Formation of lipid bilayers with high densities of membrane proteins for functional studies

Author(s): Demarche, Sophie

Publication Date: 2013

Permanent Link: https://doi.org/10.3929/ethz-a-010099161

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Formation of lipid bilayers with high densities of membrane proteins for functional studies

A dissertation submitted to
ETH ZURICH

for the degree of
Doctor of Sciences

presented by
SOPHIE DEMARCHE
Ingénieure en Biotechnologie /
Diplom Biotechnologin,
Ecole supérieure de biotechnologie Strasbourg (ESBS)

Born March 22, 1985
Citizen of France

accepted on the recommendation of
Prof. Dr. Janos Vörös, examiner
Dr. Louis Tiefenauer, co-examiner
Prof. Dr. Michael Hennig, co-examiner

2013
Acknowledgments

First of all, I owe my deepest gratitude to my doctoral thesis supervisor, Prof. Dr. Janos Vörös for giving me the opportunity to do my PhD under his supervision. I appreciated our discussions and your numerous inputs a lot. Thank you for allowing me to spend time at LBB and to make me feel as a complete LBB group member even though I was based at Paul Scherrer Institute. Your laboratory is a wonderful interdisciplinary environment, from which one emerges richer both scientifically and personally.

I would like to thank Dr. Louis Tiefenauer, my direct supervisor, for having me in his laboratory and for his guidance. I appreciated that he let me work independently, but was always available for discussions and for experimental support.

I would like to thank Prof. Dr. Michael Hennig, my co-referee for joining the meetings during the thesis work, for the good suggestions and for taking the time to review this thesis.

It is a pleasure for me to thank all the people that supported me scientifically during these four years. Thanks to: Christian Spreu for the SEM imaging of the chips; to Alexandre Larmagnac and Martin Lanz for helping me with the photoresist deposition; to Dr. Roger Dawson and his colleagues for sharing their material and knowledge with me; to Stephen Wheeler for the construction of the PMMA cells; to Dr. Orane Guillaume-Gentil for the very fruitful discussion about the PEM; to Dr. Barbora Malkova for all these hours spent at the electron microscope and for accepting to review my thesis; to David Langenegger and Dr. Andre Studer for teaching me so much, for interesting discussions and good support; to Dr. Ingrid Imhof for the time shared in the laboratory and the experimental support; to Benjamin Simona and Dr. Raphael Zahn for their help with the QCM-D; to Dr. Padeste Celestino for giving me access to his laboratory and always answering my questions; to Brian Sinnet for letting me use the DLS device at the EAWAG. I am also grateful to all the people of Laboratory of Biosensors and Bioelectronics (ETHZ) and Laboratory of Biomolecular Research (PSI) for their constant help in the laboratory. A special thanks to Ulla Sutter, Antonietta Gasperrina, Daniel Frey and Katja Bargsten.

I acknowledge the financial support of the EU Seventh Research Framework Program (FP7) ASMENA, during which I had the opportunity to work with great scientists. I am thankful to all of them. I particularly would like to thank Dr. Kaori Sugihara for everything she taught me. I appreciated a lot that she always took time to help me whether for practical or theoretical problems.

I enjoyed to spend time at LayerLab and am glad to thank Dr. Olof Andersson, Torbjörn Pettersson and Dr. Julia Hedlund for their support.
I would like to thank Dr. Gabriella Santonicola and Wilma de Groot for our collaboration. Wilma, it was nice to have you at PSI.

Many thanks to Dr. Geoff Platt and Dr. Markus Swann from Farfield Group for giving me the opportunity to spend time at Farfield. It was a very interesting time for me. I would like to thank you a lot for the wonderful support you provided me during and long after my stay!

During these four years, I met great scientists but above all great friends. Thanks to all LBB group members for making this time unforgettable. Alexandre, thanks for coming visiting me at PSI; Harald, you are a great guy, thanks for all the gossiping; Raphael and Blandine, it was really nice discussing with you; Tomaso, skiing with you is just so much fun!; Orane, c’est trop la frime!; Kaori, it was really fun with you in all the cities we went to during the ASMENA project; Norma, “drinking” beer with you was always fun, Martha, Victoria, Deborah, Juliane, Raphael, Rami, Martina, Pascal, Mike, Bernd, Pablo, Dominik, Leena, Florian, Robert, Peter, Anna, Dario, Alexandre, Esther thanks for the nice times. It was really great to get to know all of you. I spent very nice moments with you during the Taufers ski weekends, all these incredible lab retreats, the beer breaks or just in the lab.

I also would like to thank all my colleagues from PSI. Martin, thanks for running with me and for your constant support. Dominik, thanks for your very special sense of humor, I love it! Sarah, good that you joined PSI before I left. It was really fun! I loved drinking wine with you. Manuel, discussing with you was always a great pleasure, and thanks for the fun things you organized at PSI (e.g.: DönerDay©). Katja, thanks for spending nice breaks with me and always being willing to help me. Cristina, thanks a lot for your support! Jose, I’m very glad I met you, stay as you are! Sebastian, thanks for always being ready to finish my plates. Andrea, Antoine and Valérie, it was always nice discussing with you. Alexandra, thanks for all your support. I loved spending hours discussing with you. Thank you for cheering me up so many times! Bara, thanks for everything: it was very cool to have some francophone support in the lab sharing my music taste and so much more. Thanks for making me discover the Monsters’ world. Andre and David, spending time with you was very nice and seeing you leave PSI was quite sad. Dawei, it was nice sharing the lab with you. You are now free to do big parties in it.

I had hard times during these four years, but I nevertheless managed to make it until the end, thanks to the great friends who also moved from Strasbourg to Zürich: Johan, thanks for helping me releasing my aggressiveness during our Squash battles, Simon, Mirie and Martina, thanks for everything.
I also would like to thank the great people I got to know during the ESBS and the BCPST years. Thanks to all my ESBS buddies, especially Deborah, Laurent, Mattieu, Sophie, Caroline, Coline and Johan. Thanks as well to my prépa friends: Coline, such a great roommate; Armelle, my favourite mouette; Camille, who welcomed us for these great DM parties. Mathilde, Perception jovial grand Masque! Marie, we will never forget you...

I would like to thank my parents who supported me during my entire studies. I would never have succeeded without them. I thank them for leaving me the choice to do whatever I like and for always being available for discussions. I appreciated their support during the four years of my PhD, asking the eternal question: “Alors, tu trouves?” . Of course, I would also like to thank the rest of my family.

Last but not least, I would like to thank Johannes. You make my life so beautiful; you are my best friend, my best guide and my love. I can never thank you enough for the support you gave me in every situation during these four years. Thanks for always having been there to boost my confidence, give me hope and make me laugh. I’m glad we decided to be roommates in San Francisco 🥰.Ich liebe dich!
“Science never solves a problem without creating ten more.”
George Bernard Shaw (1856-1950)
Abstract

Plasma membranes are the “borders” of the cells and maintain the cells’ integrity. Protein integrated into these lipid membranes allow molecules such as nutrients and degradation products to enter or exit the cell, but also signal transduction to the cell. These membrane proteins have thus a very important biological role. Consequently, their study is of high importance, as reflected by the Nobel Prize 2012 that acknowledged the study of G protein-coupled receptors by Kobilka and Lefkowitz. Membrane proteins have also a very important pharmacological role. They represent about 60% of all protein drug targets and their deregulation can lead to diseases, e.g. cancer or cardiovascular and psychotic diseases. Therefore, functional assays to study membrane protein function and to identify novel drugs are of high importance. Nowadays, several functional assays are used in academical and in pharmaceutical laboratories out of which the patch-clamp technique is the gold standard method. However, in some cases, lipid and protein compositions need to be varied. Thus artificial lipid bilayers are the method of choice. First artificial lipid bilayers were developed in the early 1960s and a lot has been achieved since then. However, integration of membrane proteins into these artificial lipid bilayers is still a limiting step.

In this thesis, protein integration into artificial bilayers using ion channels was investigated. The most widely used technique to form artificial lipid bilayers containing membrane proteins, is painting the lipid bilayer followed by fusion of proteoliposomes, which comprise the protein of interest. This technique was successfully applied on a silicon chip. However, the inherent limitations of the system, the low protein density, directed the further work toward the formation of protein-tethered lipid bilayers. The formation of protein-tethered lipid bilayers using poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) functionalized with nitrilotriacetic acid – PLL-g-PEG-NTA was first investigated on solid support and then on a polyelectrolyte multilayer-sprayed pore. The His-tagged membrane proteins were first immobilized to the NTA group, and following the lipid bilayer was formed around these proteins. The main advantage of the use of a pore is the possible access to both sides of the membrane protein and thus the possibility of adding effectors on the extracellular or on the intracellular side of the protein. Our results suggest successful formation of a protein-tethered bilayer on a nonporous as well as on a porous surface. Furthermore, polymers, grafted on the pore wall, were used as a support. They presented the advantage to be more robust than the polyelectrolyte multilayer sprayed in the pore. In this thesis, ion channels were used to allow a test of functionality after the bilayer formation. The ion channel activity measurements were partially successful for the protein-tethered bilayer technique and further investigations are necessary. Yet,
the developed techniques present considerable application potential for the development of new versatile assays for His-tagged membrane proteins, may it be ion channels, transporters or receptors.

Résumé

Les membranes plasmiques sont les “frontières” des cellules et maintiennent leur intégrité. Les protéines contenues dans ces membranes lipidiques permettent aux molécules, tels les nutriments ou produits de dégradation, d’entrer ou de sortir de la cellule, mais également aux signaux d’être perçus et transmis à l’intérieur de la cellule. Ces protéines membranaires ont donc un rôle biologique très important et leur étude est par conséquent cruciale, comme en témoigne l’attribution du prix Nobel 2012 à Kobilka et Lefkowitz pour leurs travaux sur les récepteurs couplés aux protéines G. Les protéines membranaires ont aussi une grande importance pharmacologique étant donné que leur dérégulation ou leur mauvais fonctionnement sont à l’origine de nombreuses maladies telles que le cancer et des maladies cardiovasculaires ou psychotiques. C’est pourquoi elles représentent environ 60 % des cibles médicamenteuses protéiques et les tests fonctionnels pour étudier leur fonction et identifier de nouveaux médicaments suscitent un grand intérêt. Actuellement, plusieurs tests fonctionnels sont utilisés dans les laboratoires académiques ou pharmacologiques, mais le patch-clamp est la méthode de référence. Cependant, dans certains cas, les compositions lipidiques et protéiques doivent être variées et les bicouches lipidiques artificielles sont alors la méthode de choix. Les bicouches lipidiques artificielles ont d’abord été développées au début des années 60, et depuis, de nombreux résultats ont été obtenus. L’intégration de protéines membranaires au sein de ces bicouches artificielles reste néanmoins le facteur limitant. Dans cette thèse, l’intégration de protéines dans des bicouches lipidiques artificielles a été étudiée à l’aide de canaux ioniques. La technique la plus utilisée pour la formation de bicouches lipidiques artificielles contenant des protéines consiste à former la membrane par application de lipides dissous dans un solvant organique, puis à fusionner des protéoliposomes contenant la protéine d’intérêt. Cette technique a été appliquée avec succès à une puce en silicone; cependant les limitations inhérentes à ce système concernant la densité protéique ont orienté les futurs travaux vers la formation de bicouches lipidiques ancrées aux protéines. Dans un premier temps la formation de bicouches lipidiques ancrées aux protéines à l’aide de poly(L-lysine)-g-poly(ethylene glycol) ( PLL-g-PEG) fonctionnalisé avec de l’acide nitriloacétique -PLL-g-PEG-NTA- a été étudiée sur un support solide, puis sur un pore préalablement rempli d’une multicouche de polyélectrolytes. Des protéines membranaires contenant une étiquette poly-histidine ont tout d’abord été immobilisées sur le groupe NTA, ce qui a permis la formation d’une bicouche lipidique autour de ces protéines. Le principal avantage de l’utilisation d’un pore est l’accès possible aux deux
côtés de la protéine membranaire, et de ce fait, la possibilité d’ajouter un effecteur de son côté intra ou extracellulaire. Nos résultats suggèrent le succès de la formation de bicouches lipidiques ancrées aux protéines, à la fois sur un support poreux ou non poreux. Dans un deuxième temps des polymères entés sur la paroi des pores ont également servi de support. Ils présentent l’avantage d’être plus robustes que la multicouche de polyélectrolytes. Dans cette thèse, des canaux ioniques ont été utilisés pour tester la fonctionnalité des protéines après la formation de la bicouche lipidique. Les mesures d’activité de canaux ioniques ont montré un succès partiel et des investigations supplémentaires sont nécessaires.
Les techniques développées ont un fort potentiel d’applications pour le développement de nouveaux tests fonctionnels versatiles pour les protéines membranaires ayant une étiquette poly-histidine, que ces protéines soient des canaux ioniques, des transporteurs ou des récepteurs.
Note: This work was carried out within the EU Seventh Research Framework Program (FP7) ASMENA with various partners. If not otherwise indicated I have performed the experiments and analyzed the results myself. The involved partners and co-authors of published studies are indicated at the beginning of each chapter. I personally have written this thesis work.
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### Abbreviations

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<th>Description</th>
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<td>ABC transporter</td>
<td>ATP-binding cassette transporter</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>Ag/AgCl</td>
<td>silver/silver chloride</td>
</tr>
<tr>
<td>ASIC1a</td>
<td>acid-sensing ion channel 1a</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATRP</td>
<td>atom transfer radical polymerization</td>
</tr>
<tr>
<td>BLM</td>
<td>black lipid membrane</td>
</tr>
<tr>
<td>C</td>
<td>capacitance (Faraday, F)</td>
</tr>
<tr>
<td>CV</td>
<td>cyclic voltammetry</td>
</tr>
<tr>
<td>DDM</td>
<td>n-dodecyl-β-D-maltoside</td>
</tr>
<tr>
<td>DIB</td>
<td>droplet interface bilayer</td>
</tr>
<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
</tr>
<tr>
<td>DM</td>
<td>n-decyl-β-D-maltoside</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPhPC</td>
<td>1,2-diphytanoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DPI</td>
<td>dual polarization interferometry</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EIS</td>
<td>electrochemical impedance spectroscopy</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
<tr>
<td>G Protein</td>
<td>guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GUV</td>
<td>giant unilamellar vesicle</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal ion affinity chromatography</td>
</tr>
<tr>
<td>KCl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>LDAO</td>
<td>lauryldimethylamine-oxide</td>
</tr>
<tr>
<td>MSP</td>
<td>membrane scaffold protein</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NBD-PC</td>
<td>1-palmitoyl-2-{12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl}-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>NTA</td>
<td>nitrilotriacetic acid</td>
</tr>
<tr>
<td>OmpF</td>
<td>outer membrane protein F</td>
</tr>
<tr>
<td>OWLS</td>
<td>optical waveguide lightmode spectroscopy</td>
</tr>
<tr>
<td>PAH</td>
<td>poly(allylamine hydrochloride)</td>
</tr>
<tr>
<td>PDMS</td>
<td>polydimethylsiloxane</td>
</tr>
<tr>
<td>PEEK</td>
<td>polyether ether ketone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
</tr>
<tr>
<td>PEI</td>
<td>polyethyleneimine</td>
</tr>
<tr>
<td>PEM</td>
<td>polyelectrolyte multilayer</td>
</tr>
<tr>
<td>PLL-g-PEG-NTA</td>
<td>co-polymer of poly(L-lysine) and poly(ethylene glycol) functionalized with nitrilotriacetic acid</td>
</tr>
<tr>
<td>PMAA</td>
<td>poly(methacrylic acid)</td>
</tr>
<tr>
<td>PMMA</td>
<td>polymethyl-methacrylate</td>
</tr>
<tr>
<td>POEPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine</td>
</tr>
<tr>
<td>POPC</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>POPE</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>POPS</td>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine</td>
</tr>
<tr>
<td>Proteo-GUV</td>
<td>giant unilamellar vesicle containing membrane protein</td>
</tr>
<tr>
<td>PSS</td>
<td>poly(sodium 4-styrenesulfonate)</td>
</tr>
<tr>
<td>ptBLM</td>
<td>protein-tethered lipid membrane</td>
</tr>
<tr>
<td>QCM-D</td>
<td>quartz crystal microbalance with dissipation monitoring</td>
</tr>
<tr>
<td>R</td>
<td>resistance (R, Ohm, Ω)</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEIRAS</td>
<td>surface-enhanced infrared absorption spectroscopy</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>SI-ATRP</td>
<td>surface-initiated atom transfer radical polymerization</td>
</tr>
<tr>
<td>SiO₂</td>
<td>silicon dioxide</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>SSM</td>
<td>solid supported membrane</td>
</tr>
<tr>
<td>tBLM</td>
<td>tethered bilayer lipid membrane</td>
</tr>
<tr>
<td>TE</td>
<td>transverse electric</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TM</td>
<td>transverse magnetic</td>
</tr>
<tr>
<td>TopFluor-PC</td>
<td>1-palmitoyl-2-(dipyrrometheneboron difluoride)undecanoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TRP</td>
<td>transient receptor potential</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/w</td>
<td>weight per weight</td>
</tr>
<tr>
<td>Z</td>
<td>impedance</td>
</tr>
<tr>
<td>β-OG</td>
<td>n-octyl-β-D-glucopyranoside</td>
</tr>
<tr>
<td>α-HLY</td>
<td>α-hemolysin</td>
</tr>
</tbody>
</table>
Chapter I  Introduction

Note: Part of this chapter has been published in the review: Techniques for recording reconstituted ion channels, Demarche, S., Sugihara, K., Zambelli, T., Tiefenauer, L., Vörös, J., Analyst, 2011, 136, 1077-1089. Reproduced with permission from the Royal Society of Chemistry.
Complete or parts of several figures that are used in this chapter have been published in the review: Challenges in the development of functional assays of membrane proteins, Tiefenauer, L. and Demarche, S., Materials, 2012, 5(11), 2205-2242. (equally contributed)

I.1   Description and biological importance of membrane proteins

I.1.1  The lipid bilayer

It is essential for the cell’s survival to keep its integrity by separating the inside from the outside. It is equally important to control the transport of molecules from and into the interior of a cell. Biological cell membranes are barriers consisting essentially of a 3-4 nm thick two-layer sheet of self-assembled amphiphilic phospholipid molecules (Figure I.1A)1.

Figure 1.1: The lipid bilayer
Schematic representation of a natural lipid bilayer. (A) The two-layer sheet of self-assembled amphiphilic phospholipid molecules (orange spheres with red side chains) is represented. Red polygons represent cholesterol, interlaced between the phospholipids, and the lipids with the yellow fork-like structure symbolize the glycolipids. (B) 3D and 2D chemical structures of the four most abundant lipids of mammalian lipid bilayers: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS) and sphingomyelin. Lipid structures adapted from the Avanti Polar Lipids website.

Hydrophobic acid tails of phospholipids point toward the internal part of the sheet, while their polar head groups are located on the outside. The hydrophobic acid tails consist of fatty acids that differ in length and saturation (i.e. number of double bounds). The differences in the fatty acid composition influence lipid packing. The distribution of the different phospholipids present in the bilayer depends
on the organism, but as well on the type of membranes: mitochondrial and endoplasmic reticulum membranes have different lipid compositions\(^2,3\). Four phospholipids constitute the majority of most mammalian cells: phosphatidylcholine (PC), phosphatidyethanolamine (PE), phophatidylserine (PS) and sphingomyelin\(^1\) (Figure I.1B).

Besides phospholipids, cholesterol and glycolipids constitute the lipid part of the bilayer. The ring structure of cholesterol inserts into the bilayer close to the polar head of the phospholipids, which decreases the mobility of the fatty acids. It renders the bilayer more rigid and decreases its permeability. It also decreases the natural lateral or flip-flop mobility of the phospholipids in the lipid bilayer.

Membranes are impermeable to most of the water-soluble compounds, because the internal part of their two-layer sheet is hydrophobic. A controlled transport into and out of the cytoplasm is achieved by membrane proteins, which constitute a substantial amount of the total cell membrane composition\(^4\).

Membrane proteins are responsible for a variety of functions such as energy conversion, transport of molecules or signal transduction. The membrane proteins that transport molecules from one side of the membrane to the other are called membrane transport proteins. Other membrane proteins catalyze a biochemical reaction at the membrane, such as oxidation or reduction or transduce a signal across it: these are the membrane-associated enzymes and receptors, respectively.

### I.1.2 Different types of membrane transport proteins

In this paragraph the mechanism of translocation of ions, molecules or signals is considered as a principal criterion for classification. The discrimination between active and passive transport is generally an important issue in biochemistry: Passive transport is driven by a concentration gradient, whereas active transport occurs against a gradient, concomitant with the consumption of energy, e.g. chemical energy (adenosine triphosphate, (ATP)), oxidation or absorption of light. Such energy consuming transporters are called active transporters (Figure I.2C).

