free-standing bilayers with a natural lipid composition on nanoporous substrates are stable for at least one day, a very relevant achievement in the context of the main ASMENA objectives. By using the painting method, a high bilayer stability (> 1 day) is routinely achieved. The stability depends strongly on both the pore size and the lipid composition. For a natural soy lipid mixture the stability of lipid bilayers in 800 nm pores is in the range of half an hour, in 200 nm pores it is about 1 day. These results demonstrate the usefulness of chips with pore dimensions of a few hundreds of nanometer to support free-standing lipid bilayers. We furthermore discovered that the nature of the lipid and the method used have a tremendous influence on the stability of the free-standing bilayers. In the course of the ASMENA project the setup and the controls were further improved to corroborate earlier data.

D5.1.2 Report on measuring transmembrane ion diffusion through pore spanning lipid bilayer using ion selective electrodes (month 12)

We have demonstrated that passive diffusion of ions across stable free-standing bilayers separating two compartments can be monitored during one full day. Three major factors which limit the application for perfusion are (a) the life-time of the bilayers, (b) the spontaneous diffusion of the compound of interest across bilayers and (c) the sensitivity of its detection. In microfluidics systems the volume of the trans-compartment can be reduced to a few microliters resulting in a significant enhancement of the detection sensitivity. To detect ion diffusion or protein-mediated translocation across bilayers the total pore area should not be below about $100 \mu\text{m}^2$. Thus chips with arrays of nanopores provide a sufficiently high bilayer stability concomitant with a sufficient large diffusion area.

D5.2.1 Sensor chip functionalized with proteolipid bilayers stable for >1 day for continuous or repetitive monitoring (month 20)

A sufficiently high stability of lipid bilayers of about 1 day is one prerequisite for applications in research and drug screening. Our previous results demonstrated that the diameter of the pores of the silicon nitride membrane is one major factor determining the stability of free standing lipid bilayers (see deliverable 5.1.1). However, little information has been available on whether the stability is affected by integrated membrane proteins. The objective of this deliverable was not to perform a broad study with many variables, but to provide evidence that free standing bilayer with integrated membrane proteins are stable enough for the intended applications. Our findings demonstrate that bilayer stability is generally not

adversely affected within the timeframe needed for membrane protein assays. The main factors affecting stability are: pore diameter, lipid composition, pH-value of the puffer and electrical potential difference. The commonly used breakdown voltage test may confirm the survival of a bilayer after the measurement. However, due to the dependence on various factors, this is not a suitable method to assess proteo-lipid bilayer stability. Since the overall stability of the proteo-lipid bilayer depends on many factors, including protein density, and only two proteins have so far been tested, the stability of such functional bilayers might still have to be validated for each system.

D5.2.2 Report on monitoring insertion of alamethicin in pore spanning bilayers using EIS and voltage clamp (month 18)

The monitoring of peptide insertion into free-standing bilayers is an important method to evaluate bilayer preparations. The voltage-clamp detection technique presented here is a prerequisite to determine activities of more demanding membrane proteins.

Insertion of the fungal antibiotic peptide alamethicin (Alm) in free-standing bilayers has been monitored. The bilayers have been formed in advance by a spontaneous fusion of giant liposomes to a single nanopore. The results demonstrate that fusion of giant vesicles takes place as anticipated and that the resulting bilayers exhibit a sealing quality that is sufficiently high for measuring currents in the pA-range. A crucial prerequisite for the use of voltage-clamp techniques is a low capacitance of the supporting material, which for the total chip capacitance of the supporting chip material should be below 50 pF. The original ultrathin (300 nm) silicon nitride membranes exhibit a too high capacitance (by factor of 50) for the application of this electrochemical detection technique. Since low-capacitance silicon nitride chips were not yet generally available (they are now being developed, see WP 1), we investigated alternatives. We could demonstrate that bilayers of a reasonable stability can be formed in micropores of a thin polymer foil by painting lipids dissolved in decane. Upon insertion of Alm, transient channels are spontaneously formed and could be successfully monitored over time. The Alm channel will now be used as a simple test method for lipid bilayer preparations.

D5.2.3 Report on incorporation and multi-unit impedance measurements of cytochrome c oxidase in pore spanning lipid bilayer (month 30), changed to: Report on incorporation and electrochemical measurements of a prokaryotic model ion channel in pore spanning lipid bilayer (month 36)

Cytochrome c oxidase is a complex protein involving concerted electron transfer. This protein has been proposed, since partner 4 had long-term experience with it. However, for practical applications it is not relevant and has therefore been replaced by a more simple and relevant ion channel. All partners agreed with this decision. We have shown, that the activity of single ion channels reconstituted in lipid bilayers suspended in nanopores can be measured (A. Studer, S. Demarche, D. Langenegger and L. Tiefenauer: Integration and recording of a reconstituted voltage-gated sodium channel in planar lipid bilayers, *Biosensors & Bioelectronics*, 26, 2011).