Depending on which mechanism molecules cross the membrane, two types of passive membrane transport proteins can be discriminated: passive transporters or ion channels. Passive transport is energy-independent, hence the term “passive” (Figure I.2B). Such a transport involves several steps: Recognition of the molecule by the integral membrane protein, translocation from one side of the lipid bilayer to the other side and release of the transported molecule. On the contrary, the function of ion channels can be considered as an opening and closing of a pore through which ions pass the membrane. Each ion channel is characterized by its selectivity for an ion species and by its gating mechanism. The structural arrangement of amino acid residues at the entrance of the pore, the so-called selectivity filter, prevents the entering of all hydrated ions except for the specific species. The
opening and closing state of an ion channel can be regulated by binding of a specific ligand, by a change in the electrical potential across the membrane, or by mechanical stress applied to the membrane (Figure I.2A).

<table>
<thead>
<tr>
<th>Passive</th>
<th>Ion channel</th>
<th>Passive transporter</th>
<th>Active transporter</th>
</tr>
</thead>
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<tr>
<td>Voltage-gated ion channel</td>
<td>up to $10^8$ molecules $\cdot$ s$^{-1}$</td>
<td>10-1000 mol. $\cdot$ s$^{-1}$</td>
<td>10-1000 mol. $\cdot$ s$^{-1}$</td>
</tr>
<tr>
<td>Ligand-gated ion channel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mechanosensitive ion channel</td>
<td></td>
<td></td>
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</table>

Figure I.2: Types of transport membrane proteins
Passive transport across ion channels (A) or passive transporters (B) are driven by a chemical gradient. Ion channels are classified into three groups according to their mechanism of opening: Voltage-gated ion channels open after membrane depolarization, ligand-gated ion channels following a ligand binding, whereas mechanosensitive channels open upon mechanic stimulation. (C) Active transport by active transporters allows a transport against a chemical gradient due to consumption of energy, e.g. by ATP hydrolysis. Typical transport rates are indicated in red (mol. = molecule).

The translocation rates of ion channels are generally high whereas those of passive transporters are assumed to be much slower, since the transported ion or molecule interacts with some amino acid residues in the path across the membrane. For ion channels, rates of up to $10^8$ ions per second per channel molecule are estimated, whereas transporters or passive carriers have probably unitary rates in the range from 10 to $10^3$ molecules per second.

I.1.2.1 Types of ion channels
Alternatively, ion channels can be classified according to the transported species, the structure of the constituting proteins or their opening mechanism. A first discrimination between peptide and protein ion channels is important, since structure, stability and availability of membrane proteins are quite different to those of peptides. According to the opening mechanism, voltage-gated, ligand-gated,
and mechanosensitive ion channels can be distinguished (Figure I.2A). This subsection does not aim to present an exhaustive list of all types of ion channels, but is rather to give a general overview of the highly diverse families of ion channels.

### I.1.2.1.1 Peptidic ion channels

Peptidic ion channels are composed of monomers of maximal 50 amino acids and are either antimicrobials or toxins. The most studied ion channels are the two antibiotics gramicidin and α-alamethicin and the bee venom melittin. These peptides adsorb onto lipid membranes, insert spontaneously into them and consequently form pores (Figure I.3A). In artificial membrane systems, the use of peptides has two other advantages: they are commercially available and stable in solution. Thus, even if they are irrelevant for drug screening, transient or permanent membrane pores formed by such peptides are good models in the process of assay development for membrane proteins.

![Figure I.3: Peptidic and protein ion channels](image)

This figure illustrates the much lower complexity of peptidic ion channels in comparison with protein ion channels. (A) Gramicidin A molecule (adapted from pdb1MAG) is present as a single alpha helix structure in solution and two monomers insert spontaneously into the lipid bilayers forming a pore which spans the bilayer. (B) In contrast to peptides, membrane proteins as the pentameric acetylcholine receptor (adapted from pdb2BG9) may be obtained through a series of preparation steps and detergents are needed for stabilization. The subsequent integration of reconstituted membrane proteins into a lipid bilayer is a difficult step.

### I.1.2.1.2 Voltage-gated ion channels

Voltage-gated ion channels (Figure I.2A) represent the third largest group of signaling molecules in humans (after the protein kinases and the G-protein coupled receptors, GPCRs). Due to their high abundance they are important drug targets. Voltage-gated ion channels undergo conformational changes from a closed to an open state when excitable cell membranes are depolarized. They
allow cells to initiate and propagate action potentials and are thus crucial for neuronal and cardiac excitability. They are also involved in gene regulation and signaling pathways. Neuronal activity leads e.g. to gene expression regulated via Ca\(^{2+}\)-influx across calcium channels\(^{12}\).

The three most important families of human voltage-gated ion channels are sodium\(^{13}\) (Na\(_v\)), potassium (K\(_v\))\(^{14, 15}\) and calcium voltage-gated ion channels (Ca\(_v\))\(^{16}\). Most of these human voltage-gated ion channels have bacterial homologues, as the NaChBac from *Bacillus halodurans*, homologue of Na\(_v\)\(^{17}\) or KvAP from *Aeropyrum pernix*\(^{18}\) a homologue of human K\(_v\) channels. The biological roles of bacterial ion channels are not yet fully clear, but they are likely to be important in motility, chemotaxis and pH homeostasis\(^{19}\). Several additional smaller families of voltage-gated ion channels are known as for example the proton voltage-gated ion channels. The review of DeCoursey provides further details\(^{20}\).

I.1.2.1.3 Ligand-gated ion channels

Ligand-gated ion channels (Figure I.2A) specifically bind extracellular ligands which then induce a conformational change of the protein. Examples of such ligands are neurotransmitters or hormones. The different conformational states of the ion channels are related to different functional states: ion channels are opened, closed or desensitized (closed without the possibility of being reactivated). Specific molecules, called transmitters, increase the probability of the open state and thus change the membrane permeability for the ions for which the ion channel is specific\(^{21}\). When channels are open, the membrane potential may be changed or the concentration of a second messenger is increased. One of the best-known representative of this family is the nicotinic acetylcholine receptor (nAChR), a cationic ligand-gated ion channel of pivotal importance in neurotransmission. It is opened upon binding of acetylcholine which is released into the synaptic cleft\(^{22}\). The activity of ion channels is modulated by interactions with intracellular, extracellular, or transmembrane proteins\(^{21}\). Some ion channels are activated by intracellular ligands. For instance, ATP-sensitive channels which are present in most of excitable tissues, transform intracellular energy into a membrane potential\(^{23}\). Another example of activation is the intracellular change in concentration of a second messenger that is transduced by the cyclic–nucleotide-gated ion channels into a change of the membrane potential\(^{24}\).

I.1.2.1.4 Mechanosensitive ion channels

Current knowledge of the structure and function of mechanosensitive ion channels is rather limited. They open upon pressure changes and are important key regulators of osmotic sensing, both in bacteria and eukaryotes, as well as of complex processes such as touching or hearing\(^{25-28}\). Several members of the transient receptor potential (TRP) channels are mechanosensitive channels\(^{29}\).
Various molecular mechanisms can be involved in their activation. To give an example, a channel structure contains a domain which binds to the lipid bilayer and acts as a gating spring. If stretched along the bilayer, the open state is favored\textsuperscript{27}.

Some ion channels present in the outer membranes of the Gram negative bacteria, such as the porins, are not selective. Pore forming channels are also found in mammals, e.g. perforin which is involved in apoptosis\textsuperscript{30}.

Other ion channels do not fit into a single category, e.g. the calcium-activated big potassium channel (BK) which is both, ligand- and voltage-gated\textsuperscript{31}.

I.1.2.2 Non ion-channel membrane transport proteins

Other membrane transport proteins can be divided into facilitated transporters, primary active transporters, secondary active transporters or group translocators\textsuperscript{32}.

Facilitated transporters, also called uniporters, transport a chemical species following its gradient across the membrane. In contrary to the ion channels, the molecule binds temporarily to the membrane protein. Glucose transporters are uniporters. GLUT2 facilitates the passage of glucose from the enterocyte to the blood stream. Glucose is too large to diffuse passively through the bilayer and is carried by GLUT2 along its gradient\textsuperscript{33}.

Primary active transporters use a primary source of energy during transport. It can be driven by light absorption, mechanically, or by a chemical reaction (i.e. pyrophosphate bond hydrolysis, decarboxylation, methyl transfer or oxidoreduction). The ABC transporters belong to this class of transporters. These membrane proteins composed of two transmembrane domains and two nucleotide binding domains use the energy produced by hydrolysis of ATP to import or export a substrate. They play an essential role in cellular processes, for example in nutrient import or toxin export by the cell\textsuperscript{34}.

The transport of the secondary active transporters is driven by a gradient created by a primary active transporter. They can be an antiporter, if at least two molecules are transported in the opposite direction; or a symporter if at least two molecules are transported in the same direction\textsuperscript{35}.

The database of the Saier laboratory gives a detailed classification of transport membrane proteins, based on both phylogenetic and functional information. It contains as well some transporters of incompletely identified function, or auxiliary transport membrane proteins\textsuperscript{36}.

I.1.2.3 Non transport membrane proteins

Receptors have key functions in cells. Among them, the G-protein coupled receptors (GPCRs) constitute the most important family of membrane proteins. They are implied in most of the physiological processes of vertebrates. The signals activating GPCR are very divers: light absorption,
binding of peptides, organic molecules, lipids or hormones. There are seven transmembrane domain receptors, divided into five families in function of their structures and functionalities. Most of the GPCRs share the same signal transduction mechanism: the coupling of an agonist to the receptor is followed by the activation of a specific GTP-binding protein (G protein) leading to the modulation of some effector proteins. However, the exact mechanism of GPCRs is still under investigation and it has been found that some GPCRs do not interact with any G protein. Functional assays of GPCRs should contribute to answer these questions.

Membrane-associated enzymes, which not fully span the lipid membrane also fulfill important functions. A well known example of this membrane protein family is the Phospholipase A2. This calcium-dependent enzyme catalyzes the lipid hydrolysis specifically at the sn-2 acyl bond of phospholipids, yielding the release of free fatty acids, e.g. arachidonic acid, and lysophospholipids. The released arachidonic acid is further converted into eicosanoids, e.g. prostaglandin and leukotriene, which play a role in various physiological processes, such as the immune response. Phospholipase A2 is associated with the phospholipids of the membrane and can thus regulate other membrane proteins.

I.1.3 Structure-function relationship of membrane proteins

Each functionality of a given integral membrane protein has a structural basis. This is why structure determination of membrane proteins is a very active research field.

In this paragraph, only some basic examples based on ion channels are given. Several reviews give further information about the structure of ion channels and membrane proteins in general.

The simple fact that membrane proteins are integrated in a hydrophobic lipid bilayer and facilitate the passage of hydrophilic ions is related to their amphiphilic structure: the extracellular membrane parts are hydrophilic whereas parts within the membrane are hydrophobic.

The opening and closing mechanism of ion channels is called gating. Gating is controlled by external events, which activate and modulate the channel activity under defined conditions. Mammalian sodium voltage-gated ion channels are built up by four interlinked domains which form the channel. Each domain consists of six transmembrane helices, called S1-S6. Helix S4 contains negatively charged amino acid residues. Under normal conditions the cell membrane is polarized and the pore of the channel is closed. Through membrane depolarization the charged residues of the helix S4 move away from the symmetry axis, resulting in the opening of the channel.

An ion channel conducts preferentially one type of ions i.e. it is specific. Potassium voltage-gated ion channels translocate potassium ions 100 times more efficiently than sodium ions. This is achieved by the entry structure of the channel, the so-called selectivity filter. In case of the potassium voltage-gated ion channels, the linker between the helices S5 and S6 is forming the filter. Anions are repelled...
by negative charges which are present in the amino acid residues of the filter loop. The structure of the potassium channel pore leads to a higher energy barrier for hydrated sodium ions, whereas the entry of potassium ions\textsuperscript{43} into the channel funnel is promoted by a lower energy barrier\textsuperscript{11, 15}. Small conformational changes can thus have a high impact on ion channel function. This should be kept in mind when dealing with expressed\textsuperscript{44} or reconstituted\textsuperscript{45} membrane proteins. Solvent or other stress factors such as a high temperature could induce changes in the 3D-structure of an ion channel and lead to experimental results which do not reflect those occurring under natural conditions.

Structural information provides precious hints on the function of a membrane protein. In order to understand the function of a membrane protein, a simple model system may be helpful. For instance, the extensive studies of the channel forming peptide gramicidin have promoted the understanding of the selectivity filter of the bacterial potassium channel KcsA\textsuperscript{7, 43}.

On the other hand, structure information alone is not sufficient: a structure only represents a snapshot of a complicated process. Moreover, proteins often need to be modified in order to be crystallized. This is for instance achieved by introducing point mutations or by engineering fusions with T4 lysozyme\textsuperscript{41}.

In order to better understand functions and roles of membrane proteins, functional assays are primordial to complement results from structural studies.

### I.2 Expression and reconstitution of membrane proteins

In some cases, it is possible to study membrane protein function directly in a cell (see I.4.1), but often functional assays require the isolation of the membrane protein from the other components of the membrane (see I.4). A summary of the expression and reconstitution methods of membrane proteins is presented in the following section.

#### I.2.1 Expression of membrane proteins

In rare cases, the membrane protein of interest can be purified from native membranes, if they are present in sufficiently high densities (Figure I.4A). A prominent example is the nicotinic acetylcholine receptor (nAchR), which forms almost crystalline arrays in the membrane of the electric organ of Torpedo californica\textsuperscript{46}. However, most of the membrane proteins are present at much lower densities and need to be overexpressed. Expression of membrane proteins can be performed in various host cells\textsuperscript{47, 48}: Prokaryotic cells such as Escherichia coli (E. coli) (Figure I.4B), or eukaryotic cells such as yeast, insect cells (baculovirus system) or mammalian cells (e.g. HEK cells)\textsuperscript{49}. For an appropriate selection of the expression system many factors have to be taken into consideration: post-
translational modifications, lipid composition required for functionality and the prevention of toxicity effects emerging during expression. Overexpression is achieved by transformation (E. coli or yeast) or transfection (insects or mammalian cells) of a plasmid vector into the host cell. A recent excellent review details the different possibilities\textsuperscript{47}. Some overexpressed membrane proteins are toxic for the host cell. Different strategies are followed to overcome this problem\textsuperscript{50}. Expression can be performed using special strains selected for their tolerance to the toxicity of overexpression, like the E. coli strain C43(DE3) (Lucigen Corporation). Another way to circumvent this problem is the expression of the ion channel fused to a soluble protein, such as maltose binding protein: this attached protein is large and prevents the integration into the membrane and thus toxic effects. Such fusion proteins aggregate in inclusion bodies in the cytoplasm from which they can be solubilized and purified\textsuperscript{51}.

I.2.2 Membrane fractionation and protein solubilization
If not fused to soluble proteins, membrane proteins are expressed in the membrane of the host cell. After cell growth, the membrane is isolated. The most common way is to lyse the cell and to separate the membrane fraction from the rest using subsequent centrifugation steps\textsuperscript{52}. The ion channel molecules are solubilized by detergents (n-dodecyl-β-D-maltoside (DDM), n-octyl-β-D-glucopyranoside (β-OG), lauryldimethylamine-oxide (LDAO), etc.). Solubilization is a critical step, since the retention of functionality strongly depends on the detergent. Moreover, the choice of detergent can influence the method of reconstitution. Some detergents are not suitable for dialysis and consequently a screening is often needed in order to take a rational decision\textsuperscript{52}.

I.2.3 Membrane protein purification
The solubilized membrane protein is further purified, i.e. separated from other membrane proteins present in the solution. When using lectins, ligands or antibodies which specifically bind to the expressed protein (affinity chromatography), the purification becomes very efficient\textsuperscript{52}. The most widely used purification technique is the “Immobilized Metal Affinity Chromatography” (IMAC)\textsuperscript{53}. The recombinant ion channel contains a His-tag at the C- or N-terminus, i.e. a succession of several (six to ten) histidine (His) residues forming a complex with the transition metal ion (Zn\textsuperscript{2+}, Cu\textsuperscript{2+}, Ni\textsuperscript{2+}) of the
Figure I.4: Three methods for production of proteoliposomes

The ion channel is purified from a native membrane (A) or from a membrane of a host cell in which the protein has been overexpressed (B). In both cases, the cells are lysed and the membrane is fractionated by centrifugation. The membrane proteins are then solubilized by addition of an appropriate detergent and the ion channel of interest is purified from the other membrane proteins. Reconstitution into proteoliposomes is achieved by adding lipids to the ion channel preparation mix, followed by removal of the detergent either by addition of Bio-beads™, by dialysis or by dilution. (C) Cell-free expression: Direct transcription and translation of the deoxyribonucleic acid coding sequence for the protein of interest is achieved in vitro, optionally in the presence of lipids. Simultaneously, the detergent is removed by dialysis to directly obtain proteoliposomes.
chelator (e.g. nitrilotriacetic acid (NTA)), which is immobilized to the column material. These His-
residues of the protein occupy free coordination sites of the chelated metal ion. The protein of
interest is thus retained and, after washing out the unbound proteins, it can be eluted by adding a
stronger complexing agent such as imidazole or ethylenediaminetetraacetic acid (EDTA).

I.2.4 Membrane protein reconstitution

In some cases, these solubilized ion channels can be used directly (see I.4.2). However, in most cases,
the protein has to be reconstituted into liposomes resulting in proteoliposomes. In this stabilized
form, the protein can be studied directly (see I.4.3) or the proteoliposomes can be further integrated
into artificial lipid bilayers (see I.4.4 and I.4.5). The basic principle of any reconstitution is to replace
the detergent by lipid molecules. The purified protein is mixed with an excess of phospholipids and
the appropriate detergent to obtain micelles consisting of lipid, protein and detergent. The detergent
is removed by dialysis, by addition of hydrophobic polymer beads (Bio-beads™) or by dilution. The
choice of the method depends on the type of detergent and on how long the protein can be exposed
to the chosen detergent. The size of the resulting proteoliposomes can be tuned by ultrasonic
treatment or better by extrusion in order to achieve a homogeneous diameter.

Giant unilamellar vesicles (GUV) have diameters of 10 up to 100 micrometers and are much larger
than regular liposomes which are in the range of several hundred nanometers in diameter. Due to
their large size GUV mimic the cells better than smaller vesicles. Alternatively, such big vesicles are
useful as intermediates to form planar lipid bilayers with proteins integrated therein. For the
preparation of proteo-GUVs proteoliposomes are often a prerequisite. However, new techniques
are being developed to form proteo-GUV directly from solubilized membrane proteins or from
native cells.

I.2.5 Cell-free expression systems

Cell-free (in vitro) expression systems are emerging alternatives to conventional expression methods
(Figure I.4C). In such cell-free expression systems the transcription machinery of E. coli or of
eukaryotic cells is incubated with all of the necessary precursors (nucleotide triphosphates, transfer
ribonucleic acid (RNA), amino acids, etc.) and the double-stranded DNA-template with the RNA
polymerase or the purified eukaryotic messenger RNA. Toxic effects frequently occurring
during overexpression in cells can be avoided in such in vitro systems. Furthermore, the purification
step – often critical for membrane proteins – is very simple, and a high quantity of pure protein is
produced within a short time. Moreover, because such cell-free systems are directly accessible for
addition of stabilizers or ligands, it is possible to improve the expression or the folding of membrane
proteins. The addition of detergents or lipids to the expression mixture leads to micelles consisting of
proteins and detergents or lipids. Addition of both, lipids and detergent, to the mixture and incubation during the dialysis directly leads to proteoliposome formation (Figure I.4C).

I.3 Artificial lipid bilayer formation and integration of membrane proteins

As previously stated, membrane proteins can be studied in natural membranes, but artificial bilayers represent a more controlled system. Artificial lipid bilayers can be formed over a pore; the formed bilayer is then called free-standing or suspended lipid bilayer. They can also be formed on a solid surface; the lipid bilayer is then called a supported lipid bilayer.

I.3.1 Free-standing lipid bilayers formation and protein integration therein

Suspended lipid bilayers have several advantages over supported lipid bilayers: Both sides of the lipid bilayer are accessible. Conditions such as pH, ionic strength, effectors addition, can be varied independently on both sides of the bilayer. A solid support can eventually impede the membrane protein structure and function. Absence of a support, as when bilayers are suspended, is therefore an important advantage.

I.3.1.1 Lipid bilayer painting

The first formation of an artificial lipid bilayer has been reported in the early sixties by Müller and coworkers. It consists of applying lipids in an organic solvent over apertures of some hundred micrometers in diameter (Figure I.5A). The lipids form a monolayer at the interface of the aqueous solution and the organic solvent, and two monolayers assemble into a bilayer within the aperture. This artificial lipid bilayer is also called black lipid membrane (BLM). The advantage of this method is its simplicity. However, BLMs present one major drawback: The organic solvent used to paint the bilayer will partially remain in the membrane, having possible deleterious effects on the membrane protein. Moreover, these bilayers are stable for short time (maximum couple of hours), preventing long-term experiments.

I.3.1.2 Formation of solvent-free bilayers

The Müller-Montal method can be used to reduce the risk resulting from remaining solvent in the lipid bilayer (Figure I.5B). A lipid Langmuir-Blodgett monolayer is formed at an air/water interface, and the level of the interface is raised above an aperture. Two monolayers assemble then into a bilayer. However, the formation of high-quality bilayers using this technique is complex and requires special equipment.
An alternative to the Müller-Montal method for the formation of a solvent-free bilayer is the direct rupture of GUV over the aperture (Figure I.5C). When the vesicles reside on a pore, they open up spontaneously to form a bilayer. GUV can be moved over the aperture either manually\textsuperscript{66} or electrophoretically\textsuperscript{67}.

Two groups have recently demonstrated the formation of solvent-free suspended bilayers by direct rupture of liposomes to pores of smaller diameter, when using proper nanopore geometry and lipid composition\textsuperscript{68, 69} (Figure I.5D). However, in the first case, the bilayer formation was only demonstrated optically. Korman and al. achieved a Giga-seal\textsuperscript{69}, but the proof that the formed structure is really a lipid bilayer is questionable as the peptidic ion channel solution used to prove the functionality was added directly to the liposomes, and not after bilayer formation.

Jönsson et al. developed a new strategy consisting of first forming a bilayer on a solid surface and then driving it with shear force over a well. When using a certain pH, they observed that the bilayer spanned the well\textsuperscript{70} (Figure I.5E).

The quality of the formed bilayers can be checked by electrochemical impedance spectroscopy (EIS). It consists of applying a small amplitude AC signal of wide frequency between the working and the counter electrode and measuring the amplitude and phase shift of the response wave\textsuperscript{71}. The bilayer can then be modeled as a RC circuit, where R is the membrane resistance and C the capacitance\textsuperscript{72}.

Another proof of quality is the fluidity of the lipid bilayer which can be measured by fluorescence recovery after photobleaching (FRAP)\textsuperscript{73}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure15.png}
\caption{Different ways to form free-standing lipid bilayers
(A) Painting of lipids dissolved in an organic solvent. The annulus composed of organic solvent is represented in blue at the edges of the lipid bilayer. (B) The Müller-Montal method is a solvent-free method. (C) Formation of solvent-free bilayer by rupture of GUV. (D) Formation of solvent-free bilayer by direct rupture of liposomes. (E) Formation of free-standing bilayer by driving of a bilayer over a pore.}
\end{figure}
I.3.1.3 Stability of free-standing bilayers

One major drawback of free-standing bilayers is their short lifetime. Lipid bilayers formed over an aperture of several hundred micrometers in diameter are stable for minutes to hours. It has been demonstrated that decreasing the diameter of the aperture from hundreds micrometers to hundreds nanometers could tremendously increase the stability of the artificial lipid bilayers. This aspect should be considered when selecting a material. Nanopores have been prepared in various materials such as aluminum oxide, glass, quartz, polymer, silicon or silicon nitride. All these materials have a different thickness, which will result in a different aspect ratio of the nanopore chips, i.e., the pore length to the pore diameter. A low aspect ratio is an advantage for fast diffusion of molecules through the substrate and silicon nitride membranes are then the most suitable. On the other hand, high aspect ratios can be an advantage for optical detection. Pores can be produced using different fabrication techniques depending on the material and the desired size. First studies have mostly been carried out on polymeric materials such as Teflon or other plastic materials, in which the micrometer diameter pore was created by mechanical treatment (e.g., using a needle), which is still used nowadays. To get more precise material structures of the same order of diameter, laser is a good alternative. However, for going down in pore size, more precise techniques need to be used: photolithography, electron-beam lithography, colloidal lithography or focused ion beam. A material has to be chosen in regard of these properties (thickness, aspect ratio), but as well because of its interaction with the bilayer. Silicon surfaces have been silanized to increase the hydrophobicity and thus to successfully promote the adhesion of the lipid films to the aperture, improving the bilayer stability. The shape of the aperture can also be modulated to improve the stability of the lipid membrane. The lipid composition also influences bilayer stability. Polymerized lipids have revealed higher stability over natural lipids. However, it is also known that lipid properties can highly influence the function of a membrane protein. Some lipids have the ability to assemble in some structures, called lipid rafts, which also seem to affect protein function. This should be kept in mind when dealing with membrane proteins in artificial lipid bilayers. A compromise between lipid bilayer stability and its likeness to the natural system needs to be found.

I.3.1.4 Integration of membrane proteins into lipid bilayers

As written in I.1.2.1.1, peptidic ion channels insert spontaneously into the lipid bilayer, making them great tools for the development of functional assays of membrane proteins. Numerous studies on alamethicin, melittin, gramicidin and valinomycin have been published.
Only few membrane proteins self-integrate into the bilayer in the same way as peptides and are used as well to prove the functionality of the formed lipid membranes\textsuperscript{78, 79, 83, 94, 101}. This is the case for $\alpha$-hemolysin ($\alpha$-HLY)\textsuperscript{78, 79, 83, 95, 100} or proteins from the outer membranes of bacteria, e.g. OmpF\textsuperscript{77}. However, integration of other membrane proteins is much more complicated: it is not spontaneous and a big care must be taken not to denaturate the fragile protein (Figure I.3). Membrane proteins should be permanently surrounded by lipids and/or detergents. Several ways of bringing the protein into the bilayer have been developed.

Holden et al. successfully transferred an overexpressed integral ion channel directly from the membrane of the bacteria to an artificial lipid bilayer, using an agarose-filled tip (Figure I.6A)\textsuperscript{102}. The applications of such a system are considerable as it allows the study of membrane proteins in an artificial bilayer without the need of purification steps.

A more common method consists in fusing proteoliposomes containing the protein of interest to a preformed bilayer (Figure I.6B). Fusion is a sporadic and low probability event, but there are different ways to increase the rate of fusion events. One way is to create an osmotic gradient across the membrane either using salt or urea. The side containing the proteoliposomes should be hyperosmotic in comparison to the other side\textsuperscript{103}. To even further increase the probability of a fusion, one can combine this osmotic gradient with the so-called nystatin-ergosterol method. Pores are created in the proteoliposome membrane to increase the effect of the gradient\textsuperscript{104, 105} (for more details, see chapter IV). Such a process implies that the membrane protein can be studied in the presence of the osmotic gradient or that the bilayer is stable enough to support a buffer change. Using a particular lipid composition can as well increase the fusion rate\textsuperscript{106, 107}.

Free-standing lipid bilayers can be assembled by direct liposome and GUV rupture. Moreover, proteoliposome and proteo-GUV formation is established. One future combination will probably be the formation of suspended lipid bilayers containing membrane proteins directly from proteoliposomes (Figure I.6C) and proteo-GUV (Figure I.6D) rupture.

It should be noted that the protein density is difficult to control. It can be changed by varying the copy number of a protein in the fused liposomes or by varying the amount of proteoliposomes fused to the bilayer. However, vesicle fusion can only be controlled to a certain extent. Protein incorporation can be varied by changing the conditions of the reconstitution protocol, but is often limited to a small number of proteins per proteoliposome. A proteobilayer with only a few proteins can be used to record the function of transport membrane protein with a high transport turnover, as most of the ion channels. However, other membrane transport proteins can be recorded only if present in higher quantity.
Chapter I Introduction

Figure I.6: Method to integrate proteins into free-standing bilayers
Various methods to integrate a membrane protein into a lipid bilayer have been reported. (A) The protein can be directly transferred from a patch of a cell membrane using a glass pipette with a small orifice. For many methods proteoliposomes containing the protein of interest are used. (B) Proteoliposomes can fuse under specific conditions to a preformed lipid bilayer suspended over a pore. (C) Proteoliposomes can also be directly ruptured over a pore. (D) Giant unilamellar vesicles (GUVs), if positioned over a pore, rupture spontaneously resulting in free-standing lipid bilayers.

1.3.2 Supported lipid bilayers formation and membrane protein integration

1.3.2.1 Formation of supported lipid bilayers
Long-lasting lipid bilayers on solid supports can be prepared without the use of organic solvents by rupture of liposomes (Figure I.7A) or GUV (Figure I.7B). Conditions which promote the spontaneous rupture of vesicles on the hydrophilic glass surfaces have been widely studied\textsuperscript{108, 109}. They include lipid composition - charge or saturation of the alkyl chain- and ions or other molecules present in the surrounding buffer. For example, calcium ions are known to promote the rupture of negatively charged liposomes\textsuperscript{110}. Poly(ethylene glycol) (PEG) molecules have been proved to induce the rupture of liposomes of some composition\textsuperscript{111}. Supported lipid bilayers have also been prepared on highly doped conductive silicon-dioxide surfaces in order to directly monitor ion flow across peptidic gramicidin channels using EIS\textsuperscript{112}. The chemical property of silicon dioxide (SiO\textsubscript{2}) is similar to glass and such surfaces trigger vesicle fusion. Apart from electrically insulating silicon oxide surfaces, formation of a bilayer on many conductive sensor surfaces, used as electrodes, has also been investigated. These surfaces include indium tin oxide (ITO)\textsuperscript{113}, gold,\textsuperscript{114, 115} mercury,\textsuperscript{116} diamond,\textsuperscript{117} and carbon nanotubes\textsuperscript{118}. One major drawback of supported lipid membranes is the fact that the lower leaflet of the bilayer facing the surface is in close contact with the underlying support. This leads to a fast ion...
accumulation in the few nanometer wide space between the substrate and the bilayer, which severely hinders quantitative measurements. In addition, the surface might interact with integral membrane proteins modifying their function.

**Figure I.7: Different ways to form supported lipid bilayers**
(A) Direct rupture of liposomes. (B) Rupture of GUV. (C) Formation of tethered bilayer by rupture of liposomes on a solid support functionalized by a tether monolayer. (D) Combination of a free-standing and a supported lipid bilayer.

### I.3.2.2 Formation of tethered lipid bilayers

For about two decades, many attempts have been undertaken to solve this problem using different types of cushions or tethers in order to increase the distance between the solid surface and the lipid bilayer (Figure I.7C). The tether can consist of covalent layers as thiolpeptides, thiolipids or PEG spacers. Tethers non covalently linked to the surface have also been widely used, as the biotin-streptavidin system, polyelectrolytes, lipopolymers or non covalent PEG spacers. For example, Diaz and coworkers have created a polymer supported lipid bilayer by fusing liposomes containing PEG-conjugated lipids. The thickness of the cushion was tuned by varying the molecular weight of the PEG molecules. The larger space accommodated not only the transmembrane portion of the macromolecule, but also passivated the underlying surface and reduced unspecific adsorption of proteins. However, the bilayer resistance in such systems has to be considered critically, since the surface chemistry or the change in the charge transfer resistance can largely contribute to the observed values.

The quality of supported or tethered bilayers can be assessed electrochemically by EIS. The solid support acts as the working electrode. Atomic force microscopy (AFM) can be used to check the topology and homogeneity of the membrane. Surface plasmon resonance (SPR), dual polarization interferometry (DPI) and quartz crystal microbalance with dissipation monitoring (QCM-D) are also used to follow vesicle deposition, rupture and bilayer formation. The fluidity is, as for free-standing bilayers, checked by FRAP.

### I.3.2.3 Protein integration into supported bilayers

Most of the techniques to integrate membrane proteins into free-standing bilayers are as well applicable for the integration of membrane proteins into supported lipid bilayers. Direct rupture of...
proteoliposomes or microsomes leads to the formation of a supported (Figure I.8A) or tethered (Figure I.8B) lipid bilayer containing the membrane protein of interest. Proteo-GUV rupture will also form a lipid bilayer containing membrane proteins (Figure I.8C).

**Figure I.8: Method to integrate proteins into supported bilayers**

(A) Proteobilayer formation by direct rupture of proteoliposomes. (B) Tethered proteobilayer formation by direct rupture of proteoliposomes to a tethered monolayer. (C) Supported proteobilayer formation by opening of a proteo-GUV. (D) Protein integration into a supported bilayer by fusion-in of proteoliposomes. (E) Fusion of proteoliposomes to a supported lipid bilayer facilitated by DNA hybridization. (F) A protein-tethered bilayer is formed by protein immobilization via its His-tag (red) followed by bilayer formation around it.

Fusion to preformed monolayers or bilayers is another way to integrate a membrane protein into a membrane. This fusion can be spontaneous (Figure I.8D) or facilitated by using the SNARE proteins, which mediate vesicle fusion in living cells, or hybrid DNAs (Figure I.8E). In this last case, the bilayer and the vesicles contain a hybrid DNA sequence. Upon DNA hybridization, the liposomes and the bilayer come into contact, which promotes the fusion. These methods, which enable facilitated fusion, have not yet been applied to the formation of free-standing lipid bilayers. As for the free-standing lipid bilayers, the protein density is difficult to control.

One elegant way to tether the bilayer and simultaneously allow the integration of the membrane protein is the newly developed protein-tethered bilayer (Figure I.8F). In this method, the membrane protein is an anchor and the protein density of such a system can be controlled.
I.3.3 Emerging methods of lipid bilayers formation

I.3.3.1 Combination of free-standing and supported bilayers

As emphasized, free-standing lipid bilayers have several advantages, but also have drawbacks, e.g. their relative low stability. A combination of lipid bilayers which span a pore but are supported by a permeable material was recently demonstrated. Stabilization of a painted bilayer for 12 days has been achieved by encapsulation of the bilayer in an hydrogel by Jeon et al.\textsuperscript{152}. Recently, Sugihara et al. have created a lipid bilayer with a Giga-Ohm sealing by fusion of liposomes over a nanopore that was filled with a polyelectrolyte multilayer (PEM) (Figure I.7D). This preparation is solvent-free and lipids self-assemble into bilayers. The bilayer exhibited a long lifetime as demonstrated by monitoring single peptide ion channel activities for a few weeks\textsuperscript{99}. However, the high charge density of the polymers may impede a general application.

I.3.3.2 Further emerging methods

An alternative to free-standing, supported bilayers or a combination of both, is the droplet interface bilayer (DIB)\textsuperscript{153, 154}. Syeda and coworkers have formed DIBs between two lipid monolayer-encased aqueous droplets and monitored the transmembrane ionic currents across individual channels\textsuperscript{155}. A DIB was formed between the two lipid monolayer confined droplets, one of which was filled with the \textit{in vitro} transcription and translation mixture. \(\alpha\)-Hemolysin blockers were encapsulated in the other.

In the first droplet \(\alpha\)-hemolysin monomers were expressed and then inserted spontaneously in the bilayer between the droplets and formed a heptameric protein channel. This channel was occasionally blocked by the \(\gamma\)-cyclodextran present. Single channel activities have been recorded using two silver/silver chloride (Ag/AgCl) electrodes (one in each vesicle).

Nanodiscs are another emerging tool, which consist of a bilayer of phospholipids arranged in a discoidal structure by surrounding membrane scaffold proteins\textsuperscript{156}.

I.4 Systems for functional assays of membrane proteins

I.4.1 Native membranes

Voltage-clamp is a method which allows to measure the flow of ions across membranes as an electric current, whilst the membrane voltage is controlled (“clamped”) by a feedback amplifier\textsuperscript{157}. This electrochemical method has been applied to the membrane of a whole cell, or to a piece of a membrane. Furthermore, the type and number of electrodes can vary from two (working and counter) to four (plus standard and reference) electrodes\textsuperscript{157, 158}. The first voltage-clamp experiments were carried out in 1949 by Cole\textsuperscript{159} and Marmont\textsuperscript{160} who recorded the current of a giant squid membrane under a controlled potential. This principle was the basis for the currently well-known
voltage-clamp technique. In the fundamental experiment of patch-clamp on a whole cell\textsuperscript{161}, Neher and Sakmann approached a glass pipette with an orifice of about 3 µm in diameter to the surface of muscle fibers and measured for the first time the ion current passing through single acetylcholine receptor channels (AchR). The novelty of their work was the usage of the small pipettes to “patch” a small membrane area. Thereby, the background noise was reduced and sufficiently high resistivity achieved, which is a prerequisite for the observation of single channel activities. This pioneering achievement\textsuperscript{162} earned the two scientists the Nobel Prize in 1991.

The methodology has been improved by the same group, who achieved a “Giga(Ohm)seal”\textsuperscript{163} while the current whole-cell patch-clamp technique was established afterwards. The patch-clamp technique allows measurements on native cells, and does not require purification and reconstitution of ion channel proteins in artificial bilayers.

Although the whole-cell patch-clamp technique is still the gold standard for the investigation of fundamental electrophysiological problems in research laboratories, the method requires further improvement of high-throughput applications. There is a strong demand from the pharmaceutical industry for reliable methods in drug discovery. Especially a proper patching is a notoriously difficult hit-and-miss experiment and can only be carried out by skilled experimenters. Therefore, the automation of the system has been promoted by several research groups and companies since 1990, when NeuroSearch initiated the first automated patch-clamp instrument called NeuroPatch. There are several informative reviews covering automated patch-clamp methods\textsuperscript{164, 165}. All systems are similar to the manual patch-clamp techniques in terms of data quality. However, only few cell lines are suitable for a successful patching. Furthermore, the low success rate of patching and the relatively fast decrease of membrane resistance are limiting factors. Therefore, although automated patch-clamp devices have a good signal quality so far, improved methods are required.

The advantage of patch-clamp is that there is no need of purification of the ion channel. The assay can be done using a natural cell or an overexpressing cell. However, all kinds of proteins remain present around the studied protein, and it is therefore difficult to distinguish the function of the membrane protein of interest from the others. Moreover, the composition of the lipids is given and in most cases unknown. Because of such drawbacks and because patch-clamp is only suitable for the study of ion channels, other concepts of functional assays have been followed.

In some of these assays the studied membrane is neither the native membrane, nor an artificial system, but is made of some vesicles purified from a native membrane, the so-called microsomes. Because these microsomes can be mixed with artificial lipids, the system is more controllable than the patch-clamp on native membranes. However, the presence of other membrane proteins cannot be excluded\textsuperscript{128, 166}. 
I.4.2 Non-sealed setups

There are two difficult steps to obtain a proteoliposome; first, the expression and purification of the membrane protein of interest and second, its reconstitution into a proteoliposome. To avoid the difficult second process, it is possible to employ the purified membrane protein directly, without reconstitution. It has been shown that some membrane proteins retain at least a part of their activity in detergents only\textsuperscript{167, 168} or in mixture of lipids and detergents\textsuperscript{169}. Some other membrane proteins, as the P-glycoprotein, are known to lose their function with loss of the interaction between the lipids and the protein\textsuperscript{170}. Functional assays of these proteins cannot be performed in lipid-free or lipid micelle systems, but are possible in nanodiscs\textsuperscript{171} where their functional activity is retained.

Such systems allow functional studies\textsuperscript{172}. Nowadays, a lot of functional assays are thus done on purified but not reconstituted membrane proteins. This especially includes binding assays to detect, for example, agonist binding. Such assays can be optical as with SPR\textsuperscript{167, 169, 173}, radio-ligand based\textsuperscript{174} or enzymatic\textsuperscript{171}.

The results give precious information about the membrane protein function, for example about the binding of agonist or the modifications of the protein under different conditions\textsuperscript{174}. In the case of enzymatic membrane proteins, even direct function can be measured\textsuperscript{175}. Such methods are thus suitable for non transport membrane proteins, like GPCRs.

However, the information they provide on transport membrane proteins are incomplete. In the case of the P-glycoprotein, non-sealed environments provide functional information and information on the ATPase activity, but no information on the direct transport of the substrate. This is the major limitation of these non-sealed setups (Table I.1).

I.4.3 Giant unilamellar vesicles, liposomes and droplet interface bilayers

To study direct transport of membrane proteins, closed compartments are necessary, as in proteoliposomes, proteo-GUV or DIB.