D5.2.4 Report on electrochemical characterisation of nAChR containing nanopore spanning lipid bilayers, changed to Report on electrochemical characterization of pharmaceutically relevant eukaryotic membrane protein containing nanopore spanning lipid bilayers

Partner 5 has extracted this highly relevant membrane protein and offered membrane fragments in relatively high purity. The main difficulty of such preparations of not reconstituted proteins is to integrate them into planar lipid bilayers. Since isolated proteins don't have a His-tag the attractive reconstitution method developed for His-tag binding to NTA-polymers as shown above can't be applied. Furthermore, our nystatin/ergosterol fusion method needs a specific lipid composition which is obviously not given for natural extracts. To the end of the ASMENA-project we have established a method by which negatively charged liposomes consisting 50% of POPS can be fused into preformed positively charged bilayers. This method is potentially suitable to integrate nAChR in planar bilayers. We have decided to focus on methods for bilayer preparations in the last part of the ASMENA project and therefore we performed electrochemical measurements of simpler ion channels.

D5.2.5 Report on ammonium transport across planar AmtB-bilayers using ammonium-ISE and pH-electrodes (month 30), changed to Report on a pharmaceutically relevant membrane protein activity measured by alternative means as control (month 34)

Beside ion channels, transporters are highly relevant membrane proteins. We included two representatives in the ASMENA project: an bacterial ammonium transporter and aquaporins. The transport mechanism of AmtB has been investigated in the lab of partner 2 based on mutant proteins and using electrochemical methods and therefore has been included in the original program. It was clear from the beginning that transport rates are very low (100 ammonium molecules per second per AmtB-transporter molecule) and electrochemical measurements consequently requires a high protein density (about 1 %) in the lipid bilayer. This density cannot be achieved by fusion methods. However, the His-tag proteins can bind to a high density to NTA and this will be the method of choice for the future.

The activity of the second mentioned transporter by optical method has been demonstrated by partner 3 (D5.3.2).

D5.3.1 Over expressed human AQP proteoliposomes and putative drug leads for the same (month 18)

Three AQPs have been successfully over-expressed in *Pichia pastoris*, purified and found to be functional and active when reconstituted into proteoliposomes. Functionality has been tested by measuring light scattering using stopped-flow spectroscopy after exposure of proteoliposomes to a hypertonic medium. Light scattering is monitored upon proteoliposomes shrinkage and compared to liposomes without inserted aquaporin protein that shrink less quickly. The three isoforms produced are SoPIP2;1, a plant aquaporin, and human AQP5 and AQP8.

These three AQPs are now being produced on a regular basis in mg quantities and stored at -80°C. One batch of one of the AQPs has been delivered to partner Chalmers. A set of putative inhibitors to one human AQP isoform was identified prior to the start of the ASMENA-project and the efficiency of these substances are being investigated.

D5.3.2 Report on measurements of aquaporin activity using hole-tethered vesicles or nanopore spanning lipid bilayer membranes (month 28)

Sub 10 ms liquid exchange times required sub 50 $\mu\text{m} \times 10 \mu\text{m}$ measurement areas (see D2.2.2). This microfluidic device was compatible with LSPR and fluorescence readout, but not with conventional SPR readout, in which case the liquid exchange time is limited to 100 ms. However, fabrication of LSPR active substrates with sufficiently extended evanescent fields ($\sim 100 \text{ nm}$) to probe the interior of tethered vesicles, and hence solute transport, was not successful. Therefore, measurements of aquaporin activity were performed using tethered vesicles (the kit was provided from Layerlab AB) with fluorescence (sub 10 ms switching times) and conventional SPR (sub 100 ms switching times) as the mode of readout. Using SPR, the glycerol aquaporin PfAQP from the malaria parasite *Plasmodium falciparum* was explored, demonstrating a new means to rapidly probe transport of sugar alcohol compounds (glycerol, xylitol and sorbitol) that occurs on the order of a couple of seconds [REF: Branden M, Tabaei SR, Fischer G, Neutze R, Hook F: Refractive-Index-Based Screening of Membrane-Protein-Mediated Transfer across Biological Membranes. *Biophysical Journal* 2010, 99:124-133]. Using a surface-based fluorescence quenching assay, water transport measurements were verified for human aquaporin (AQP5) reconstituted proteoliposomes, obtained from partner 13, with single proteoliposome resolution [REF: Olsson G, Tabaei S, Jonsson U, Kjellbom P, Tegenfelt J, Hook F: Sub 10 ms liquid exchange for permeation monitoring of single proteoliposomes. *Lab on Chip*, in writing.]

1.3.6 WP 6: APPLICATION FOR DRUG SCREENING

WP Leader: Michael Hennig, Roche

In this WP we made use of the materials and methods acquired in previous WPs to develop a prototype system for a functional assay for drug screening against membrane protein targets. In a first phase emphasis was put on quantitative measurements of membrane protein, allowing automated control of electrochemical and optical detection of effector compound binding and its effect on drug target activity. In the next phase integrated microfluidics developed in WP1 were used to establish a prototype system to monitor membrane protein activity under screening of libraries of effector compounds. Additionally, the same setup was planned to be explored as platform for investigating drug lead-membrane interaction and permeation. To ensure relevance of the prototype design tests will be carried out together with partner Leister with primary interest in commercialising the drug screening setup and with partner Roche with primary interest as end user of instrumentation for drug screening.

Summary of progress

Regular reviews of the progress of the work have been done. In order to enable quantitative measurements of membrane proteins, the task of partner 14 (Roche) was to provide an isolated, highly purified and active preparation of a pharmaceutical relevant ion-channel. It has to be emphasized that this was a high risk project given the fact that not many ion channels have been isolated and characterized in a cell free preparation. The activities were focused on two pharmaceutical relevant proteins: The hERG channel and the ASIC1a channel.

hERG channel is a well-known off-target protein often involved in unwanted side-effects of drug molecules. hERG testing with cell based assays is mandatory for compounds during lead optimization and clinical candidate selection. Various constructs of hERG were expressed in different expression system. Despite the support from an external group Roche failed to express and purify the protein in sufficient amounts and activity. Material could only be isolated in low yields. It showed no activity in a Biacore based binding assay. To our knowledge the hERG channel could so far not be expressed and functionally purified by any other scientific group.