Various electrochemical methods can be used to monitor direct transport. The conventional patch-clamp technique has been used on proteo-GUV to observe ion channel activity\textsuperscript{176}. Less conventional electrophysiology methods have also been developed. The solid supported membrane (SSM)-based electrophysiology methods consist of absorbing proteoliposomes to a SSM and to activate them using a rapid substrate concentration jump\textsuperscript{177}. The charge translocation is measured via capacitive coupling of the supporting membrane. This method has been applied for both, transporters\textsuperscript{178, 179} and ion channels\textsuperscript{177}. The disadvantage of this method is that no voltage control is possible.

Optical methods are as well used to study the direct transport of a substrate into a proteoliposome. To cite only two examples, ion channel activity was assessed by using a fluorescent ion indicator\textsuperscript{180} and ATP synthesis of an ATP synthase was followed thanks to an enzymatic reaction\textsuperscript{181}. SPR has also
been used to study membrane protein activity in proteoliposomes\textsuperscript{182}. Radioactivity assays are also a method of choice because of their very high sensitivity. In this case, the substrate can be radiolabelled and its uptake followed\textsuperscript{63}. However, the use of radioactivity requires special equipment and formed experimenter.

Optical and electrochemical methods can be combined. Heron and coworkers have measured the transport of Ca\textsuperscript{2+}-ions across a single $\alpha$-HL channel in a DIB using an optical and an electrochemical method simultaneously\textsuperscript{183}. Downsizing the volume of investigation to a 50 nL vesicle allowed them to optically detect Ca\textsuperscript{2+}-ions by monitoring a dye confined within the vesicle, while electrodes positioned inside and outside of the vesicle recorded the ion current. Such parallel monitoring using two methods is potentially useful in order to reveal mechanistic details and to understand how ion channels function.

These sealed-lipid setups have several drawbacks: It is difficult to determine and to control the number of proteins per liposome, GUV or DIB. The internal volume is very small, making quantitative measurements difficult. Moreover, the internal volume is not accessible (Table I.1).

\subsection*{I.4.4 Supported bilayers}

Electrochemical measurements are the best way to study membrane proteins integrated into a supported bilayer. The formed bilayer acts as an electrical insulating platform. EIS is the measurement of choice. The solid support acts as the working electrode\textsuperscript{71}. EIS is used to first characterize the lipid bilayer, and then to monitor the insertion or the activity of the membrane protein\textsuperscript{181, 184, 185}. An active ion channel in the bilayer decreases the resistance of the system by allowing ion flux. Cyclic voltammetry (CV) is used to measure activity whenever redox reactions are involved\textsuperscript{186-189}.

Optical methods can as well be used on membrane proteins integrated into supported lipid bilayers. For example, SPR\textsuperscript{127, 167}, optical waveguide light spectroscopy (OWLS)\textsuperscript{190} or surface-enhanced infrared absorption spectroscopy (SEIRAS)\textsuperscript{191, 192} have been employed. In several cases, these optical detections are combined with electrochemical detection\textsuperscript{190}.

Supported lipid bilayers allow a lot of functional studies of membrane proteins, but still have several drawbacks. Even if a tether is used to increase the space between the bilayer and the surface, the problem of the support remains: only one side of the bilayer is accessible. Moreover, the volume between the supported bilayer and the support is very small, hindering good quantitative measurements.
I.4.5 Free-standing lipid bilayers

Functional assays of membrane proteins in free-standing lipid bilayers have the big advantage that both sides of the membrane proteins are free, and thus the system mimics best the natural state. Moreover, effectors can be added to both sides of the protein. Conditions, as pH, ionic strength can as well be set independently. The volume on each side of the bilayer can be easily varied and adapted to the need of the experiment. Free-standing bilayers are thus the system, which allows the most complete studies of membrane proteins.

Voltage-clamp methods were frequently used to monitor self-inserting membrane peptides into artificial membranes for the validation of new detection methods\(^\text{76, 77, 83}\). The progresses in purification and integration of ion channels into artificial bilayers enabled more studies on reconstituted protein ion channels by voltage-clamp techniques\(^\text{82, 193, 194}\). EIS is also very often used in these systems\(^\text{72}\).

Electrochemical methods on free-standing bilayers are not limited only to ion channels, but might as well be applicable to every transport membrane protein whose substrate is charged. For example, proton pumps have already been studied in free-standing bilayers\(^\text{195}\).

Some functional assays of membrane proteins in free-standing bilayers have optical detection methods. Ganesan and Boxer have developed a novel optical interferometer enabling indirect monitoring of ion channel activities. They determined the change in the curvature of the planar lipid bilayers spanned over micro-wells\(^\text{196}\) that resulted from an increased osmotic pressure due to the transport of Na\(^+\)-ions across gramicidin channels.

The turnover of active transporters is much lower than the one of ion channels. To study their function, a sufficiently high copy number of a protein in a lipid bilayer is needed. However, it is not always possible to enhance the protein density in a lipid bilayer. Studying a larger bilayer area would be a way to increase the copy number of proteins, but it would as well make a proper detection more difficult. In the case of supported bilayers, it will decrease the electrical insulation properties of the system. In the case of free-standing bilayers, it will dramatically decrease the lifetime of the bilayer. The advantage of free-standing bilayers using arrays of several small diameter bilayers is a conserved stability concomitant with an increased copy number of the studied protein\(^\text{66, 83, 197}\) (Table I.1).
<table>
<thead>
<tr>
<th>Detection</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>native membranes, microsomes</td>
<td>- Electrochemical: patch-clamp</td>
<td>- No need of purification and reconstitution</td>
</tr>
<tr>
<td>lipid free, lipid/micelles, nanodiscs</td>
<td>- Optical</td>
<td>- No need of reconstitution (except nanodiscs)</td>
</tr>
<tr>
<td>liposomes, GUV, DIB</td>
<td>- Electrochemical: SSM-electrophysiology, patch-clamp</td>
<td>- Possible study of direct transport</td>
</tr>
<tr>
<td>supported lipid bilayer, tethered lipid bilayer</td>
<td>- Electrochemical: EIS, CV, Optical</td>
<td>- Study of direct transport, Highly robust, relative ease of formation</td>
</tr>
<tr>
<td>free-standing lipid bilayer</td>
<td>- Electrochemical: EIS, voltage-clamp, Optical</td>
<td>- Possible study of direct transport, Access to both sides, Membrane protein free from both sides, Possible arrays</td>
</tr>
</tbody>
</table>

Table I.1: Comparison of the advantages and disadvantages of different functional assay systems for membrane proteins

Microfluidics is becoming increasingly important in functional assays of membrane proteins. Lipid bilayers can be formed by the painting method in microfluidic systems. Microfluidic systems have the advantage that the volume of painting solution and its localization can be precisely controlled. Moreover, once the bilayer is formed, the solutions can be easily exchanged and effectors can be added without the rupture of the bilayer or the introduction of too high noise.

I.4.6 Application of functional assays of membrane proteins

I.4.6.1 Application in life science

The importance of functional assays in biology has been demonstrated by the description of numerous biological processes they are implied in (see I.1). They will contribute to understand the regulation of cellular uptake and release of molecules and related to this, the transduction of information in multicellular organisms.
I.4.6.2 Application in drug discovery

The biological relevance of membrane proteins, as outlined above, explains why membrane proteins are also of high interest for drug discovery. It can be estimated that about 60% of all protein drug targets are membrane proteins (Figure I.9).

Receptors constitute the biggest group of drug targets; they represent 32% of all protein drug targets. Among them, GPCRs are about 19% of the protein drug targets (Figure I.9) and about 36% of the marketed drugs target GPCRs. Because of their ubiquitous role in physiological function and their presence in most of our cells, their deregulation can cause several diseases: i.e. allergy, cardiovascular and psychotic diseases\textsuperscript{199} or cancer\textsuperscript{200}. Non GPCR receptors represent as well a big class of drug targets, especially for cancer therapy\textsuperscript{201}.

Ion channels are abundant in neural\textsuperscript{202}, cardiac\textsuperscript{203} and muscle cells, and establish ion gradients at the cell membranes resulting in electrochemical potentials. An appropriate action potential is a prerequisite for signal transmission controlling contraction of cardiac and muscle cells. Dysfunction of ion channels can thus lead to diseases, such as cystic fibrosis, osteoporosis, muscular dystrophy, which are generally called channelopathies\textsuperscript{204}. Ion channels are also involved in nociception. Targeting ASIC1a has for example be explored in pain relief therapy\textsuperscript{205}. Furthermore, their importance in cancer therapy is being increasingly acknowledged\textsuperscript{206}. Therefore, ion channels are highly relevant in medical therapies, representing about 17% of all drug targets\textsuperscript{207} (Figure I.9).

Quantitative assays measuring a specific ion channel activity are highly desired in drug discovery. One of the common side effects caused by drug candidates is cardiac arrhythmia, in particular the so-called \textit{torsades de pointe}. The human potassium ion channel hERG is inhibited by compounds causing this syndrome. To identify a lead in the drug discovery process, side effects should be recognized early. At present cell-based assays measuring hERG activity are frequently used to exclude molecules from the list of potential drugs\textsuperscript{208,209}.

Transporters represent only 5% of drug targets. However, their role is of major importance in drug discovery. They have for example an important role in drug resistance. During cancer therapy, ABC transporters are overexpressed, causing an efflux of drugs from the cell which results in drug resistance. ABC transporter inhibitors play thus an important role in cancer therapy\textsuperscript{210}. Moreover, these transporters regulate the absorption and excretion of numerous drugs, modulating their pharmacokinetics\textsuperscript{211}.
Human membrane proteins as drug targets

Human membrane proteins represent about 60% of all protein drug targets. GPCRs are most prevalent, followed by ion channels and receptors. Membrane-associated enzymes, solute carriers and transporters are also important drug targets. The different classes are illustrated presenting a typical protein structure. GPCRs: adenosine receptor (adapted from pdb3RFM); ion channels: ASIC1a (adapted from pdb2QTS); receptors: toll-like receptor (adapted from pdb3J0A); solute carriers and transporters: P-glycoprotein (adapted from pdb3g61); membrane-associated enzymes: fatty acid amide hydrolase (adapted from pdb1MT5); non membrane protein: myoglobin (adapted from pdb3RGK)

I.4.6.3 Other applications

Gramicidin ion channels have also been used as the sensing element in biosensors for the detection of particular compounds. For example the so-called “ion channel switch” biosensor has been developed for the detection of influenza A. An extension of this concept is the use of chemically synthesized organic compounds which mimic ion channel function. They offer the advantage to be far more robust than protein ion channels in lipid bilayers and could therefore be also used as sensing elements— for example for “terrorist agents” or large-scale chemical separators for desalination of sea water. Mutated pore proteins channels have also been used successfully to sequence DNA.
Chapter II  Scope of the thesis

In chapter I, the importance of membrane proteins both in biological processes and drug discovery was emphasized. Among the functional assays, free-standing lipid bilayers have important advantages (see section I.4.5). The intra- and extracellular domains of the protein are unhindered, the lipid composition can be controlled and both sides of the bilayer are accessible. One major difficulty remains the integration of membrane proteins into a lipid bilayer and the control of the quantity of protein molecules, which is often limited. The resulting functional assays can be used to measure the function of ion channels, but not the function of active transporters or other membrane transport proteins with a low turnover.

In this thesis, several new procedures and methods have been developed and validated to improve the preparation techniques of functional membrane proteins integrated into artificial bilayers.

In chapter IV, two procedures to integrate membrane proteins into a free-standing lipid bilayer are presented. The protein was integrated into a painted lipid bilayer by fusion of proteoliposomes, with the use of a salt gradient or the nystatin-ergosterol method. Ion channel activity was measured on plastic foil, and, in order to avoid the formation of lipid plugs on a well defined micropore in a silicon chip. However, in both cases, the protein integration remained limited.

In chapter V, a way to tremendously increase the protein density was explored, i.e. the creation of a protein-tethered bilayer on a co-polymer of poly(L-lysine) and poly(ethylene glycol) functionalized with nitritotriacetic acid, adsorbed on solid support. Several techniques proved the successful immobilization of the oriented membrane protein followed by the formation of a lipid bilayer. Antagonist binding proved the functionality of the resulting protein-tethered bilayer.

Free-standing bilayers are less stable than supported bilayers and their formation without solvent is difficult. Whilst supported bilayers are easier to form in the absence of a solvent, their main disadvantage is the limited access to only one side of the bilayer and the presence of a support which potentially changes protein function. Combination of supported and free-standing lipid bilayers is a good compromise. The bilayer is not completely free, but is accessible from both sides. It is supported by a flexible material, lowering the risk of loss of protein function in comparison with supported lipid bilayers. Chapter VI showed further improvement of the functional assay. The protein-tethered bilayer presented in chapter V was formed over a polyelectrolyte multilayer-filled
nanopore. The formation of the combined protein-tethered bilayer was successful and some results showed possible ion channel activity. However, the low sealing did not allow sufficient reproducibility. The use of the polyelectrolyte multilayer could explain a part of the observed variation.

In chapter VII a new platform is presented, where the supporting cushion in the pore was assembled in a more reproducible way. The protein-tethered bilayer was formed on polymers covalently grafted in the pores. Preliminary results showed the successful formation of a protein-tethered lipid bilayer on this new support.

In chapter VIII general conclusions on the results achieved in this thesis are drawn and remaining challenges are presented.
Chapter III  Materials and methods

III.1  Chemicals, buffers and supports

III.1.1 Chemicals
4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid (HEPES), sodium phosphate monobasic (NaH₂PO₄), potassium phosphate monobasic (KH₂PO₄) and dibasic (K₂HPO₄), sodium acetate (NaCH₃CO₂), tris(hydroxymethyl)aminomethane (Tris), sodium chloride (NaCl), potassium chloride (KCl), ethylenediaminetetraacetic acid (EDTA), imidazole, nystatin, ergosterol, decane, methanol, chloroform, nickel(II) sulfate hexahydrate (NiSO₄), nimodipine (#N149), nifedipine (#N7634) were purchased from Sigma-Aldrich (Switzerland). Sucrose was purchased from Merck Millipore (Switzerland). Acetone and isopropanol were purchased from Fischer Scientific Inc. (USA).

III.1.1.1 Polyelectrolytes
The co-polymer of poly(L-lysine) and poly(ethylene glycol) functionalized with nitrilotriacetic acid (PLL-g-PEG-NTA) was obtained from SuSoS AG (Switzerland). Polyethyleneimine (PEI # 408727), poly(allylamine hydrochloride) (PAH, #283223), and poly(sodium 4-styrenesulfonate) (PSS, #243051) were purchased from Sigma-Aldrich (Switzerland).

III.1.1.2 Detergents
n-dodecyl-β-D-maltopyranoside (DDM, #D3105) and n-decyl-β-D-maltoside (DM, #D3225) were purchased from Affymetrix (USA).

III.1.1.3 Lipids
1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC, #850356), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS, # 840034), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, #850457), 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (POEPC, #890705), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE, #850757), 1-palmitoyl-2-[12-[(7-nitro-2,1,3-benzoazadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphocholine (NBD-PC, #810132) and 1-palmitoyl-2-[dipyrometheneboron difluoride]undecanoyl-sn-glycero-3-phosphocholine (TopFluorPC, #810281) were purchased from Avanti Polar Lipids (USA) and stored at -20 °C.

III.1.2 Buffers
All buffers were prepared with ultrapure water (MilliQ, Millipore Corporation, Germany) and filtered before use with a 0.2 µm filter. pH of NaCl containing buffers was adjusted with NaOH (5 M) and HCl (32 % (v/v)); pH of the KCl containing buffers was adjusted with KOH (3 M) and HCl.
III.1.3 Supports
For the measurements on a plastic foil, a hole was punched into a projector plastic foil using an insect needle.

Silicon chips (Leister, Switzerland) were manufactured$^{92}$ and silanized$^{219}$ as previously published.

III.1.3.1 Photoresist deposition
Before photoresist deposition (VI.4.2), the chips were cleaned for 15 min with oxygen plasma (PDC-32G, Harrick, USA). A 50 nm chromium layer was then deposited on the trans side by vapor deposition. A drop of Shipley 1805 (Microchem. Corporation, USA) was deposited in the middle of the chip and spin-coated at 2000 rpm for 60 s. The chip was incubated for 10 min at 80 °C and the trans side was illuminated 22 s in a mask aligner (Süss, MicroTec AG, Germany). The chip was then dipped for 3 s in Microposit developer solution (Microchem. Corporation, USA) and rinsed with MilliQ water.

III.2 Preparation protocols

III.2.1 Protein expression

III.2.1.1 His-NaChBac
His-NaChBac was expressed and purified following the published protocol$^{220}$ with the following modification: Superdex 200, instead of Superdex 75 was used during the gel filtration (GE Healthcare, Germany).

III.2.1.2 His-mCherry-NaChBac
His-mCherry-NaChBac was expressed using the same protocol as for His-NaChBac and using the nucleotide sequence corresponding to the following sequence inserted into a pET plasmid (plasmid from R. Jaussi, PSI):

```
MEARQKQNFSFTSKMQIKVHNRAFTFTVIALILFNALIVGIETYPRIYADHKWLFYRIDLVLWIFTIEIAMRFLASNPKSFAFRSSWNWFDFLIJAVA
GHIFGAGOFVTVRLRLVRVAISVPSLRRLDLYMTAPIALGNILMSIFFYFAVGTMFLQHV/SPFYFNQLQLSLTLTFQVTVLESWAGV
MRPFAEVPWSWLYFVSGFTTIFILNFIVGINVNEKAELODNEEDGEADGLKEISALRKDVKALSDKSMLKQSGKESVSGGEDNMAIKFMRF
KVMHMEGSGNHEFIEIIEGEEGRFEGQYGTQATKLKVTGQPPLPFWDIILPSQOMYSGKAYVKHPADIPYKLSFPEGKWERVMNFDGGVT
VTQDSSLQDGFIYKVLQRTGNPFSDGVPMQKKTMBASSERMYPEDGALKGEIKQRLKLDDGGHYDAEVKTITYAKSKPVLPGAYNVIK
LDITSHNEDYTVGEQYERAEGRHSTGGMDELYKKHHHHH
```

The red part corresponds to m-Cherry and the blue part to the His-tag.

III.2.1.3 His-KvAP
His-KvAP was purified by I. Imhof (PSI) following the published protocol$^{89}$. 
III.2.2 Liposome preparation

Lipids in chloroform were mixed in a test tube and dried with a nitrogen stream for a minimum of 3 h. The lipid film was hydrated for 1 h with the liposome buffer to a final lipid concentration of $10 \text{ mg} \cdot \text{mL}^{-1}$ and subsequently vortexed. The solution was extruded 31 times through polycarbonate filters with the desired mesh size of 800, 400, 200, 100 or 50 nm (Nucleopore Track Etched membranes, GE healthcare) using a mini-extruder syringe (Avanti Polar Lipids, USA).

III.2.3 Proteoliposome preparation

III.2.3.1 His-NaChBac proteoliposome preparation

Lipids in chloroform were mixed in a test tube and dried with a nitrogen stream for a minimum of 3 h. The lipid film was hydrated for 1 h with a mix of the protein, liposome buffer and DDM (end concentration 0.03 % w/v) to a final lipid concentration of $10 \text{ mg} \cdot \text{mL}^{-1}$. Glass beads were added to the test tube and stirred until the lipid film was dissolved. The solution was dialyzed against a DDM-free buffer during 3 days using a 14 kDa membrane (Spectra/Por®, Spectrumlabs.com, USA). The solution was extruded 31 times through polycarbonate filters with the desired mesh size of 800, 400, 200, 100 or 50 nm using a mini-extruder syringe.

III.2.3.2 His-KvAP proteoliposome preparation

Liposomes were previously formed following the procedure described in III.2.2. Liposomes were rotated overnight at room temperature with DM to a final concentration of 5 mM. Protein was incubated for 1 h to this solution of DM and liposome. Bio-beads™ (SM2, Bio-rad Laboratories Inc., USA) were added every hour for 4 h to reach a final concentration of $500 \mu\text{g} \cdot \mu\text{L}^{-1}$. After further rotation at 4 °C overnight, the proteoliposomes were separated from the Bio-beads by sedimentation.

III.2.4 Polyelectrolyte multilayer spraying

Polyelectrolytes were dissolved at a concentration of $1 \text{ mg} \cdot \text{mL}^{-1}$ in 10 mM HEPES and 150 mM NaCl, pH 7.4 buffer and filtered before use with a 0.2 µm filter. Polyelectrolyte multilayer (PEM) was sprayed using a home-built robot (D. Textor, ETHZ)\textsuperscript{221}. The robot consists of four airbrushes (Paasche VLS airbrushes, Paasche Airbrush company, USA) – one per electrolyte plus one for the buffer - connected to a compressor and a pressure controller and controlled by a Lego Mindstroms NXT robot (Lego Group, Denmark). During spraying, a constant pressure of 1 bar was applied. The spraying was programmed as followed: polyelectrolyte spraying for 5 s, pause for 15 s, buffer spraying for 5 s, and pause for 15 s. This sequence was repeated until the final number of polyelectrolyte bilayers was achieved.
Before spraying, the silicon chips were cleaned for 15 min on each side with oxygen plasma. The chips were placed on a tray at a distance of 19 cm from the airbrushes, tilted from 45°. PEI\((\text{PSS/PAH})_{28}\) multilayer was sprayed. After spraying, the chips were stored until use in buffer and at room temperature.