ASIC1a (Acid Sensing Ion Channel) is a potential target for many neurodegenerative disorders (multiple sclerosis, Huntington's disease, Parkinson's disease, ischemic brain injury), neuropathic and inflammation pain and anxiety. The homomeric ASIC1a is a proton-gated Na⁺ selective channel. Psalmotoxin, a 40 aa long peptide, is a specific antagonist for ASIC1a. SF9/baculovirus was used as the expression system to analyse various different constructs of human and chicken ASIC1a. The quantification and functional analysis of expressed protein was achieved by fluorescence-based size exclusion chromatography in detergent solution and reverse phase HPLC. The assays were indispensable for the optimization of the expression in SF9 cells as well as for the optimization of the solubilization and purification of the protein. A binding assay based on Biacore technology was developed and used to characterize the binding of the specific, peptide antagonist Psalmotoxin to isolated ASIC1a. Finally, Partner 14 (Roche) succeeded in producing highly purified, homogenous protein that showed binding of Psalmotoxin in the expected nano-molar range. 1 mg of protein (solubilized at a concentration of 1.25 mg/ml) was produced and delivered together with commercially available Psalmotoxin to the ASMENA team in February 2011.

This was followed by an enormous effort by several project partners to enable the incorporation of this pharmaceutically relevant membrane protein into pore spanning bilayers. Unfortunately, mostly due to difficulties with reconstitution, these efforts so far have not been successful.

Main significant results

As a result of collaboration between several project partners, a new method has been invented for the optical and electrochemical quantification of membrane transport processes. (D6.1.1) In addition, the proposed method for the aquaporin system could not only be demonstrated but also a small scale screening of a compound library was performed with outstanding success.(D6.2.2) Although many difficulties were faced with the ion-channels, finally an isolated, highly purified and active preparation of a pharmaceutical relevant ion-channel drug target protein for the establishment of a label-free binding assay

for quantitative measurements could be delivered by partner 14 (Roche). Several promising approaches are being followed at three partners' laboratories even after the project end but so far we could not report on the successful electrochemical characterization of this system

Deviations from Annex I

WP6 is the integration of all project results into a close to product application. As all risk factors accumulated around this effort and the original planning was highly ambitious we had to continuously update the target goals throughout the project. Even as such we were not able to completely reach all project goals.

The project anticipated the use of an ion-channel drug target protein for the preparation of a quantitative assay to measure the interaction of drug candidate molecules and, subsequently, to enable screening at medium to high throughput. The work towards this goal of the project was delayed therefore set-up of the assay with ASIC1a could only start at the very end of the project. This resulted in two important deviations from Annex I. The drug compound library test was only possible on the aquaporin system, i.e. D6.2.2 but it could not be performed on a transporter nor on an ion channel so D6.2.1, D6.2.3 and D6.2.4 could not be fulfilled.

Achieved results and Deliverables

D6.1.1 Drug permeation assay for representative drugs using a nanopore spanning lipid bilayer (month 36)

As a prerequisite for drug permeation assays a microfluidic device enabling the automated formation of stable lipid bilayers was required. Such a device was designed and manufactured as prototype together with the PSI and an academic partner. It was shown that using the device allowed indeed automated lipid bilayer formation. This device was primarily used for ion channel measurements using single or multiple pore chips, proving its suitability for these kinds of analyses (see WP5). Permeation measurements, however, were not really possible for this first prototype, since it is not suitable for standard optical readouts. For this, an advanced prototype would be necessary, based on the gained experience from its predecessor, which permits simple optical analyses in the UV/VIS range, depending of course on the drugs to be tested. These analyses could not be executed within the project time frame.

Note: In the current status, the question of the chip design is still not answered. The approach of a chip-based permeation assays makes only sense, if current technologies are either improved in terms of the "biological added value" that the new approach would provide, or by reducing significantly the current assay costs. Thus, beside of showing the feasibility of drug permeation assays, the question of the chip design (and its resulting costs) weighs heavily on this task.

However, a new methodology for drug permeation assay has been invented and patented during the ASMENA project. [Membrane permeability sensor with a sensitive porous reservoir, PCT #####] This method enables the detection of membrane transport process through a lipid membrane coated porous film such as a polyelectrolyte multilayer or a thick polymer membrane by any sensor detection mechanism. It has so far only been demonstrated using OWLS technique but partner 8 has licensed it for electronic measurements. [Simultaneous OWLS and EIS monitoring of supported lipid bilayers with the pore forming peptide melittin; Kaori Sugihara, Marco Delai, Istvan Szendro, Orane Guillaume-Gentil, János Vörös, and Tomaso Zambelli; Sensors & Actuators: B. submitted in 2011 and master thesis of Marco Delai; OWLS assay techniques for investigating membrane-bound ion-channel activities; Inna Székács, Katalin Erdélyi, István Szendrő, Nóra Kaszás, Pál Gróf, Emilia Madarász, in preparation].