III.2.5 Poly(methacrylic acid)-nitriotriacetic acid functionalization
The functionalization was carried out by W. de Groot (Twente University, the Netherlands). Poly(methacrylic acid) was grafted to the pore wall of the silicon chip following the published protocol\(^{22}\). Nitrilotriacetic acid was further functionalized by incubation in N-ethyl-N’-[dimethylamino)propyl]carbodiimide hydrochloride : N-hydroxysuccinimide (EDC-NHS, 1 : 1) for 30 min, followed by rinsing and further incubation for 1 h in 0.1 M aminobutyl-NTA. The probes were further rinsed with MilliQ water and stored in a nitrogen-rich environment.

III.3 Characterization

III.3.1 Protein characterization

III.3.1.1 SDS-Page
Samples were mixed with loading buffer and heated up to 95 °C for 5 min. They were subsequently loaded on a 12 % acrylamide gel and electrophoresis was run for 1.5 h at 180 mV (Hoefer mini VE, Hoefer Inc., USA). The gel was stained for a minimum of 30 min in staining solution (0.05 % Coomassie R250, 25 % isopropanol, 10 % acetic acid) and destained in 40 % EtOH and 10 % acetic acid in H\(_2\)O.

III.3.1.2 Western-blot
After SDS-Page, the SDS-gel was placed on a blotting membrane (#RPN 203D, Hybond FCL, Amersham Biosciences, Switzerland) and the proteins were transferred for 1.5 h at 102 mA (Hoefer semi dry transfer unit, Hoefer Inc). The membrane was developed in a Processor plus instrument (Amersham Biosciences, USA) using the following protocol. Washing for 5 min in 10 mL tris-buffered saline (TBS), followed by blocking for 30 min in 10 mL blocking buffer (3% milk powder in tris-buffer saline-tween (TTBS)). Two times 5 min incubations in 10 mL TTBS and a 5 min incubation in 10 mL TBS followed. The antibodies were then incubated for 30 min: 10 µL of anti-Penta-His antibody (#34660, Qiagen, USA) and 2.5 µL of alkaline phosphatase conjugated secondary antibody (#A3562, Sigma Aldrich) were mixed in 10 mL blocking buffer. The membrane was washed two times 5 min in 10 mL TTBS and 5 min in 10 mL TBS. For the development, the membrane was incubated 30 min in
10 mL buffer mixed with 100 µL nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate (#585 002001, Roche Diagnostics, Switzerland and # N6876, Sigma-Aldrich).

III.3.2 Liposome and proteoliposome characterization

III.3.2.1 Dynamic light scattering
Dynamic light scattering (DLS) measurements of the proteoliposomes were performed on a DynaPro™ temperature controlled micro sampler (Wyatt Technology Corporation, USA) using the DYNAMICS software.

The DLS measurements in chapter V were carried out using a Zetasizer Nano ZS (Malvern instruments, UK) and analyzed using the Zetasizer software.

III.3.2.2 Thin layer chromatography
Chloroform (65 mL), methanol (25 mL) and acetate buffer pH 4.0 (4 mL) were used for the mobile phase. The samples were spotted (5 µL) on a TLC silica gel 60 (#1.05721.0001, Merck, Germany) at 3 cm distance from the lower edge and carefully blow dried. Lipid stock solutions in chloroform were used as reference (2 µL). After migration of at least 5 cm, the plate was blow dried again and sprayed with molybdatophosphoric acid solution (#100.480.0100, Merck). After blow drying, the background was bleached by exposition of the plate to ammonia gas (25 % ammonium solution) for some minutes.

III.3.2.3 Sucrose gradient
80 %, 30 %, 20 %, 10 % and 5 % (w/v) sucrose solutions were prepared and complemented with a protease inhibitor cocktail (Complete, Roche, Switzerland). 2 mL of each solution was carefully poured in a polayallomer tube (#331362, Beckman coulter inc., USA), the 80 % solution at the bottom and the 5 % solution on top. The sample was placed at the top of the discontinuous gradient. The tube was ultracentrifugated for 19 hours at 39 000 rpm and 4 °C (SW41 rotor, Beckman Coulter Inc.). A hole was punched at the bottom of the tube and fractions of 1 mL were collected and analyzed by Western blot and thin layer chromatography.

III.3.2.4 Electron microscopy (chapter V)
Liposome solution (200 nm) was diluted to 1 mg · mL⁻¹ in the presence of either 0.1 %, 0.05 % or 0.03 % DDM and incubated for a minimum of 1 h before use. Holey carbon film-mounted grids (Quantifoil® R 2/1, Quantifoil Micro Tools GmbH, Germany) were treated by plasma for 75 s at 25 mA. A 3 µL drop of sample was deposited on the grid, which was blotted (4 s with offset of -3) and plunge-frozen in liquid ethane using a Vitrobot cryostation (FEI, USA). Grids were transferred to a FEI
Chapter III Materials and methods

Tecnai F20 FEG (FEI) electron microscope and images were recorded at 200 kV and 40 000 x nominal magnification, unless otherwise indicated.

III.4 Analysis

III.4.1 Painting

III.4.1.1 Painting solution

POPE/POPC (7/3 (w / w/)) chloroform solutions were mixed in a test tube and dried under a nitrogen stream for 1 h minimum. The dried lipid film was dissolved by vortexing in decane to a final concentration of 10 mg · mL⁻¹.

III.4.1.2 Painting on the foil (chapter IV)

The hole was pretreated with 2 µL of painting solution. The foil was sealed between two compartments of a Teflon® horizontal chamber. One silver/silver chloride (Ag/AgCl) home-made electrode (Ag/AgCl wire, Warner Instruments), which was previously chlorinated, was placed in two separated compartments filled with buffer. These compartments were linked to the two sides of the foil using home-made agar bridges. A 20 µL pipette tip was shortly dipped in painting solution and the lipids were applied to the aperture by creating an air bubble in this tip.

III.4.1.3 Painting on the chip (chapter IV)

The silicon chip (chapter IV) was placed between two identical polymethyl-methacrylate (PMMA) compartments of 3 mL each sealed by two silicon rings. Hg/HgO reference electrode (XR400, IG Instrumenten Gesellschaft, Switzerland) and platinum wire (#010285, Alfa Aesar, Germany) were placed on each side of the chip for the 4 electrode configuration. A 20 µL pipette tip was shortly dipped in painting solution and the lipids were applied to the aperture by creating an air bubble in this tip.

III.4.2 Proteoliposome fusion to the painted bilayer by salt gradient

Proteoliposomes were formed as described in III.2.3. The bilayer was formed on a foil as described in III.4.1. Proteoliposome solution (2 µL) and 3 M KCl solution (2 µL) were injected close to the bilayer. If no activity was observed within 5 min, the bilayer was mechanically broken and painted again.
III.4.3 Proteoliposome fusion to the painted bilayer using the nystatin-ergosterol method

Proteoliposomes were formed as described in III.2.3 using the 20 mM NaCH₃CO₂, 150 mM NaCl and 450 mM KCl pH 5.5 buffer, unless otherwise stated. Subsequently, the proteoliposomes were activated by nystatin: 10 µL of 500 µg · mL⁻¹ nystatin in methanol was added to 90 µL of proteoliposomes and the solution was stored at 4 °C for a minimum of 1 h.

The bilayer was formed as described in III.4.1.3 using 20 mM NaCH₃CO₂, 150 mM NaCl and 450 mM KCl at pH 5.5 on the cis side and 20 mM NaCH₃CO₂ and 150 mM NaCl at pH 5.5 on the trans side. Activated proteoliposome solution (10 µL) was added to the cis side.

III.4.4 ptBLM formation

III.4.4.1 ptBLM formation on glass slide (chapter V)

The glass surface was activated for 5 s by atmospheric-pressure helium plasma (PZ1, Reinhausen Plasma, Germany) and a polydimethylsiloxane (PDMS) ring was deposited on the glass to form a well of about 50 µL volume. Working buffer contained 10 mM HEPES and 150 mM NaCl at pH 7.4 for His-NaChBac ptBLM formation; 20 mM HEPES and 150 mM KCl at pH 7.4 for His-KvAP ptBLM formation; 20 mM Tris and 150 mM NaCl at pH 8.0 for His-ASIC1a ptBLM formation. PLL-g-PEG-NTA with a final concentration of 0.1 mg · mL⁻¹ in working buffer was incubated for 1 h and the well was rinsed 3 times with buffer. NiSO₄ (20 mM in working buffer) was incubated for 10 min and the well was rinsed 15 times with buffer. His-protein solution was diluted to 250 µg · mL⁻¹ in the following buffers: 20 mM KH₂PO₄ and 500 mM KCl at pH 8.0 with 0.2% DM for His-KvAP; in 50 mM NaH₂PO₄ and 300 mM NaCl at pH 8.0 with 0.03 % DDM for His-NaChBac and in working buffer with 0.05 % DDM for His-ASIC1a, and incubated for 1 h in the PDMS well. The well was rinsed with the buffer used during protein incubation. Solutions of liposomes of 200 nm in diameter were diluted to 1 mg · mL⁻¹ in the presence of either 0.1 %, 0.05 % or 0.03 % DDM and incubated for a minimum of 1 h before use. Each liposome solution, from the highest detergent concentration to the lowest, were incubated for 1 h in the PDMS well and replaced by the next solution without rinsing. Liposomes at 1 mg · mL⁻¹ were then incubated for 1 h and the well was rinsed 15 times with working buffer.

III.4.4.2 ptBLM formation on the chip (chapter VI)

The silicon chip was placed between two identical PMMA compartments of 200 µL volume each, sealed with two silicon rings. Both compartments were filled with working buffer and one Ag/AgCl electrode was placed in each compartment (WPI reference electrode, Lot-Oriel AG, Germany). The same protocol as explained before (III.4.4.1) was used.
III.4.3 ptBLM formation in the microfluidic system (chapter VI)
The microfluidic system consisted of two PDMS slabs containing 500 µm wide and 250 µm high channels and a space for the silicon chip, a PMMA plate and a microscopy slide. All these elements were sonicated (Sonorex, Bandelin electronic, Germany) for 10 min in ultrapure water and exposed to atmospheric-pressure helium plasma for 10 s. The two slabs were pressed against each other, on one side with the glass slide, and on the other side with the PMMA plate. The system was connected to a Nemesys pump (Cetoni GmbH, Germany) equipped with 500 µL syringes (#2624915, Innovative Labor Systeme GmbH, Germany) via polyether ether ketone (PEEK) tubings (#1531132, Ercatech AG, Switzerland). The ptBLM was formed as explained in III.4.4.1, but each solution was pumped through the microfluidic system using a speed of -0.3 µL · s⁻¹ for the buffer, -0.04 µL · s⁻¹ for the protein solution and -0.03 µL · s⁻¹ for the lipid solutions.

III.4.5 Electrochemical methods

III.4.5.1 Electrochemical impedance spectroscopy (EIS)
After painting, the bilayer was checked by electrochemical impedance spectroscopy using Autolab PGSTAT 12 (Metrohm AG, Netherlands) equipped with a FRA module. EIS spectra were recorded from 1 MHz to 0.01 Hz at 0 V offset potential, applying a 10 mV signal amplitude.

III.4.5.2 Breakdown voltage (chapter IV)
A linear sweep voltammetry potentiostatic procedure was applied using the Autolab with a starting potential of 0 V, an end potential of 900 mV and a scan rate of 0.13 mV · s⁻¹.

III.4.5.3 Voltage-clamp
Chronoamperometry was applied at a fixed potential, which is defined in the description of each experiment, using the Autolab. The automatic ranging option and the ECD module were used and measures were taken every 100 ms.
KvAP activity was measured with a single channel amplifier (Axopatch 200B, Molecular Devices, USA) at alternating potential defined in the description of each experiment.

III.4.6 Fluorescence recovery after photobleaching
Liposomes prepared with 3 % NBD-PC as fluorophore were prepared as explained in III.2.3. ptBLM formation was conducted as explained in III.4.4.1. After the last lipid incubation, the sample was rinsed 30 times with buffer and placed in a Ludin chamber (Life Imaging Services, Switzerland) which was mounted with a sample coverslip of thickness #1 (Menzel-Gläser, Germany).
The confocal images were acquired with a Leica microscope TCS SP5 (Leica Microsystems, Germany) equipped with an argon laser (488 nm) and a 63 X glycerol objective (#11506193, Leica Microsystems). Images were analyzed with the FRAP wizard of the LAS AF software (Leica Microsystems). The coefficient of diffusion was calculated according to Soumpasis et al. 73.

III.4.7 Quartz crystal microbalance with dissipation monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D) is a technique widely used to characterize the properties of layers between a solid support and a bulk solution 144, 223. QCM-D for lipid bilayer characterization has been established 143, 144. The QCM-D experiments were performed in a QCM-D Qsense-E4 apparatus (Q-sense AB, Sweden) using gold crystals with a fundamental frequency of 5 MHz (#QSX 301, Q-sense AB). Prior to experiments, the crystals were cleaned for 30 min in a 2 % sodium dodecyl sulfate (SDS) solution, rinsed with MilliQ water and cleaned for 30 min in a UV/ozone cleaner. The ptBLM was formed as described in III.4.4.1 by injecting each solution (minimum of 300 µL) into the QCM chamber.

III.4.8 Dual polarization interferometry

Dual polarization interferometry (DPI) is a recently developed optical method, which allows to gain information about biological layers 142, 224, 225. The sensor chip is a slab-waveguide which is made of both, reference and sensing layers, which form a Young's interferometer when the waveguided light, excitated by a polarized laser beam, leaves the chip (Figure III.1A). The interference pattern is recorded with a CCD camera. When a layer is deposited within the evanescent field of the waveguide, the change of the refractive index causes a fringe shift. Two orthogonal polarizations are used to excite the chip (exciting TE, transverse electric, and TM, transverse magnetic, waveguide modes), which result in two separate measurements of fringe shifts (TM and TE phase, see Figure III.1B). For an isotropic layer, \( n \), the refractive index of the added layer and \( d \), the layer thickness, can be determined by solving the Maxwell’s equation of electromagnetism for both, TM and TE waveguide modes. For an anisotropic layer, e.g. a lipid bilayer, the refractive index parallel and perpendicular to the surface are different and their difference is called the birefringence 142. The data can be fitted by assuming a constant thickness or the average refractive index of the layer (Figure III.1B2).
Figure III.1: Principle of dual polarization interferometry
(A) Upon illumination by a laser, the chip is excited by two polarizations (TM and TE) and two fringe shifts are generated. (B) (1) Isotropic layer. Thickness and refractive index of the added layer are determined. (2) Anisotropic layer. Data are fitted either by fixing the thickness or by averaging the refractive index of the layer.
Adapted with permission from Farfield.
A way to monitor the birefringence change without making any assumption is to plot $\Delta(\text{TM-TE})/(\text{TM+TE})$, the so-called anisotropy. The anisotropy increases proportionally to the birefringence.

The mass of the adsorbed layer can be calculated using the de Feitjer formula:

$$m = d \times \frac{(n_{iso} - n_{buffer})}{dn/dc} \tag{Eq. III.1}$$

- $m$: mass per unit area
- $d$: thickness of the adsorbed layer
- $n_{iso} = \left(\frac{n_{TM}^2 + 2n_{TE}^2}{3}\right)^{1/2}$ with $n_{TE}$ and $n_{TM}$ the effective refractive index respectively in the plane of the waveguide or orthogonal to the plane of the waveguide.
- $dn/dc$: refractive index increment

The experiments were performed on an Analight 4D instrument (Farfield Group Ltd., UK) equipped with a 635.8 nm laser and an unmodified silicon oxynitride “Anachip Plus” waveguide.
The chip was cleaned with a 2 % SDS solution, rinsed with MilliQ water and cleaned for 20 min in UV/ozone cleaner (Procleaner) and left overnight for stabilization. After mounting in the Analight
device, two solutions of known refractive index were injected to the chip: 80 % EtOH and MilliQ water in order to calibrate the waveguide chip. All solutions were previously degassed. ptBLM was formed following the protocol explained in III.4.4.1.

PcTX1 (#4435-5, Peptide Institute Inc., Japan) was dissolved in MilliQ and stored at -20 °C.

For graphical representation, the change of TM phase and anisotropy were followed.

For calculation, the isotropic refractive index of the lipid film was approximated to be 1.47 and $dn/dc$ was fixed to 0.135 cm$^3$ · g$^{-1}$ for lipids and 0.182 cm$^3$ · g$^{-1}$ for proteins$^{142}$. 
Chapter IV Integration of membrane proteins to free-standing lipid bilayers by fusion of proteoliposomes

The easiest method to create a free-standing lipid bilayer is painting. However, painting is performed using an organic solvent, which potentially denaturates the proteins. It is thus unfeasible to integrate the membrane protein while painting the bilayer. Several methods of insertion of membrane proteins after bilayer formation have been developed. The use of proteoliposomes is the method of choice. Membrane proteins are reconstituted in proteoliposomes in which they are stable for up to several weeks.

IV.1 Membrane proteins used in this study

A versatile concept for functional assays can be applied to different classes of membrane proteins. However, some transport membrane proteins exhibit a very low turnover, making the detection of the transported species very challenging. Ion channel activities are easily detected by electrochemical methods and are thus well suited to evaluate new functional assays.

In this study, two voltage-gated ion channels were used: KvAP from *Aeropyrum pernix* and NaChBac from *Bacillus halodurans*.

KvAP is a K⁺ channel, of which the 3D structure was first determined in 2003¹⁸ (Figure IV.1A). The structure of this ion channel is very close to eukaryotic Kv channels. It is a tetramer of about 100 kDa that stays in its tetrameric form in the presence of selected detergents. Each of the monomers consists of six hydrophobic transmembrane segments and the tetramer assembly leads to the formation of a central ion conduction pore¹⁸ (S5-S6, Figure IV.1B). The selectivity filter is very similar to the one of other potassium channels. The mechanism of selectivity has been clearly described in the article of Zhou et al.²²⁶. Four carbonyl oxygen atoms are present at the selectivity filter. The hydration’s shell of the potassium ions is displaced by these atoms and the dehydrated potassium molecule is moved along the pore via the interaction with other oxygen atoms. The voltage-sensor (S4) appeared to be very flexible and to move across the membrane²²⁷, ²²⁸. The positively charged arginine residues of this sensor structure (Figure IV.1B, blue residues) are exposed to the lipid bilayer and are able to interact with the phosphate groups of the phospholipids⁹⁵. This explains why the lipid composition of the membrane has such an influence on the gating behavior of KvAP⁹⁵, ¹⁹⁴. It is also important to note that upon depolarization of the membrane, a part of these channels is inactivated; upon several series of depolarization steps, the resulting current is decreasing over time¹⁹⁴. KvAP midpoint of activation is around -40 mV⁹⁵ and its unitary conductance is of about 170 pS²²⁹.
NaChBac is a prokaryotic Na⁺ channel first characterized by Ren et al in 2001\(^{230}\). It is a tetramer of about 128 kDa and also formed of six transmembrane segments\(^{230}\). The S5-S6 segments are the pore forming region and S4\(^{231, 232}\) is, as for KvAP, the voltage-sensor region (Figure IV.1C). The mechanism of voltage sensing is probably similar to the one of KvAP\(^{11}\). However, the selectivity filter is different: for NaChBac, the side chains and not the oxygen atoms of the main chain carbonyl are the key elements of selectivity\(^{233}\). Its X-ray structure is still unknown, but the crystal structure of an orthologue has been solved\(^{234}\). It is known that NaChBac shares structural similarities with Na\(_v\), K\(_v\), and Ca\(_v\) channels\(^{235}\). This fact explains why NaChBac is sensitive to calcium channel inhibitors, such as nimodipine and nifedipine\(^{230}\). Information about NaChBac activity will thus also contribute to understand the function of Na\(_v\), K\(_v\), and Ca\(_v\), voltage-gated channels.

NaChBac has an opening threshold of -80 mV\(^{232}\) and a midpoint of activation of -24 mV. The conductance of an individual channel is 12 pS and its selectivity for sodium is quite high\(^{230}\): \(P_{Na}/P_{Ca} = 72\), whereas \(P_{Na}/P_{K} = 171\).