D6.2.1 Report on quantitative measurement of reconstituted ion channel and/or ion transporter activity subject to drug lead (month 32)

When the ASIC channel became available the general assembly has made a decision that the most promising technologies from the various partners' laboratories should be attempted in parallel in order to fulfil this deliverable. Several joint projects and technology transfers took place but despite of these concentrated efforts of the consortium results of quantitative measurements with reconstituted ion channel as well as potential drug molecules (molecules that modulate the activity of the ion channel and lead to a dose related response) are not available today. Here we present one of the most promising approaches (a collaborative effort of partner 1, 2, 9 and 11) based on the in situ reconstitution of an ion-channel at the sensor surface. The solubilised and immobilized His-proteins could be integrated into a self-assembling lipid bilayer, when the detergent molecules are removed. This procedure comprising removal of detergents and concomitant replacing with lipid molecules needs time and has been monitored by QCM. The EIS investigation as well as the FRAP-investigations confirmed that an intact lipid bilayer has formed. (See Figure 6.1) The diffusion rates of dyed lipids after photo-bleaching were in the same order of magnitude as free-standing bilayers, but always somewhat lower probably due to interactions of the bilayer with the supporting polymer. However, the diffusion of ions across the supporting polymer is still good. We could demonstrate the formation of NaChBac-lipid-bilayer in single pores. The moderate sealing quality can be caused by open ion channels or imperfect sealing. This system will be tested also with drug leads and a report will be created by partner 2 but the necessary experiments will be performed only after the project end.

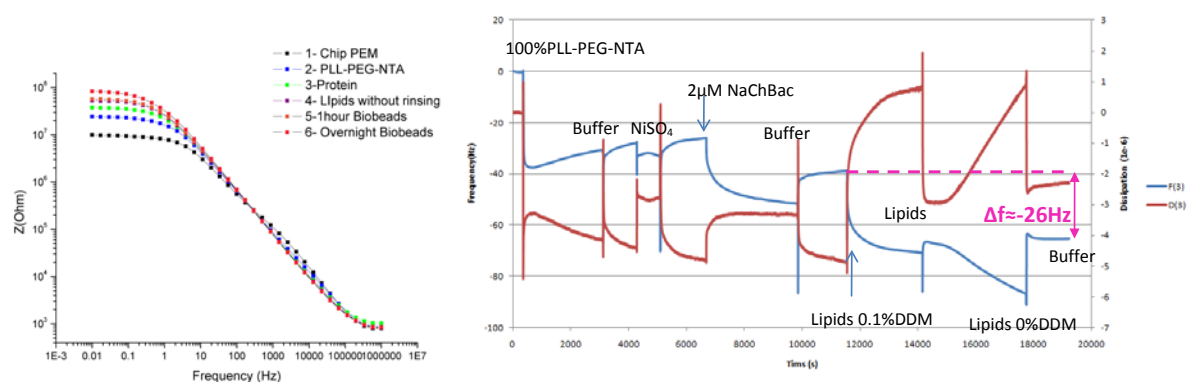


Figure 6.1: Left: Bilayer formation around surface immobilized ion channels with his-tag monitored by EIS. Right: The in situ reconstitution process monitored by QCM-D.

D6.2.2 Report on quantitative measurement of AQP activity subject to drug lead (month 36)

Partner 3 (Chalmers) has contributed to the screening of 23 drug leads against the glyceroaquaporin from the malaria parasite *Plasmodium falciparum* (see D5.3.2). In this work, a new class of inhibitors of the *Plasmodium falciparum* glyceroaquaporin was developed using in silico high throughput screening. The crystal structure of the PfAQP protein was used in conjunction with the virtual high-throughput screening programme (vHTS) eHiTS to identify a small library of putative inhibitors of PfAQP. To test these compounds in vitro, SPR-based screening of translocation of uncharged solutes across lipid membranes was used (see D5.3.2). In this way, rapid screening of the functionality and the degree of inhibition of the channel reconstituted in artificial proteoliposomes for multiple potential inhibitors was successfully performed. Inhibitory values (IC_{50}) for the best out of the 23 investigated compounds were found to be in the range of 1-10 μM . The initial hit compound was further tested in an in vivo assay to determine its activities against intraerythrocytic *Plasmodium falciparum*. [Fischer G, Moberg A, Sjöhamn J, Tabaei S, Brändén M, Höök F, Hedfalk K, Fishwick C, Johnson P, Neutze R, Simmons K: Discovery of Novel Small Molecule Inhibitors of the Aquaglyceroporin of *Plasmodium falciparum* using Virtual High-throughput

D6.2.3 Report on a pharmaceutically relevant membrane protein activity in nanopore spanning lipid bilayer subjected to a drug compound (month 36)

Here we report one example of a promising approach among several that we followed. Partner 1 in collaboration with partner 2 could successfully form a bilayer from proteoliposomes directly derived from cells using the optimized protocols developed in WPs1-4. The system was then introduced to partner 9 and an impedance measurement was performed to test the electronic properties and the drug effect. These initial results are promising and the partners will continue to follow them up also after the project end. (See Figure 6.2)

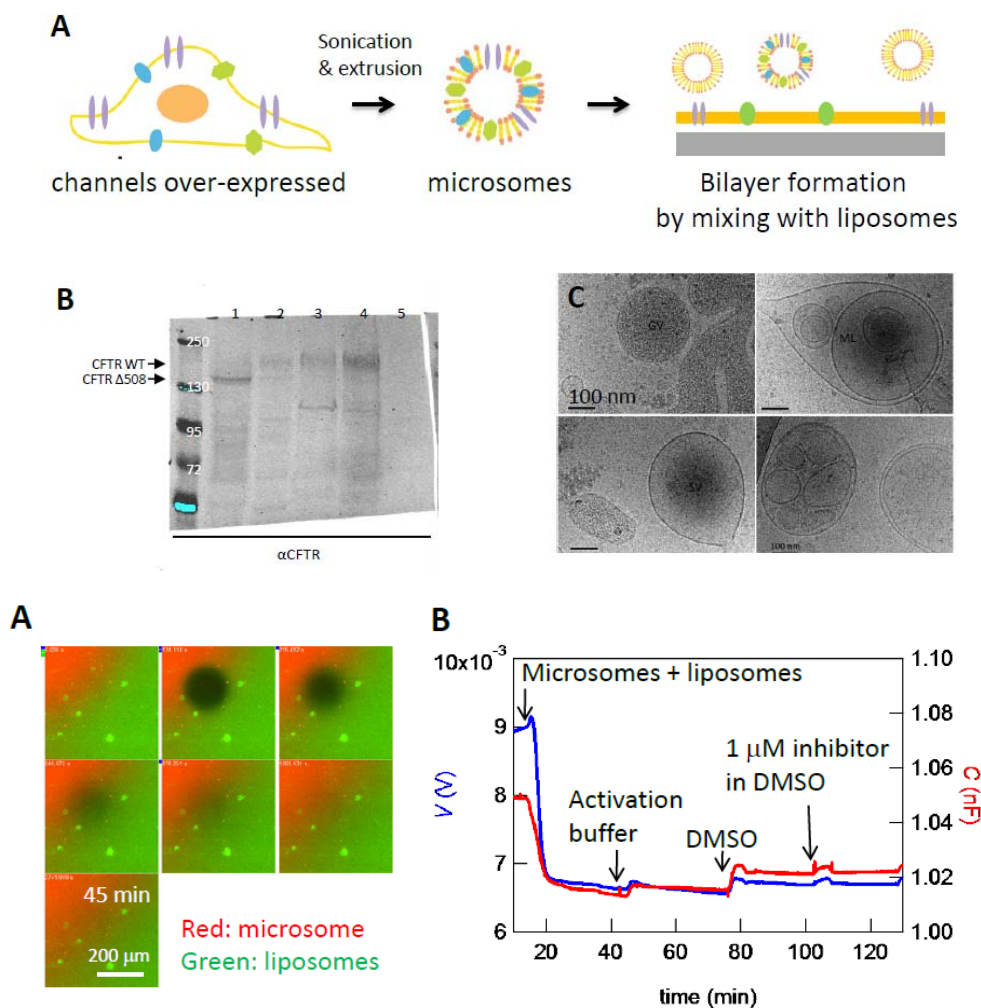


Figure 6.2: Top: The microsome approach: A) A schematic of the procedure. B) A western blot, showing the successful expression of CFTR proteins. C) cryoTEM images of the microsomes. Bottom: A) fluorescence recovery after photobleaching (FRAP) and B) the electrical sealing of the bilayer made of microsomes.

D6.2.4 Drug compound library test (month 36)

Due to the fact that the results from D6.2.1 and D6.2.3 were only becoming available towards the very end of the project, a reliable and validated assay system could not be implemented and testing of a compound library was out of scope for the remaining time of the project.

1.4 The potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and exploitation of results

At the start of ASMENA, we were presented with the following situation:

The report “Intellectual Property in the Nanotechnology Economy” (2007) from Nanoforum, a pan-European network funded by the EU analyzing the nanotechnology field, claims that nanotechnology in the health and personal care market has an estimated worth of EUR 535 billion and is growing at 7-9% per annum. Patents and scientific papers have grown exponentially in this sector over the last two decades; with Europe being the leading region in the number of filings. The “Outcome of the Open Consultation on the European Strategy for NANOTECHNOLOGY” (2005) selected “Nano structured sensors” and “Sensors based on biological molecules” as areas most important for EU investments in nanotechnology. Bionanotechnology is an area of nanoscience where Europe has a relative position of strength, but it is also an area of rapid growth where further investment and research is seen as crucial to future success and large market share.

According to “Nanotechnology and its Implications for the Health of the EU Citizen” (2004), the in vitro diagnostics market, which mainly includes microarrays and sensors, was worth approximately US\$25 billion in 2004 with strong growth expected. The same report estimated the world wide pharmaceuticals market to US\$ 400 billion in 2002 and at a current growth rate of 8%. Since more than 50% of all interesting drug targets are membrane proteins [94] the impact and market share of new technologies addressing specific problems of drug screening on membrane proteins is obviously huge. It can be expected that new research tools making it possible to screen function of membrane drug targets – like cell surface receptors – will open up new avenues for original drug development. On the other hand, the X-ray structure of only a few membrane proteins is available which hinders the use of computational methods for the identification of drug targets. At the same time, the drug discovery process demands high-throughput techniques (HTS) for the validation of the large amount of potential drug molecules offered by combinatorial chemistry. Although, the over 2500 publications about proteomics in 2004 indicate the enormous interest of the scientific community in using proteomics to answer biological questions, no good method existed for high-throughput investigation of membrane proteins, the most important class of proteins.