**Figure IV.1: KvAP and NaChBac**
(A) Top view of a KvAP tetramer, made from its X-ray structure. Each monomer is represented in a different color (adapted from pdb1ORQ). (B) 2D model of one KvAP monomer. (C) 2D model of one NaChBac monomer. For (B) and (C), the six transmembrane domains are numerated S1-S6. S5-S6 form the pore region, S4 is the voltage-sensor. The charged arginine residues are shaded in blue. The 2D topology graphs were performed using TEXtopo\(^{236}\).
IV.2  Fusion of proteoliposomes to painted lipid bilayers using an osmotic gradient

IV.2.1 Theoretical considerations
Fusion of proteoliposomes to integrate a membrane protein into a free-standing lipid bilayer was first described 30 years ago\(^\text{237}\). Since then, it has been probably the most used method to study ion channel activity in BLM\(^\text{238}\). From an energetic point of view, movements of lipids are favored between a structure with a high surface tension and a structure with a lower surface tension\(^\text{239}\). Fusion between a lipid bilayer and a vesicle is thus favored when the surface tension of the vesicle becomes sufficiently high\(^\text{239}\). The surface tension of a vesicle can be linked to the hydrostatic pressure with the Laplace’s law\(^\text{240}\):

\[
\gamma = \frac{P r}{2}
\]

where \(P\) is the hydrostatic pressure (pressure on the concave side of the vesicle – pressure on the convex side of the vesicle), \(r\) is the radius of the vesicle and \(\gamma\) is the surface tension. One direct method to increase the surface tension of the vesicle is thus to increase the hydrostatic pressure, which can be achieved by applying an osmotic gradient\(^\text{103, 237, 238}\), salt or urea can be used. The concentration should be higher on the side of the bilayer where the vesicles are added (cis). Proteoliposomes that come close enough to the lipid bilayer will share a part of their membranes. Because of the osmotic gradient, water will enter the proteoliposomes to equilibrate the concentration between the trans side and the internal solution of the proteoliposomes. In turn, ions enter the vesicle to equilibrate the salt concentration with the concentration in the cis, which favors the entrance of water into the vesicle. Consequently the hydrostatic pressure is enhanced, which leads to the fusion of the proteoliposomes to the bilayer.

IV.2.2 Direct fusion of KvAP proteoliposomes and activity measurements
In this study, a Teflon device containing two compartments separated by a horizontal cavity was used. A plastic foil with a hole of about 100 \(\mu\)m diameter was sealed over this cavity and a bilayer was painted with a POPE/POPC (7/3, w/w) solution. KvAP proteoliposomes (3 \(\mu\)L) were added to the cis side together with 3 M KCl solution to create a salt gradient over the bilayer. Electrochemical measurements were done by a two electrodes voltage-clamp using Ag/AgCl electrodes and agar bridges (see chapter III for more details). Activity was observed rapidly after the addition of the proteoliposome solution.
KvAP of different lipid composition successfully fused to the painted bilayer: only zwitterionic phospholipids, 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC); entirely positively charged phospholipids: 1-palmitoyl-2-oleoyl-sn-glycero-3-ethylphosphocholine (POEPC) or a mixture of zwitterionic and positively charged lipids (20 % POEPC and 80 % POPC). The conductance of one ion channel molecule (unitary conductance) was between 100 and 170 pS, which is consistent with the published values. Schmidt et al. showed that KvAP is functional only if surrounded by zwitterionic lipids. It was also proved that even when reconstituted in lipids in which the ion channel was not functional, the functionality was recovered after fusion into a bilayer containing zwitterionic lipids. This explains why activity of KvAP could be recorded after fusion of proteoliposomes formed of 100 % POEPC. A trace of monitoring of activity resulting from the fusion of proteoliposomes consisting of 20 % POEPC and 80 % POPC is shown in Figure IV.2. Ion channel activity is measured at applied voltages of 150 mV, 125 mV and 100 mV (Figure IV.2D), but not at -150 mV (Figure IV.2A). The measurement indicates that all ion channels have the same orientation after fusion and that voltage-dependence was conserved. The calculated unitary conductance was 170 pS (from Figure IV.2C). Interestingly, the same proteoliposomes were still active after a cycle of freeze/thawing, but with a unitary conductance of only 75 pS (data not shown).

**Figure IV.2:** KvAP activity after fusion of proteoliposomes to a painted bilayer
(A) Current trace at 150 mV after fusion of proteoliposomes to a painted bilayer on a foil. Proteoliposomes were formed of 20 % POEPC and 80 % POPC. (B) Zoom of the squared area in A. Single ion channel opening could be observed. Three currents levels could be identified, indicating the presence of 3 active KvAP ion channels. (C) Normalized counts in function of current for the current trace at +150 mV showed in A. The peaks are separated by 26 pA, which corresponds to a conductance of about 170 pS. (D) Voltage dependence of the KvAP. Results from the same bilayer as used for graphs A-C.
In summary, fusion of KvAP proteoliposomes to a painted free-standing bilayer was successful and allowed the integration of ion channels into the bilayer.

**IV.2.3 Direct fusion of NaChBac proteoliposomes**

Fusion of NaChBac proteoliposomes was also investigated but without success. Three reasons could explain such a result. (1) The protein is not present in the liposomes. (2) The reconstituted protein is not active. (3) The proteoliposomes do not fuse to the bilayer. As explained before, the hydrostatic pressure on the liposome is built up upon equilibration of the salt concentration between the interior of the proteoliposome and the trans side, which causes water to flow into the liposomes. If there is no open channel with sufficient conductance in the vesicle, the hydrostatic pressure might not increase enough to induce the fusion.

To verify if the liposomes contained the membrane protein, a sucrose gradient purification of the proteoliposomes was performed. The proteoliposomes were loaded on a discontinuous sucrose gradient and ultracentrifuged at 39 krpm for 19 hours. The sample was then fractionated and each fraction was tested for the presence of the protein by Western blot and for the presence of lipids by thin layer chromatography (TLC). The Western blot proved that the protein was present in high quantities in the high density fractions where lipids were absent (Figure IV.3B, red dashed rectangle).

![Western blot analysis of NaChBac proteoliposomes](image)

**Figure IV.3: NaChBac proteoliposomes purified by a sucrose gradient**

(A) DLS analysis of proteoliposomes (100 % POPC, 1:50 000 protein to lipid ratio (mol/mol), 250 nm diameter) (B) The proteoliposomes were placed on top of a discontinuous sucrose gradient (1 to 1.16 g · cm⁻³) and ultracentrifugated for 19 hours. After centrifugation, the sample was carefully fractionated from the bottom (higher density) to the top (lower density). Each fraction was analyzed by Western blot, using an anti-Penta-His antibody to detect NaChBac and by TLC to detect the lipids. The lipids could be detected in the two lowest density fractions with about 43 % of the protein (blue dashed square). The free protein was found in fractions of higher density (red dashed line).
However, both, proteins and lipids were detected in the lowest density fractions (Figure IV.3B, blue dashed rectangle). Lipids were shown to form liposomes of about 250 nm diameter (Figure IV.3A). These results indicate that a big fraction of the total protein was integrated into the proteoliposomes. The percentage of integrated protein was quantified to be about 43%.

The protein was thus present in the proteoliposomes and two potential reasons remain that could explain the absence of activity. Either the protein present in the proteoliposomes lost its activity or the proteoliposomes did not fuse to the bilayer. It is not possible to clearly monitor the insertion of the protein into the free-standing bilayer, which is a limitation of the method. Moreover, only few protein molecules could be integrated. Another method to insert membrane protein in a more controlled way was thus investigated.

**IV.3 Fusion of proteoliposomes to free-standing bilayers using the nystatin-ergosterol method**

*Note*: Part of this work has been published in the article: Integration and recording of a reconstituted voltage-gated sodium channel in planar lipid bilayer, Studer, A., Demarche, S., Langenegger, D., Tiefenauer, L., Biosensors and Bioelectronics, 2010, 26(5), 1924-1928.

This work was initiated by A Studer. He established the nystatin-ergosterol method in our laboratory. The recording of the nimodipine inhibition was done by A. Studer. S. Demarche contributed to the assessment of the reproducibility of the method and tried to increase the fusion rate. The measurements shown are from S. Demarche.

**IV.3.1 Principle and advantages**

As explained in section IV.2, an open channel in the proteoliposomes is a necessary condition for the enhanced fusion of proteoliposomes to the bilayer. One way to achieve this condition is adding an open channel into the liposomes, which would increase the fusion rate. Ideally, this supplementary channel will disappear from the bilayer after fusion. The nystatin-ergosterol system, first developed by Woodbury fulfills these requirements\(^{105}\). Nystatin is an antifungal polyene that forms pores of conductance of about 5 pS in sterol-rich bilayers\(^{241}\). Steroids as all lipids can diffuse freely within a membrane. Nystatin forms pores in sterol-rich proteoliposomes. These pores increase the swelling of the proteoliposomes, which share a membrane with the bilayer, and thus increase the fusion rate (Figure IV.4A2). The resulting bilayer contains the protein of interest, ergosterol and nystatin. As the bilayer is free of other sterols, the ergosterol will diffuse within the membrane, resulting in the dissociation of the nystatin channels\(^{242}\) (Figure IV.4A4). The temporary pores formed by the polyene channel in the bilayer can be recorded by voltage-clamp as decaying current peaks (Figure IV.4A3 and B). Thus, the nystatin-ergosterol method has two advantages: It allows monitoring of the proteoliposome fusion events and it increases the fusion rate.
Figure IV.4: Principle of the nystatin-ergosterol system

(A) Proteoliposomes are added close to the bilayer in the presence of an osmotic gradient. 
1) Proteoliposomes attached to the bilayer will share a part of the membrane with the bilayer. 
2) Water molecules enter the proteoliposome to equilibrate the concentration difference between the trans side and the inside of the proteoliposomes. This will in turn lead to the influx of ions through the open nystatin channels, which will again promote flow of water into the liposome. 
3) Subsequently, the proteoliposome fuses to the bilayer, integrating the ion channel, the nystatin channels and the ergosterol. 
4) Ergosterol diffuses away and the nystatin channels dissociate. 
(B) Under voltage-clamp, a fusion event can be monitored as a current peak.

IV.3.2 Fusion of NaChBac proteoliposomes to a painted bilayer on a foil

IV.3.2.1 Recording of NaChBac activity

This study was carried out in vertical cells, with a four electrode system (Figure IV.7). For the nystatin-ergosterol method, proteoliposomes consisting of 40% POPC, 40% POPE and 20% ergosterol, 1:500 protein to lipid ratio (mol/mol) and 200 nm in diameter were used. Sucrose gradient purification confirmed that 27% of the protein was integrated (same method as Figure IV.3). Effects after addition of NaChBac proteoliposomes close to the bilayer painted over a pore in a plastic foil were monitored electrochemically (Figure IV.5A). Three fusion events were identified (Figure IV.5A, red stars) followed by a slow decay of the current. Following these three fusion events, discrete current levels could be detected, confirming ion channel activity. The current distribution (Figure IV.5B) shows a conductance of 110 ± 20 pS and the current steps lasted for up to several...
seconds. The observed current is similar to what has been observed in the group, but one factor of magnitude higher than what has been previously reported for NaChBac$^{230}$. The addition of 15 µM of nimodipine, known to be a NaChBac inhibitor$^{230}$, showed a specific inhibition of the 12 pA steps (data not shown$^{220}$), which confirmed that the observed current steps are specific to NaChBac activity.

**Figure IV.5:** NaChBac activity after fusion of proteoliposomes to a painted bilayer using the nystatin-ergosterol method

(A) Trace of current for a voltage-clamp of 100 mV. Three fusion events can be identified (red stars), followed by current steps, i.e. NaChBac ion channel opening and closing. The baseline is slowly decreasing, which is due to the nystatin pores decay. After 1100 s, some further fusions were observed, followed by a breakdown of the bilayer. (B) Current distribution of the signal observed in A. Omitting the first current level, all the distribution peaks are separated by 11 ± 2 pA, corresponding to a conductance of 110 ± 20 pA (= 12 pA / 100 mV). The difference between the first peak (-24 pA) and the second (11 pA) is 35 pA, which is a multiple of the difference between the other distribution peaks. This separation could thus correspond to three open ion channels.

Pavlov demonstrated that the NaChBac inactivation time can vary from 50 ms to 100 s, depending on the applied voltage and its duration. In that kinetic study, the pore domain of NaChBac was mutated. The authors demonstrated that NaChBac inactivation is regulated at the pore site. It is the so-called C-type inactivation, different from the much faster N-type inactivation, i.e. the “ball and chain” mechanism described for the *Shaker* K+ channel$^{244}$. Opening times up to several seconds are thus possible for NaChBac.

The higher conductance observed for NaChBac could be explained by one or several of the following reasons.

(1) NaChBac conductance has been shown to be dependent on the depolarizing voltage. However, the observed conductance were then only up to 4 times the conductance of 12 pS observed by Ren$^{245}$ and not in the higher range observed in our study. (2) Other factors can vary the conductance: a single ion channel conductance was twice as high in a free-standing bilayer compared to a patch-clamp experiment$^{246}$. This difference could be explained by the different ion concentrations.
Moreover, the lipid composition of the surrounding bilayer also plays an important role in ion channel conductance; it influences both the ion concentration in the vicinity of the pore and the kinetic behavior of the ion channel. (3) The observed higher conductance could be explained by the assumption that the ion channel did not assemble correctly during reconstitution. If one considers the Hille equation, which relates the pore radius of the ion channel and its conductance:

\[ \frac{1}{g} = \left( l + \frac{r \pi}{2} \right) \frac{\rho}{\pi r^2} \]

Eq. IV.2

where \( g \) is the conductance; \( l \) the length of the channel, estimated to be 4 nm; \( \rho \) the resistivity of the solution, estimated to be 0.74 \( \Omega \cdot m \) and \( r \) the radius of the pore, a conductance of 120 pS would correspond to an ion channel pore with a radius three times longer than for the pore of the original ion channel.

IV.3.2.2 Improvement of the fusion rate

With the nystatin-ergosterol method, fusion was achieved only in 24 % of the experiments (Table IV.1) and activity of the ion channel in only 3 % of the cases. One experiment is defined as a voltage-clamp on the painted bilayer at 100 mV during 1400 s. If no activity or fusion was observed after this period, the bilayer was broken and painted again.

<table>
<thead>
<tr>
<th></th>
<th>Number of exp.</th>
<th>No signal (in %)</th>
<th>Fusion (in %)</th>
<th>Breakdown &gt;900 mV (in %)</th>
<th>Fusion rate corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard conditions</td>
<td>68</td>
<td>50 (74 %)</td>
<td>16 (24 %)</td>
<td>28 (56 %)</td>
<td>40 %</td>
</tr>
<tr>
<td>pH 7.4</td>
<td>31</td>
<td>18 (58 %)</td>
<td>4 (13 %)</td>
<td>13 (72 %)</td>
<td>22 %</td>
</tr>
<tr>
<td>Horizontal device - standard</td>
<td>32</td>
<td>21 (66 %)</td>
<td>6 (19 %)</td>
<td>18 (86 %)</td>
<td>43 %</td>
</tr>
<tr>
<td>Horizontal device – sucrose and POPS</td>
<td>13</td>
<td>11 (85 %)</td>
<td>1 (8 %)</td>
<td>5 (45 %)</td>
<td>13 %</td>
</tr>
</tbody>
</table>

Table IV.1: Nystatin-ergosterol experiments on a painted bilayer on a foil

100 mV voltage was applied to the painted bilayer for 1400 s. “Number of exp.”: Number of repetitions is indicated. “No signal”: Number and percentage of attempts where no signal and no activity were observed are presented. “Fusion”: Number of successful fusions is displayed. Sum of the two percentages in these columns is not always 100 %, because some bilayers broke during voltage-clamp. “Breakdown >900mV”: Number of “No signal” experiments with breakdown voltage higher than 900 mV is indicated. “Fusion rate corrected”: Percentage of fusion calculated using the difference between “Number of exp” and “Breakdown >900mV”.

Conditions were varied in order to increase the fusion rate.

(1) Published nystatin-ergosterol experiments were performed around pH 7. The pH was therefore increased from 5.5 to 7.4. However, the fusion rate slightly decreased at neutral pH (Table IV.1).
(2) In a further series of experiments, the measurement device was changed to an horizontal device, to improve the accessibility of the painted bilayer. However, a decrease of the fusion rate was observed in this new device (Table IV.1).

(3) Proteoliposomes were loaded with 300 mM sucrose, formed with negatively charged lipids and added to the bilayer in the presence of calcium. The higher density of the sucrose causes the proteoliposomes to sink towards the bilayer. Combination of POPS and calcium ions is known to be a fusion condition. Even these conditions did not improve the fusion rate (Table IV.1).

After each experiment of 1400 s, the bilayer was subjected to a breakdown voltage experiment. An increasing potential amplitude was applied to the membrane and a sudden increase of current indicated the breakdown of the bilayer. Higher breakdown voltages signify higher stability of the bilayer. However, if no breakdown is observed over a certain voltage ($\approx 900$ mV), it is very probable that the structure formed over the pore is not a lipid bilayer, but rather a lipid plug or an air bubble. Interestingly, for the painted bilayer on the foil, a lipid plug or an air bubble was formed in up to 86% of the experiments. This could explain the low fusion rate: No fusion can occur on a lipid plug, or if it does occur, it cannot be recorded. If experiments, for which the putative bilayer did not break down at voltages higher than 900 mV, were not considered anymore, the fusion rate was about 40% (Table IV.1).

Bilayer quality is of pivotal importance and as it was an issue in this study, the development was continued on silicon chips which contained more defined pores.

IV.3.3 Fusion of NaChBac proteoliposomes to a painted bilayer on a chip

A major drawback of painted bilayers is their lower stability as supported bilayers. As explained in section I.3.1.3 of the introduction, a smaller pore diameter leads to higher stability. Silicon chips (Figure IV.6) with single pores or pore arrays of diameters ranging from 200 nm to 25.6 µm have been previously developed by our project partner (Leister) and are also used in this study (Table IV.2). They consist of a 6*6 mm silicon square chip (Figure IV.6A) with a 0.5 mm square window of 300 nm thick silicon nitride in their center.

<table>
<thead>
<tr>
<th>Pore size (µm)</th>
<th>Number of pore</th>
<th>Distance between pores (µm)</th>
<th>Total pore area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single pore 25.6 µm</td>
<td>25.6</td>
<td>1</td>
<td>514.719</td>
</tr>
<tr>
<td>Single pore 12.8 µm</td>
<td>12.8</td>
<td>1</td>
<td>128.680</td>
</tr>
<tr>
<td>4 pores 12.8 µm</td>
<td>12.8</td>
<td>4</td>
<td>27.2</td>
</tr>
<tr>
<td>Single pore 800 nm</td>
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<td>0.503</td>
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<tr>
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<td>16 085</td>
</tr>
<tr>
<td>Single pore 200 nm</td>
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<td>1</td>
<td>0.031</td>
</tr>
<tr>
<td>Pore array 200 nm</td>
<td>0.2</td>
<td>16 384</td>
<td>512</td>
</tr>
</tbody>
</table>

Table IV.2: Specification of the different chips used in this study

Chips with single pore, four pores or pore arrays, with pore diameters from 25.6 µm to 200 nm were used.
The chips were rendered hydrophobic by a silanization procedure. On this material, a Giga(Ohm)seal bilayer, stable for several hours, could be painted (Figure IV.7). In the presented EIS spectra, the obtained lipid bilayer has a resistance of 3.5 GΩ and a capacitance of 3.5 nF. The high capacitance could be explained by the presence of a bilayer over the whole silicon nitride window. The capacitance would then be 0.4 µF · cm⁻², which is the expected value.

**Figure IV.6: Silicon chips used in this study**
(A) Picture of a silicon chip. The green square in the middle is the silicon nitride membrane. (B) Scheme of the side view of the silicon chip. (C) SEM picture of the top view and side view (D) of a 200 nm diameter pore array.

**Figure IV.7: Painting of a silicon chip**
(A) The chip was placed between two PMMA compartments and voltage-clamped with four electrodes. Reprinted from reference²²⁰ with permission from Elsevier. (B) EIS spectra of a single 25.6 µm diameter pore before (blue) and after (red) painting. The impedance spectra (plain line) and the phase (dashed line) are shown. The data before painting were fitted with the model $R_{\text{buffer}}(R_{\text{chip}}C_{\text{chip}})$. The model $R_{\text{buffer}}(R_{\text{chip}}C_{\text{chip}})(R_{\text{bilayer}}C_{\text{bilayer}})$ was used to calculate the lipid membrane resistance and capacitance. The bilayer had a resistance of 3.5 GΩ and a capacitance of 3.5 nF, which is compatible with the presence of the bilayer over the whole silicon nitride area.
The nystatin-ergosterol method presented in section IV.3.1 was applied to bilayers painted on the chip. The painting procedure was more effective than on the foil, as shown by the small number of experiments for which the breakdown voltage was over 900 mV, which show the presence of a lipid plug. This could be for the reason that smaller amount of lipids were used during painting.

<table>
<thead>
<tr>
<th>Number of exp.</th>
<th>No signal (in %)</th>
<th>Fusion (in %)</th>
<th>Breakdown &gt; 900 mV (in %)</th>
<th>Fusion rate corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.6 µm pore - standard conditions</td>
<td>18</td>
<td>13 (72 %)</td>
<td>7 (39 %)</td>
<td>0 (0 %)</td>
</tr>
<tr>
<td>12.8 µm pore - standard conditions</td>
<td>47</td>
<td>44 (94 %)</td>
<td>1 (2 %)</td>
<td>6 (14 %)</td>
</tr>
<tr>
<td>4 pores 12.8 µm – 50 nm proteoliposomes – pH 7.4</td>
<td>14</td>
<td>13 (93 %)</td>
<td>1 (7 %)</td>
<td>1 (7 %)</td>
</tr>
<tr>
<td>25.6 µm pore – 50 nm proteoliposomes – pH 7.4</td>
<td>10</td>
<td>8 (80 %)</td>
<td>2 (20 %)</td>
<td>1 (13 %)</td>
</tr>
</tbody>
</table>

Table IV.3: Fusion rate using silicon chip as a support of the painted bilayer

It was possible to observe fusion events and ion channel activity in free-standing lipid bilayer on the chip (Table IV.3). For the pore with the largest diameter of 25.6 µm, the fusion rate was comparable to the rate on the foil, when lipid plugs are not considered for calculation. For the 12.5 µm pore, the fusion rate was more than ten times lower. Moreover, the comparison of experiments using 50 nm liposomes either on a single pore of 25.6 µm diameter or on four pores of 12.8 µm diameter suggest that the fusion rate does not depend on the total pore area. Despite the same total pore area, the fusion rate was seven times lower on the 4 x 12.8 µm pore chip. This could indicate that either a certain liposome area, pore area ratio is necessary for a successful fusion, or that fusions cannot happen on bilayers below a certain diameter. However, more experiments are needed to statistically confirm this trend and to conclude on the reason of the different fusion rates.

Ion channel activity on the micropore chips was observed after fusion of 50 nm proteoliposomes to a painted bilayer over a single 25.6 µm pore. The observed conductance was 50 pS (data not shown). The pH during the experiment was pH 7.4, which could explain why the observed conductance was lower than the previously observed 110 pS (Figure IV.5).

The nystatin-ergosterol method allowed a protein activity measurement either on a plastic foil, or on the more defined silicon chip. However, in both cases, only up to five ion channel molecules could be integrated and detected.
IV.4 Conclusion and outlook

In conclusion, ion channel activity was successfully recorded for two different types of ion channels. Fusion of proteoliposomes in preformed lipid bilayer was facilitated by a salt gradient, which was sufficient in the case of the KvAP proteoliposomes. NaChBac proteoliposomes did not efficiently fuse in the painted bilayer and the nystatin-ergosterol method was necessary to induce fusion and observe ion channel activity. Activity of this membrane protein could then be observed using a silicon chip, which contained a micropore. However, the fusion conditions could not be improved to achieve a reasonable fusion rate. Due to the low number of fusion events, a systematic study of an ion channel would be rather difficult. For studying membrane proteins with a lower turnover a high copy number of these proteins is required, which cannot be achieved by any fusion method. As the aim of this study is the development of a method applicable to as many membrane transport proteins as possible, the focus of the rest of the study was put on increasing membrane protein density.
Chapter V Formation of supported protein-tethered lipid bilayers with high densities of membrane proteins

Note: The dual polarization interferometry (DPI) experiments were carried out by S. Demarche in collaboration with Dr. G. Platt and Dr. M. Swann from Fairfield Group. S. Demarche and Dr. G. Platt acquired the DPI data with His-NaChBac. S. Demarche and Dr. G. Platt designed the ASIC experiments. S. Demarche prepared the material and G. Platt acquired the ASIC1a experiments. Dr. M. Swann and Dr. G. Platt performed the simulations. The ASIC1a protein was courtesy of R. Dawson and colleagues, from Roche. The cryo-EM micrographs were acquired by Dr. Barbora Malkova (PSI) and S. Demarche.

The integration of membrane proteins into artificial bilayers remains the limiting factor of functional proteobilayer formation. As shown in chapter IV, this integration is possible by fusion, but remains difficult. In this chapter, the focus is put on controlling the amount of protein in the bilayer using a solid, nonporous support. The best way to control the amount of protein in the bilayer is to first immobilize the protein and to subsequently form the bilayer.

V.1 State of the art

It has been known for several years, that mixing detergent-solubilized membrane proteins with micelles of lipids and detergent, followed by the removal of the detergent, leads to a successful reconstitution of membrane proteins in proteoliposomes\(^54, 181, 249\). Depending on the type of detergent and on the membrane protein, the detergent can be removed using different techniques: dialysis, addition of Bio-beads or successive dilutions\(^54\). This strategy has also been followed by several groups for the formation of supported lipid bilayers, the so-called protein-tethered bilayers (ptBLMs). In contrary to other tethered bilayers, here the tether is not the lipid, but the membrane protein itself. First, the membrane protein solubilized in detergent is immobilized and second, the bilayer is formed by addition of micelles of lipids and detergent.

Several techniques are available to immobilize the protein during a ptBLM formation. The first published method was an amine coupling\(^250\). The drawback of this technique is the random orientation of the protein molecules, due to the possible coupling of any free amine group of the protein. An immobilized antibody has also been employed. However, this implies the availability of a specific antibody directed against the membrane protein. Recombinant membrane proteins are often engineered with a His-tag at their C- or N-terminus so that they can be purified by IMAC. The method of choice for the immobilization of the membrane protein is thus the use of an NTA group to form a complex with the His-tag. This strategy has been extensively studied\(^151, 187, 191, 250-256\). In all these studies, the NTA group is covalently linked to the solid surface, either by alkylation\(^254, 255\), by creation of a carboxamide linkage between a 3,3'-dithiodipropionic acid di(N-hydroxysuccinimide
ester) (DTSP) monolayer and N-(5-amino-1-carboxypentyl) iminodiacetic acid (ANTA) or by covalent linkage of an NTA-functionalized hydrogel. This implies the presence of a chemically active group capable of forming a covalent bond and limits the choice of materials that can be used. The membrane protein is immobilized to the NTA-functionalized surfaces by forming a chelate of the His-residues with a metal ion, i.e. Cu\(^{2+}\) or Ni\(^{2+}\). The next step is the formation of the lipid bilayer using micelles of lipids and detergent. Removal of the detergent is either achieved by rinsing, by dialysis or by addition of Bio-beads.

It is important to verify that the bilayer has been formed. One method to do so was monitoring of the thickness of the deposited film by SPR. Alternatively, resistance increase was recorded by EIS. Surface-enhanced infrared absorption spectroscopy (SEIRAS) was also employed to follow the composition of the bound layer at a molecular level. Formed bilayers were imaged by AFM or infrared microscopy. Such ptBLMs were used to study the membrane protein structural characteristics or their activity.

In most of the cases, the immobilized protein was an enzyme involved in an oxidoreduction reaction and its activity was probed by cyclic voltammetry. For such a measurement, it is not necessary that the bilayer is perfectly sealed. The activity of other membrane proteins was demonstrated by a ligand binding study. No membrane transport protein has been studied by this technique so far.

This chapter describes a versatile method to immobilize the membrane protein. Furthermore, the chapter contains characterization of the lipid bilayer formed around the immobilized protein.

### V.2 Membrane proteins used in this study

The goal of this part of the thesis work was to establish a technique that allows an assessment of membrane transport proteins activities. Ion channels were studied because of their high transport rate (see chapter IV). NaChBac was chosen because of the availability of an inhibitor. It was used to demonstrate a proof of principle. Consequently, the acid-sensing ion channel 1a (ASIC1a), which is a pharmacologically more relevant ion channel was investigated.

ASIC1a is expressed in the nervous system and involved in nociception, since tissue acidosis is related to pain. ASIC1a is a 180 kDa trimeric ion channel gated by extracellular protons. It conducts cations, and, particularly sodium ions. Each monomer is formed by two transmembrane domains and by a big extracellular loop. The proton binding sites are located at a large distance from the transmembrane domain. ASIC1a is activated by pH changes as small as -0.4 and the gating mechanism can be modulated by several compounds. One of them is the spider toxin PcTX1, which stabilizes the desensitized state of the ASIC1a. The co-crystallization of ASIC1a with PcTx1 revealed the binding of the toxin to the acidic pocket. Recently, Gouaux et al. published a structure
of ASIC1a in complex with PcTX1 that revealed the structure of ASIC1a when the pore is open\textsuperscript{261, 262} when the pore is open (Figure V.1).

![Figure V.1: ASIC1a structure](image)

(A) Side view and (B) top view of the ASIC1a - PcTx1 complex at low pH in ribbon representation. Each monomer is represented in a different color (orange, blue and green) and PcTx1 peptides are represented in surface representation (in gray). Adapted from the pdb structure 4FZ0\textsuperscript{263}.

### V.3 Protein immobilization on nitrilotriacetic acid-functionalized poly(L-lysine)-g-poly(ethylene glycol)

The covalently NTA-functionalized devices showed successful and specific protein immobilization\textsuperscript{151}. However, chemical steps are involved in the surface functionalization and decreasing the NTA coverage lead to unspecific binding\textsuperscript{187}. The use of a co-polymer, consisting of a poly(L-lysine) (PLL) backbone and poly(ethylene glycol) (PEG), functionalized with NTA – PLL-g-PEG-NTA would solve these two drawbacks. The addition of PLL-g-PEG-NTA to a surface is very simple and does not involve any chemical step. PLL-g-PEG copolymers spontaneously adsorb to divers surfaces, such as metal-oxides\textsuperscript{263} and polystyrene\textsuperscript{264} by electrostatic interactions. Moreover, PLL-g-PEG-NTA forms a very efficient non-fouling surface and this at different grafting ratio\textsuperscript{265}.

In this chapter, the use of PLL-g-PEG-NTA as a pBLM support was investigated. PLL-g-PEG-NTA was deposited on a QCM crystal, chelated by nickel and exposed to His-NaChBac diluted in its buffer of gel filtration (Figure V.2). A decrease in frequency of 18.6 Hz was observed, indicating successful protein immobilization. To prove the specificity of the immobilization, imidazole was added. Imidazole is an aromatic heterocycle that displaces the His-tag nickel complex by...
competition. This procedure restored the frequency level before protein addition (± 3 Hz) meaning that it released almost all the previously immobilized proteins. This shows the specificity of the immobilization. A second addition of His-NaChBac decreased again the frequency to 18.9 Hz, indicating a second successful protein immobilization.

Figure V.2: QCM experiment showing the specific immobilization of His-NaChBac to PLL-g-PEG-NTA
Shift of the resonance frequency (Δf, blue line) and energy dissipation (ΔD, red line) at the third overtone. Running buffer was 10 mM HEPES and 150 mM NaCl at pH 7.4. PLL-g-PEG-NTA was incubated at 0.1 mg · mL⁻¹ (1 h) and rinsed with buffer. NiCl₂ was injected for 10 min at 20 mM and rinsed with buffer. His-NaChBac was injected at 250 µg · mL⁻¹ in running buffer containing 0.03 % DDM and rinsed with the same buffer. The protein shifted the frequency from -18.6 Hz. 500 mM Imidazole was injected and rinsed with buffer. The frequency came back to the level before protein addition. His-NaChBac was injected for the second time at the same concentration, which caused a similar frequency shift as during the first injection (18.9 Hz).

DPI was used to determine the mass of the immobilized membrane protein. QCM-D gives information about the “wet mass” of the immobilized proteins: it includes trapped solvent. DPI can be used to estimate the “dry mass”. About 49 ± 8 ng · cm⁻², i.e. 0.38 pmol · cm⁻² of His-NaChBac was immobilized to the PLL-g-PEG-NTA. If one considers NaChBac as a 10 nm diameter sphere, 49 ng · cm⁻² would correspond to about 20 % of the area occupied by immobilized proteins.

His-ASIC was immobilized at a higher density: 184 ± 102 ng · cm⁻² or 1.02 pmol · cm⁻². If His-ASIC is modeled as a 7.4 nm diameter sphere, about 26 % of the surface was occupied by the protein.
The observed difference in surface density between NaChBac and ASIC1a immobilization could be explained by the different quality of the membrane protein preparations. After purification, some of the NaChBac aggregated. The presence of aggregates in the solution could hinder a proper immobilization due to steric effects.

The calculated coverage values are two to three times lower than what has been published for His-GFP\textsuperscript{265} immobilized on PLL-g-PEG-NTA (about 60\% of the area covered by His-GFP). This difference could be explained by the fact that in this experiment, the membrane proteins are surrounded by the detergent and that the complex of the protein with detergent is larger than just the protein molecules.

PLL-g-PEG-NTA has already been shown to be a successful platform to immobilize His-tagged proteins. Here it has been shown that it can also serve as a platform for the specific immobilization of membrane proteins in the presence of detergent.

V.4 Lipid bilayer formation around the protein

V.4.1 Lipid bilayer formation

In the following, the formation of a lipid bilayer around the immobilized protein was investigated. The lipids were added at 1 mg · mL\textsuperscript{-1} with DDM at 0.1\% (w/w), which is far above the critical micelle concentration (cmc, 0.009\%) and is sufficient to solubilize all the lipids as micelles (Table I of the reference\textsuperscript{54}). DDM was chosen, because both NaChBac and ASIC1a were solubilized in DDM.

V.4.1.1 Choice of the lipids and the detergent removal method

Different detergent removal methods were compared. These were direct rinsing, Bio-beads addition and detergent dilution. The latter method comprises in successive incubation of solutions of lipids and detergents with the same lipid concentration, but with an increasing ratio of lipid over detergent. Dialysis is not compatible with the use of DDM, because of its low cmc.

Lipids containing 3\% of the NBD-PC fluorophore were incubated 1 hour with the His-NaChBac immobilized on PLL-g-PEG-NTA in the presence of 0.1\% DDM. The surface was either directly rinsed with buffer (direct rinsing), or incubated 1 hour with Bio-beads. For the third removal method, the solution of lipids and 0.1\% DDM was replaced by a solution with the same lipid concentration, but with 0.03\% DDM for 1 hour and for the next 1 hour with a lipid solution without DDM. After rinsing, FRAP was conducted to calculate the coefficient of diffusion (D). FRAP is a technique commonly used to test the quality of lipid bilayers, by assessing lipid mobility and thus fluidity of the lipid bilayer\textsuperscript{266, 267}. As an example, FRAP can be used to distinguish between the presence of intact liposomes or bilayers on a surface. Liposomes will have a coefficient of diffusion close to zero and a lipid bilayer spread over a solid surface will have a coefficient in the range of μm\textsuperscript{2} · s\textsuperscript{-1} (see Table I of the
The diffusion coefficients (D) for different preparations were found to be the following: after rinsing: \( D = 0.41 \pm 0.26 \mu m^2 \cdot s^{-1} \), after Bio-beads addition: \( D = 0.67 \pm 0.24 \mu m^2 \cdot s^{-1} \) and after detergent dilution: \( D = 0.64 \pm 0.35 \mu m^2 \cdot s^{-1} \) (Figure V.3). All diffusion coefficients were lower than the ones reported for supported lipid bilayers on solid surfaces, which are in the range of 3 to 10 \( \mu m^2 \cdot s^{-1} \). However, tethered bilayers (tBLM) have lower reported diffusion coefficients (from 0 to 5 \( \mu m^2 \cdot s^{-1} \)), depending on the density of the tethers\(^{268}\). Wagner and al. designed a supported bilayer with a PEG group covalently linked to lipids and reported a coefficient of diffusion of 0.5 \( \mu m^2 \cdot s^{-1} \) for cytochrome b5 containing tethered bilayers\(^{269}\). With the use of PLL-g-PEG-NTA, the lipids are not linked to the polymer, but a similar effect can be expected, as the recovery is hindered by the presence of immobilized proteins.

The dilution method to generate lipid bilayers showed a smaller coefficient of diffusion. Moreover, the cmc of DDM is low. Therefore, it can be expected that the lipid deposition after direct rinsing will be rather low. Bio-beads and successive incubation both gave similar results. A protocol using Bio-beads has the advantage of being faster, but is not applicable for every measurement because the Bio-beads are too big to be injected in QCM or DPI measurement flow cells. Detergent was thus further removed using the successive dilutions method.

**Figure V.3:** *FRAP experiment obtained using the detergent dilution method*

Picture just right after the bleaching (A1) and 7 min later (A2). (B) Normalized intensity in function of the time. The measured points are in black and the fitting function is in red. The calculated coefficient of diffusion was 0.3 \( \mu m^2 \cdot s^{-1} \).

NaChBac is originally found in *Bacillus halodurans*, an alkalophilic strain. The membranes of these alkalophilic strains contain up to 60 % of negatively charged lipids\(^{270}\). The influence of the negatively
charged lipids on the lipid bilayer quality was investigated by FRAP: lipid bilayers were formed with POPS ratio from 0 to 50 % and no major differences were found. A composition of 50 % negatively charged lipids and 50 % neutral lipids was used (50 % POPC and 50 % POPS). Because no major influence on lipid bilayer quality was found. ASIC1a was studied in POPC, the neutral phospholipid of highest abundance in human plasma membrane.

V.4.1.2 Further characterization of the lipid bilayer
FRAP experiments indicated successful formation of a lipid bilayer. In order to further confirm this result, QCM and DPI experiments were performed. His-NaChBac was immobilized to a PLL-g-PEG-NTA layer which was adsorbed on a gold QCM crystal. Further, lipids were incubated for 1 hour in the presence of 0.1 % DDM (Figure V.4). The detergent was diluted twice, whilst the lipid concentration was kept constant. The decrease of frequency between lipid addition and final rinsing was about 25 Hz. This value corresponds to the value expected for lipid bilayer formation on a crystal by liposome rupture and is another indication of the formation of a lipid bilayer.

![Figure V.4](image)

**Figure V.4: ptBLM formation characterized by QCM-D**
(A) Schematic representation of the different steps of the ptBLM formation. (B) Shift of the resonance frequency (Δf, blue line) and energy dissipation (ΔD, red line) at the third overtone. Running buffer was 10 mM HEPES and 150 mM NaCl at pH 7.4. PLL-g-PEG-NTA was injected at 0.1 mg · mL⁻¹ and rinsed with buffer. NiCl₂ was injected at 20 mM and rinsed. His-NaChBac was injected at 250 µg · mL⁻¹ and rinsed with running buffer with 0.03 % DDM. The lipid injections followed. Lipids with 0.1 % DDM, followed by lipids with 0.03 % DDM and lipids with 0 % DDM. After rinsing with running buffer, the decrease of frequency due to lipids was found to be 25 Hz (gray double arrow).
For QCM, the Sauerbrey equation links the decrease of frequency with the mass of the adsorbed layer:

$$\Delta f = \frac{n}{C} \cdot m_f = \frac{n}{C} \cdot \rho_f \cdot h_f$$  \hspace{1cm} \text{Eq. V.1}$$

where $m_f$ is the areal mass density of the adsorbed film, $\rho_f$ and $h_f$ are the density and the thickness of the adsorbed film, $n$ is the overtone, $\Delta f$ the decrease of frequency and $C$ is equal to 18 ng · cm$^{-2}$ · Hz$^{-1}$ (fixed value for the 5 MHz crystal used in this experiment). This equation is valid for rigid films, i.e. for $\Delta D_n/(-\Delta f_n) < 4 \cdot 10^{-7}$ Hz$^{-1}$, which is the case in our measurement. 25 Hz corresponds to the mass of lipids deposited during the formation of a complete lipid bilayer. In this ptBLM experiment the proteins are already immobilized; thus one could expect a lower decrease of frequency. However, the equation assumes a uniform layer, which does not reflect the reality as the layer is formed of both, lipids and proteins. Moreover, the formed lipid layer might be thicker, due to the presence of proteins. In conclusion, the 25 Hz do not allow a precise calculation of the amount of deposited lipids, but shows that the results are consistent with lipid bilayer formation.