The purpose of the ASMENA project was to contribute to the competitiveness of European Industry by developing a technology that impact three of the main five processes of the pharmaceuticals industry (see Figure 17): lead identification, lead optimisation and permeability assays. Our industrial partners had and have interest both as suppliers of screening technology for the processes below – a market of millions of chips per year – and as suppliers of measurement technology to the larger, but lower throughput, research market. Finally, the pharmaceutical partners have an interest as end-users of the technology, where increasingly information rich assays and well defined measurement technology will save time and cost of carrying out the above mentioned processes as described in more detail below. In particular, lead identification is the bottleneck in the drug development process. Highly accurate and specific screening methods are required to identify active substances, which bind to a pre-selected drug target and for the assessment of their potential adverse effects. Functional assays for membrane proteins as ion channels, GPCRs and transporters are therefore highly demanded.

Although, ASMENA could not solve all the complex issues that are required for the automated screening of membrane proteins by the end of the project period, the concentrated efforts of the partners have made a major contribution both in terms of advancing technology in the right direction and towards understanding the remaining challenges. E.g. it is the first time that spontaneously formed lipid membranes could be achieved over nanopores resulting in extreme stable and reliable sensor architectures that are compatible with laboratory automation tools.

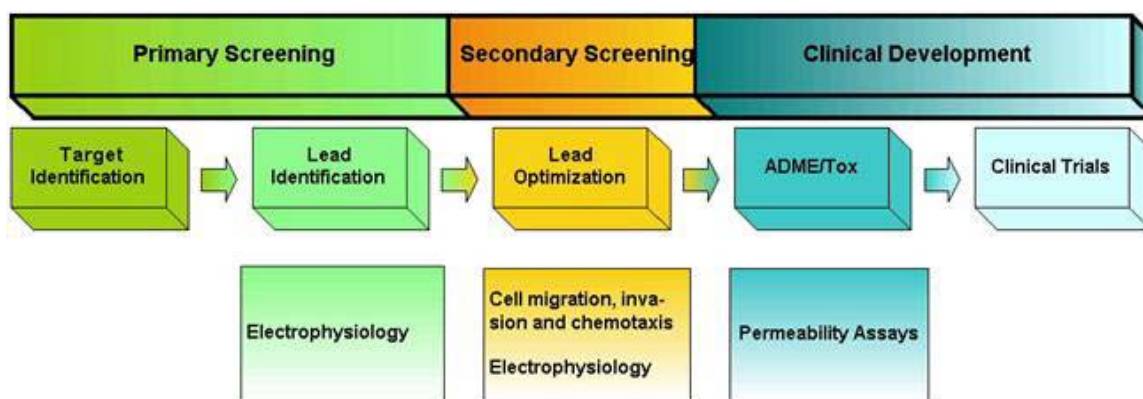


Figure 17. Work flow for developing leads in the pharmaceutical industry.

The primary use of the developed technology will be for analytical profiling, which is the reliability step before screening in drug lead discovery. Since the technology has enabled new ways to characterise drug leads some of the major impact is primarily expected in the medium to long run. Today, drug leads targeted against membrane proteins are primarily evaluated in vitro based on binding assays. In most cases not even affinity constants are determined, since they require multiple measurements in a range of various concentrations when measured by fluorescence binding assays. Thus, several of the novel approaches that were developed in the course of this project for studying the effect of ligand binding on the membrane target currently lack competitive technology, which could cross-validate the new results. Building a database which correlates the new information rich assays with actual in vivo performance will take additional time, but is a prerequisite for a wide-spread revolution in analytical profiling and drug screening within the pharmaceutical companies. Thus, the impact can be divided in at least two time horizons: short (impact at the completion of the project) and long (impact after dissemination and acceptance of the knowledge generated related to the assays).

1.4.1 Economic and Social Impacts

Important for creating impact with the proposed research is that it not only addresses relevant problems in drug-membrane target interactions, but also that it does so in a format which is **relevant to industrial standards and work processes**. In order to ensure that the developed tools have impact on screening on relevant drug targets and work flows already adopted in the pharmaceuticals industry an industrial partner (partner #14) has been involved in the final testing of the measurement techniques and assay formats employed here. Although it is clear that the developed technologies still have a long way to go until they can be accepted by the stringent requirements of real pharmaceutical screening, at the last meeting of the Industrial Board of ASMENA all industry partners have agreed that they benefited tremendously from the project either via the new knowledge and better understanding that was generated or via new technologies that could be implemented as new application notes of the SME partners.

The testing of the analytical profiling tools targeted against ion channels and transporters will still be performed on a highly relevant ion-channel (hERG) frequently used in drug screening as early indicator for side effects after the project end in collaboration with partner #14. If successful, impact on research on other classes of membrane drug targets can also be obtained by similarly demonstrating correlation of results with existing model systems for those classes. Once a sufficient number of significant correlations have been established impact on the methodology and speed of early drug screening is expected to be large and enduring, due to the introduction of completely new tools with enhanced information content.

Overall, the outcome of ASMENA has provided a variety of new materials and methods for researchers to establish their own dedicated assays and contribute to understanding of the function of known and new membrane proteins.

Faster, higher and more enduring impact by having one of the industrial partners (partner #8) as leader of the chip and instrumentation development was one of the main goals of ASMENA. By tying the scientific developments to production of industrial prototypes for their implementation it will now be possible to

quickly exploit and disseminate the generated intellectual property to a wider market. Although the greatest novelty and impact would be generated by establishing the core nanopore chip for electrochemical functional and local surface plasmon resonance affinity and liquid pumping measurements on npsLB as standard tools in the industry, some of the IP generated on membrane functionalisation of sensor substrates are expected to impact on other sensor platform formats as well. WP4 included two partners with interest in such dual exploitation. Current affinity sensors like surface plasmon resonance and waveguide spectroscopy have yet to market tools which consistently offer enough sensitivity, reproducibility and ease of use for membrane proteins. Our approach addressed those issues in several ways. The combination of the technology for membrane protein immobilization contributed by, *e.g.* partner #5 and #9, with membrane sensing formats developed for highly sensitive LSPR and waveguide spectroscopy instruments had significant impact on technology for membrane-ligand interaction. By using the generic approaches developed by the academic partners and also tailoring those specifically for the instrument developers' systems, the outcome of the research can now have a direct impact on the market for drug screening tools. By filling this gap in the market for analytical tools benefit will accrue not only to the consortium partners aiming to commercialising the instrumentation and chips, but also to the substantial European drug industry who will be end users of the analytical tools.

Even if not all goals have been fully reached, an important step has been taken so that the European public in the future will benefit from potentially more rapid and cheaper drug development by the same companies.

The successful implementation of these drug discovery tools will have direct and immediate relevance to the study of any membrane process, with obvious relevance for the wider study and understanding of many different disease mechanisms such as viral susceptibility or neurodegenerative diseases such as Alzheimer's or prion diseases.

As hoped, the project has moved us to a more thorough addressing of the structure-function relationship of membrane proteins. The structure-function relationship is a central paradigm of molecular life sciences. Structural resolution of membrane proteins has advanced in the last few years; however, assay systems for these membrane proteins are still scarce and mostly based on proteoliposomes. There are few tools available and therefore few studies consistently addressing this important area of research. By focusing on generating the chain from fundamental understanding (theoretical and experimental) of membrane assembly to specialised instrumentation for measuring membrane component functionality via development of thorough understanding of biosensor response to membrane-small molecule interaction we think we had some impact on both fundamental and applied research in the area. The high scientific impact of allowing investigation of structure-function relationships for membrane proteins in real time with standard sensor techniques will now help greatly in promoting acceptance of the technology developed by our commercial partners. By establishing protocols and formats for studying these effects on the molecular scale it will be possible to develop future industrial standards for utilising such measurements for improved drug screening. As such our technology, like any technology that enhances our understanding on the structure-function relationship, is expected to have high impact. For example, it is plausible that in the future, direct measurements of structure and/or function *in vitro* will make it possible to earlier detect promising drug leads based on this information alone (before biological tests) and by the complementary assays also early detect toxic effects of the same drug leads. Such early profiling would greatly reduce time and cost for drug screening.

There is already customer demand experienced by the biosensor partners for sensor chips which are functionalized for studying biological membrane interactions. Robust, generic protocols for the immobilization of supported and tethered lipid membranes of a variety of compositions containing a high percentage of transmembrane proteins had been unavailable and will now transform the ability of the commercial instruments to address many new aspects of membrane and membrane protein based measurements. This will of course be true for dual polarization interferometry (DPI) and optical waveguide lightmode spectroscopy (OWLS) (marketed by the partners) which both benefited largely from the new application notes and product lines that could be launched thanks to this project, but also other biosensing

tools will now benefit from opening this new range of applications, as the technology is robust and easily transferrable. This will enable the technologies (both surface chemistry and instrumentation) to undergo a transition from interesting research system to standard assay in these areas, with the associated order of magnitude increase in commercial implementation and impact.

The establishment of the technique as a robust assay method for characterizing the mechanistic details of drug/membrane interactions is expected to have a significant impact on measurements of membrane permeability and physico-chemical characterization for Adsorption, Distribution, Metabolism, Excretion and Toxicity (ADMETox) studies, which are key steps in the drug discovery process. Determination of the sensitivity of structural effects observed for membrane proteins using DPI have shown how widely applicable across other optical waveguide platforms the developments are. Impact of this will be to significantly simplify the drug discovery and optimization process for membrane proteins.

Current standard formats in use in the pharmaceutical industry rely on pipetting for liquid handling of targets and drug leads. By including development of on-chip microfluidic handling with corresponding *in situ* surface functionalisation we have provided an integrated alternative to that approach. Integrated chip microfluidics has potential to impact the industry by allowing use of smaller volumes and more advanced multiplexing assay formats. Thus reducing time and cost, as well as increasing reliability and information content for the same measurement. On the scientific level the implementation of microfluidics for studying membrane proteins can allow accessing very short time scales to study transient response of membrane proteins to environmental changes, which could together with other techniques developed in the project be extended to single-molecule studies. Such studies would greatly enhance our understanding of how membranes function and respond on the molecular level.

Summary of short-term impact:

1. Tools for localization of biomolecules (particularly lipids) using nanoscale chemical and topographical structures of high relevance to the scientific and industrial communities
2. Theoretical and experimental tools for analyzing lipid membrane and membrane protein interaction with small molecules and other environmental factors.
3. Demonstrators, prototypes and intellectual property rights in various fields of interest for our industrial partners:
 - i. Patent for membrane permeability measurement. EP09008858.4
 - ii. Membrane protein sorting, handling and immobilization technology for biosensor systems. (partner #8 and #9)
 - iii. New compounds for functionalisation of biosensor and biointerface applications. Specifically suitable for membrane and protein interaction with patterned substrates. (partner #10)
 - iv. Microfluidic tools for lipid membrane and membrane protein handling and drug screening against such targets. (partner #8 and #13)
 - v. Sensor platform for studying aquaporins and for drug screening on aquaporin targets. A new method for the efficient screening of drug leads acting on aquaporins. (partner #12 and #13)
 - vi. Waveguide based platform for affinity measurements and possible conformational changes of membrane proteins in analytical profiling. (partner #11 and #12)
 - vii. Electrochemical platform/instrumentation for functionality measurements on membrane proteins. (partner #1 and #8)

Summary of long-term impact:

1. New and better methods to address structure-function relationships of membrane proteins integrated in lipid membranes.
2. A better understanding of membrane proteins, especially tunable activity through allosteric effects. New tissue specific drugs acting on allosteric sites will have less side-effects and be tissue-specific. The expected gain for future diagnostics and therapies will be a significant step towards molecular medicine.
3. Novel profiling and screening methods in the pharmaceutical industry to score hits and remove toxic drug leads, leading to standardized *in vitro* testing of membrane protein targets and reducing number of biological tests.
4. Establishment of a European biosensor industry as leading cluster in membrane protein analytical profiling and drug screening.
5. Improved public health through the discovery of novel drugs acting on membrane proteins that are involved in many different disease mechanisms such as viral susceptibility or neurodegenerative diseases such as Alzheimer's or prion diseases.

1.4.2 Main dissemination activities and exploitation of results

Fast publication of research results after considering legal protection of intellectual property was a main objective of the dissemination strategy from the outset. In addition to fulfilling the academic requirements, fast dissemination of the scientific results in high impact journals was an integral part in maximising the impact of the created technology, by creating wide awareness and acceptance in the community for the novel approaches to drug discovery for membrane protein targets.

The consortium has been very successful in doing so, overall, 46 peer reviewed papers have been published or are being submitted, with an additional 15 publications that are currently being written.

Another goal of ASMENA was the dissemination of results by participating in established international conferences in the topic areas of the project, which typically include ACS, AVS, Gordon and ESF and international nanotechnology and biosensor conferences where several of the PIs regularly attended as invited and keynote speakers. Altogether the partners presented the project related results at over 100 conferences and in various public journals and radio interviews.

In addition, as planned, partner #4 has organized a biannual workshop (TETHEM) on biomembranes, and partner #1 a major conference Nanobio2010 for which this project constituted a core topic and was also regularly presented to a worldwide audience of the leading research groups in the field.

Partners #1 and #3 had also been involved in organising a highly successful biannual ESF conference series on Biointerface science, which has, as planned been organised for the fourth time in 2011.

All these conferences had been used to as platforms to disseminate results from the project and promote awareness of key technological developments to a leading industrial and scientific core audience.

Results have been disseminated and exploited in several ways:

- Publication of research papers (see list of publications)
- Production of application notes and protocols by/for use by industrial partners (see ASMENA web-page and the partners' web-pages)
- Patenting of key technologies (see patent list)
- Production of commercially available assay kits, specific chips, and new instruments by industrial partners.

Occurrence of IPR

Preparation for patents had started already from the onset of the project to ensure that dissemination of results to the public could proceed as quickly and smoothly as possible. Early patenting of the key concepts for guided self assembly and the sensor platform technology had also ensured that as much as possible of the developed platform(s) is owned by partners of the consortium, which have an established relationship.

Some key patents were already in possession of the partners and provided the foundation for add-on patents specific for the research to be carried on for membrane proteins. Among these can be noted patents on application of nanopore array chips for functional assays of membrane proteins had been patented by partners #2 and #8; patents on the waveguide instrumentation used by partners #11 and #12; patents on molecules for surface coatings with specific biological interactions on oxides by partner #10; patents on protein and liposome tags for sorting and liposome constructs by partners #5 and #9 and patents on use of various targets and drug leads to be used in the project by partners #13 and #14.

Thus, these participants had and have a strong interest to transfer the findings of this project into products.

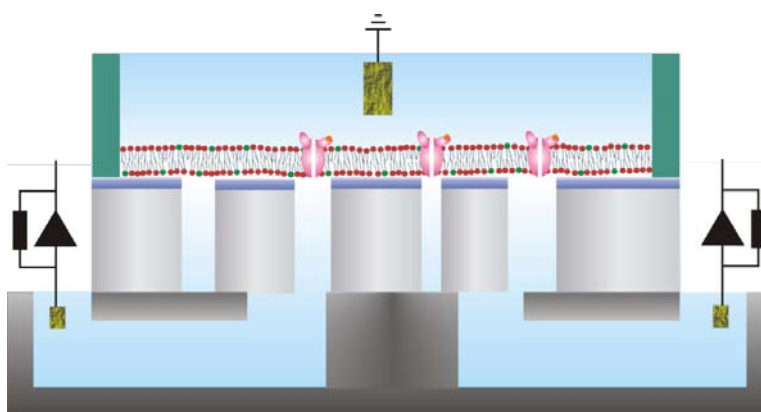
Further 5 patents of technical key steps have been created, including the production of nanoporous membrane chips, membrane assembly and surface functionalisation over nanoporous coatings towards membrane transport monitoring, a method for fluidic device making and a platform for drug screening.

Furthermore, a project web-site with access to the most important results of the project has been created. See below:

Website and logo:



<http://www.asmena.ethz.ch/>



“Functional assays for membrane protein on nanostructured supports” (ASMENA)