To gain more knowledge on the mass and the structure of the deposited layer, the same experiments were repeated in a DPI device for NaChBac and ASIC1a. At the end of the process, the layer of protein and lipids was modeled as a uniform layer with a refractive index (RI) of 1.47.

For NaChBac, at the end of the lipid addition, a mass of $300 \pm 30$ ng · cm$^{-2}$ was obtained for the layer of proteins and lipids (Figure V.5 A), from which $256$ ng · cm$^{-2}$ can be estimated to come from the lipids. It was calculated before (see V.3) that about 20 % of the surface is occupied by the membrane protein. The creation of a perfect lipid bilayer would thus correspond to the deposition of $360$ ng · cm$^{-2}$, i.e. 80 % of the value expected for a perfect bilayer (450 ng · cm$^{-2}$). This result could be explained by (1) a coverage of the surface by the lipids and by the proteins only by 77 % or (2) by the underestimation of the surface covered by the protein because of the presence of aggregates.

The birefringence value of the DPI provides information about the orientation of the molecules at the surface. After rinsing, the birefringence was $0.0225 \pm 0.0007$, which is consistent with the presence of a lipid bilayer (Figure V.5A).

As for protein immobilization, lipid absorption during ptBLM formation with ASIC1a was higher. After the liposome injection, the calculated mass of the lipid bilayer was higher than expected. Therefore, water was injected into the system in order to cause an osmotic shock to all the intact liposomes that could possibly remain attached. After this injection, a mass of $570 \pm 160$ ng · cm$^{-2}$ was calculated for the layer of lipids and proteins (Figure V.5B). It can be approximated that $387$ ng · cm$^{-2}$ results from the lipids, after subtraction of the protein mass. From the DPI experiment on ASIC1a immobilization,
it was approximated that ≈ 26% of the area is occupied by the protein (see V.3). From the crystal structure, it is known that around one third of this surface corresponds to the transmembrane domain. Consequently, a full coverage of the free surface by the lipids would correspond to ≈ 90% of the full chip surface. A POPC supported lipid bilayer\textsuperscript{142} has a mass of 440 ng · cm\textsuperscript{-2}. Thus, 90% of a complete POPC bilayer would be 396 ng · cm\textsuperscript{-2}, which is very close to the found 386 ng · cm\textsuperscript{-2}. This estimation shows that the obtained data are consistent with the presence of a complete lipid bilayer. The birefringence at the end of the process (0.0182 ± 0.0031) is close to the published value for a POPC supported lipid bilayer (0.0216)\textsuperscript{142}. The anisotropy increases upon detergent dilution (Figure V.5C, red curve). This result is also consistent with the formation of a lipid bilayer, the detergent is removed and replaced by well ordered lipids by applying the different detergent dilution steps. All these results confirm the presence of a lipid bilayer. In the case of NaChBac, the coverage was incomplete, whereas for ASIC1a it was closer to a perfect complete bilayer.

Figure V.5: ptBLM formation followed by DPI

(A) Summary of the DPI data obtained for ptBLM with His-NaChBac as an anchor. (B) Summary of the DPI data obtained for ptBLM with His-ASIC1a as an anchor. (C) Example of a DPI experiment. The TM phase shift (TM1, blue line) and the anisotropy ((TM1-TE1)/(TM1+TE1), red line) are followed. The running buffer was 20 mM Tris and 150 mM NaCl, pH 8.0. Before t = 0, PLL-g-PEG-NTA was adsorbed at 0.1 mg · mL\textsuperscript{-1} and chelated with nickel. At t = 0, His-ASIC1a was injected at 143 µg · mL\textsuperscript{-1} and rinsed with running buffer containing 0.05% DDM. Liposomes of 100% POPC mixed with 0.1% DDM were injected, followed without rinsing by an injection of lipids with 0.05% DDM and lipids with 0.03% DDM, which led to a high increase in mass deposition. After the injection of liposomes without detergent, the system was rinsed with buffer. Water was injected to cause an osmotic shock to all the liposomes that remained intact.
V.4.1.3 Need of membrane proteins as anchoring points

Based on the differences in lipid deposition for ASIC1a and NaChBac, it seems that there is a dependency between the number of membrane proteins immobilized to the NTA groups and the amount of deposited lipid. A trend, rather than a strong correlation was observed for the different ASIC1a experiments. Moreover, when no membrane proteins were immobilized, no lipid bilayer could be formed, as observed in the QCM experiment presented in Figure V.6. This confirms that the membrane proteins act as anchoring points for the lipid bilayer formation.

Interestingly, when the same lipids were injected on PLL-g-PEG-NTA surfaces chelated with nickel but without protein and in the absence of detergent, a strong adsorption was observed by confocal microscopy, QCM and DPI (data not shown). The detergent possibly adsorbed to the NTA-nickel layer and shielded the charges.

\[ \Delta f(3) \text{ and } \Delta D(3) \]

**Figure V.6: The membrane protein is necessary for lipid deposition**

QCM-D experiment. Shift of the resonance frequency (\(\Delta f\), blue line) and energy dissipation (\(\Delta D\), red line) at the third overtone. PLL-g-PEG-NTA was injected at 0.1 mg · mL\(^{-1}\) and rinsed with buffer. NiCl\(_2\) was injected at 20 mM and rinsed. No protein was injected, only buffer in detergent. Then, the usual lipid solutions with different detergent concentration were injected: 0.1 % DM, 0.05 % DM, 0.03 % DM and 0 % DM. Similar results were observed with DDM as detergent. The frequency, dissipation and time do not start at 0 because the first 70000 s were used to form a polyelectrolyte multilayer (PEM) on the crystal (not shown). Similar results were observed without this PEM formation. The lipid additions only led to a frequency decrease of 7 Hz, showing that, without membrane proteins, no lipid bilayer is formed.
V.4.1.4 Characterization of the solution of lipid and detergent

In order to better understand the system, the different solutions of lipid and detergent used during the detergent dilution process were analyzed by dynamic light scattering (DLS) and cryo-electron microscopy (cryo-EM). Based on the DPI experiments, the lipids deposited most efficiently for 0.05 and 0.03 % DDM (Figure V.5). To determine which structure the lipids form under these conditions, 100 % POPC liposomes at 1 mg · mL⁻¹ with various detergent concentration were investigated by DLS and cryo-EM.

**Figure V.7**: DLS study of the different lipid/detergent solutions used during the ptBLM formation
(A) DLS experiments on 1 mg · mL⁻¹ POPC solution with DDM concentrations ranging from 0.1 % to 0 %. (1) Without DDM, liposomes were found. (2) For DDM concentrations between 0.005 % and 0.5 %, structures of the size of the original liposomes as well as sub-micrometer structures were observed. (3) For higher DDM concentrations, only small structures were observed. (B) Possible explanation for the observed structures. The higher DDM concentrations would contain only micelles (3), concentrations of DDM between 0.5 % and 0.005 % would lead to the formation of both liposomes (possibly also containing DDM) and thread-like structures (2).

Four different situations were observed (Figure V.7 and Figure V.8). (1) In the absence of detergent, liposomes of about 230 nm diameter were found. Cryo-EM showed that the liposomes were either multilamellar or unilamellar (Figure V.8 (1)). (2) For detergent concentrations between 0.005 % and 0.05 %, two kinds of structures were found: one with size ranges of the starting liposomes and the other in the sub-micrometer range. In the case of 0.03 % DDM, cryo-EM revealed the presence of both, liposomes and liposomes that adopted a tubular form (Figure V.8 (2a)). In the presence of 0.05 % DDM, thread-like micelles can be seen in the cryo-EM micrographs (Figure V.8 (2b)). The thread-like micelles showed the tendency to aggregate and some of them had a length of several hundred nanometers. Similar structures have already been observed by cryo-EM²⁷²-²⁷⁵. The sub-micrometer structures observed by DLS could correspond to these thread-like micelles.
Figure V.8: Cryo-EM micrographs of mixtures of liposomes with different concentration of DDM
(1) Liposomes without DDM. Unilamellar (black arrowhead) and multilamellar (white arrowhead) liposomes were present. (2a) Liposomes incubated with 0.03 % DDM. Smaller liposomes were also found (black arrowhead), some liposomes were opening (white asterisk) and others adopted a vesicular shape (white arrowhead). (2b) Liposomes incubated with 0.05 % DDM. Fewer liposomes were found (black asterisk). Thread-like micelles were abundant (white arrowhead) and had the tendency to aggregate. Some thread-like micelles were very long (black arrowhead). The transition between liposomes and thread-like micelles was observed (white asterisk). (3) Liposomes incubated with 0.1 % DDM. Mainly small micelles were observed. Some intact liposomes were present (not shown).
(3) For a DDM concentration of 0.1 % and higher, mostly smaller structures were visible. These were assumingly micelles. They formed aggregates on the cryo-EM grid (Figure V.8 (3)), but the DLS results tend to prove that these aggregates are not present in solution (Figure V.7A).

The most efficient deposition could occur when thread-like micelles are present because of the increased surface-area of these structures.

V.5 Activity measurements of ion channels in ptBLM

The functionality of NaChBac and ASIC reconstituted in ptBLM was studied by binding assays and DPI. As already mentioned in chapter IV, nifedipine, initially a calcium channel inhibitor, is also an inhibitor of NaChBac\textsuperscript{230}. It was proved to bind to the transmembrane domain IV of the calcium channel\textsuperscript{276} and it can be assumed that it also binds to the S4 of NaChBac. For ASIC1a, binding of an antagonist in the acidic pocket of the extracellular domain, PcTx1, was used as an assay. These molecules were added to the ptBLM and the effect on mass and birefringence was measured.

Nifedipine addition to NaChBac ptBLM did not result in any measurable mass increase. However, a negative change of -0.0004 in the birefringence could be observed, i.e. binding of nifedipine caused a decrease in the order of the lipids. This decrease could have two origins. First, it could be the result of a direct interaction between nifedipine and the bilayer. Second, the binding of the nifedipine to NaChBac could cause a conformational change of NaChBac and thus indirectly affect the order of the lipids. However, in an experiment with the addition of nifedipine to only lipids, similar changes were observed. This indicates that it was the interaction of nifedipine with the lipid bilayer that was detected and not the interaction with the protein. Nifedipine is a hydrophobic drug, and these drugs are known to interact with lipid bilayers. Thus, no convincing functionality data on NaChBac could be obtained with this ptBLM.

However, the addition of PcTx1 to ASIC1a ptBLM resulted in changes presented in Figure V.9. Specific binding of PcTx1 to ASIC1a could be observed. Nevertheless, the calculated \( K_0 \) from an equilibrium analysis was 90 nM, which is two orders of magnitude higher than what has been found by SPR for ASIC1a immobilized in the presence of micelles\textsuperscript{169} (2 nM). It would be interesting to repeat the same experiment on SPR to exclude that the observed \( K_0 \) difference results from the detection method. However, the most probable reason would be that part of the immobilized ASIC1a was partially denaturated during the detergent depletion process or during water injection.

No changes in the birefringence were detected, which would be expected as the PcTx1 binding site is about 50 Å up away from the transmembrane region and does not have large effects on the transmembrane helices\textsuperscript{169}. 
Chapter V Formation of supported protein-tethered lipid bilayers with high densities of membrane proteins

Figure V.9: Antagonist binding to ASIC1a ptBLM monitored by DPI

The graph shows the changes in the mass upon PcTx1 injection to a chip containing a ptBLM with ASIC1a. This graph was obtained after subtraction of the mass changes upon the same injections to a chip containing a ptBLM with NaChBac. Consequently, the signals resulting from the bulk or from the interaction with the lipids were subtracted. PcTx1 binding can clearly be detected from a concentration of 150 nM. The negative value for 75 nM is due to an unusual signal observed on the NaChBac channel.

The $K_D$ was calculated to be 90 nM ($\chi^2 = 1.43 \times 10^{-3}$) from the equilibrium analysis of the values of this graph, excluding the 75 nM value.

V.6 Conclusion and outlook

In conclusion, QCM, FRAP and DPI experiments indicated successful formation of ptBLM using PLL-g-PEG-NTA as support and an ion channel as anchoring protein. Both ion channels used in this study, a voltage-gated ion channel NaChBac and a ligand-gated ion channel ASIC1a, formed ptBLM successfully. The fluidity of this lipid layer formed by this method is comparable to those of tethered lipid bilayers. Furthermore, the thickness and amount of lipids of such ptBLM as estimated by QCM and DPI is consistent with the presence of a lipid bilayer. Birefringence data are also very similar to those published for lipid bilayers. The coverage by the lipids appears to depend on protein density and might be imperfect in the case of NaChBac. However, for optical detection, this is not an issue. The advantage of DPI over SPR is the possibility to follow the birefringence, a parameter giving information about the order of the adsorbed layer. It was possible to detect an effect of the inhibitor nifedipine on the ptBLM. However, this effect was not protein specific, but it rather appeared to be related to an interaction of the inhibitor with the lipids. Binding of PcTX1 to ASIC1a was successfully detected following the mass change. However, the $K_D$ was two factors of magnitudes higher than the one published by SPR. Further investigations are needed to understand this difference. Moreover, PcTX1 had no effect on birefringence.
It could be assumed that the described ptBLM system will allow to gain additional information to those provided by the existing ptBLM systems. It has the potential to be formed over any surfaces on which PLL-g-PEG-NTA can be adsorbed. Moreover, this is the first that ptBLM formation was verified using DPI. Based on the effect of drugs on membrane proteins, it will allow to determine if the binding of a drug has an effect on the protein structure. In this study, the use of PcTx1 was not optimal because it binds far away from the transmembrane area and does not induce big conformational changes. The fact that the protein is successfully integrated into a lipid bilayer is of high relevance, as the bilayer structure has effects on membrane protein function\textsuperscript{277} and because some membrane proteins need a lipid bilayer to keep their functionality\textsuperscript{171}.

In the last part of this thesis, it was investigated if the procedure developed in this chapter could be applied to form a ptBLM resulting from a combination of a supported and a free-standing bilayer, i.e. a bilayer suspended in a pore and supported by a permeable support, in order to allow direct electrochemical recording of ion channel activity.
Chapter VI Formation of ptBLM suspended in a nanopore filled with a polyelectrolyte multilayer

Note: K. Sugihara taught the spraying method to S. Demarche and helped to establish the equation of the coverage calculation. A. Larmagnac and S. Demarche developed the photoresist protocol. A. Larmagnac and M. Lanz prepared the photoresist protected chips with the help of S. Demarche. The microfluidic system was developed by I. Imhof in collaboration with the Fachhochschule Buchs SG. S. Demarche developed the use of this microfluidic system with the silicon chips over prolonged time in the presence of detergent, and its possible use with voltage-clamp.

Chapter V describes a system for the formation of artificial bilayers including a high density of oriented membrane proteins, which is a necessary condition to study the activity of membrane transport proteins with a low turnover. The protein is anchored to an NTA group present on PEG chains. Thus, this technique overcomes one of the main problems of tethered bilayers, which is the small distance between the bilayer and the support that is potentially problematic for protein function. However, the method retains the second disadvantage of all tethered bilayer, since the bilayer and the incorporated protein can be accessed only from one side. The fact that experimental conditions, such as presence of the membrane protein effectors, can only be varied on one side of the bilayer, hinders the study of certain membrane proteins. As emphasized in the introduction, free-standing lipid bilayers provide a way to solve this problem, but have drawbacks, such as their low stability. Recently, Sugihara et al. were able to form a lipid bilayer with a Giga-Ohm sealing by fusion of liposomes over a nanopore that was filled with a polyelectrolyte multilayer (PEM)\textsuperscript{99}. The positively charged PEM promoted the rupture of negatively charged liposomes and thus the formation of a bilayer. This preparation is solvent-free and self-assembling, but requires 100\% negatively charged liposomes, which is very different from a natural system.

In this chapter, the protein-tethered bilayer is formed on a nanopore filled with polyelectrolyte multilayer.

VI.1 Characterization of the polyelectrolyte multilayer-sprayed chip

The bilayer formation over PEM was performed using silicon chips with a single pore of 800 nm diameter (Figure IV.6 and Table IV.2).

The same PEM as used by Sugihara et al., composed of polyethyleneimine (PEI), poly(allylamine hydrochloride (PAH) and poly(sodium 4-styrenesulfonate (PSS): PEI(PAH/PSS)\textsubscript{28} was sprayed from the trans side of the chips. It was demonstrated to be suitable for measurements of peptidic ion channels\textsuperscript{99}. Sugihara and al. characterized the sprayed nanopores by AFM and EIS. The resistance of
the PEM sprayed pore was found to be $23.5 \pm 6.5$ M\(\Omega\) and the AFM pictures showed a complete filling of the pore, with some PEM accumulated on the edges of the pores\(^{278}\). Melittin activity was recorded during up to two weeks, indicating long-term stability of the bilayer.

In this work, PEM stability was further investigated because it is a crucial requirement for the viability of the system. Therefore, it was tested by assembling the sprayed chips in the device presented in Figure IV.7 and by acquirement of EIS spectra for several days (Figure VI.1A). Overall, the \(\text{PEI(PAH/PSS)}_{28}\) is stable for up to 50 days, with the exception of small variations of resistance over the time of the measurement. These findings confirmed that this technique is suitable for long-term experiments.

The measured resistance of a pore after spraying was much more variable than previously reported (Figure VI.1B). The available literature shows that the pore should be completely filled following the spraying of 28 polyelectrolyte bilayers\(^{278, 279}\). Sugihara and al. estimated the PAH/PSS PEM resistivity\(^{278}\) to be about 4.4 k\(\Omega \cdot \text{cm}\). For this estimation, the PEM was modeled, based on an AFM study, as a cylinder of 800 nm diameter and 275 nm length. The variation of the resistances as represented in Figure VI.1B could result from differences in PEM thickness.

Only the chips that exhibited a resistance (after spraying) between 10 M\(\Omega\) and 70 M\(\Omega\) were used. Lower resistances can be assumed to represent cylinders that are less than 100 nm high and thus do not reach the cis side of the chip pores. On the contrary, a too high resistance could be a problem for the electrochemical measurement of ion channel activity.

Figure VI.1: Spraying of a PEM in the pore of the silicon chip

(A) Normalized resistance in function of time for 4 different chips in 10 mM HEPES and 150 mM NaCl at pH 7.4. Resistance values were calculated using the model \(R_{\text{buffer}}(R_{\text{chip+PEM}}C_{\text{chip+PEM}})\) and normalized to the value at \(t = 0\). Some variations were observed in both senses, i.e. either decrease or increase of resistance. Interestingly, a decrease of \(R_{\text{chip+PEM}}\) could be followed by an increase. The PEM was stable for up to 50 days.

(B) PEM resistance after spraying. Only chips with a PEM resistance between 10 and 70 M\(\Omega\) were used for ptBLM formation.
VI.2 Protein-tethered bilayer formation on polyelectrolyte multilayer

In order to investigate a possible combination of PEM spraying and ptBLM formation, the assembly of the ptBLM was tested on a solid support on which a PEM was previously assembled. PEI(PSS/PAH/PSS) was assembled on a QCM crystal (Figure VI.2). The last polyelectrolyte of the PEM was the negatively charged polyelectrolyte PSS to allow adsorption of the PLL-g-PEG-NTA polymer via the positive charges of its backbone. The decreases of frequency were similar to the ones observed during ptBLM assembly directly on a solid support, confirming that the ptBLM forms similarly on PEM and on solid surface (Figure V.4). DPI experiments also showed a similar ptBLM formation over PEM (data not shown).

**Figure VI.2: ptBLM formation over a PEM characterized by QCM-D**

(A) Schematic representation of the different steps of the ptBLM formation. (B) QCM data of a ptBLM formation with His-NaChBac over a PEM. Shift of the resonance frequency (Δf, blue line) and energy dissipation (ΔD, red line) at the third overtone. The running buffer was 10 mM HEPES and 150 mM NaCl at pH 7.4. PEI(PSS/PAH/PSS) was assembled over the QCM crystal by successive incubation of the different polyelectrolytes at 1 mg · mL⁻¹, with buffer rinsing between each step (b on the graph). Each polyelectrolyte addition resulted in a decrease of frequency between 10 and 20 Hz. PLL-g-PEG-NTA was then adsorbed from a solution of 0.1 mg · mL⁻¹. The decrease of frequency of about 30 Hz after rinsing is consistent with previous experiments. NiSO₄ incubation and buffer rinsing followed. His-NaChBac resulted in a decrease of 15 Hz which is in the same range that was observed in Figure V.4. A frequency decrease of 24 Hz was observed after lipid additions followed by detergent removal.
Based on this experiment, it can be assumed that protein immobilization and lipid bilayer formation will also take place on a PEM-sprayed chip. As described in chapter V, binding of about 54 ng · cm⁻² of NaChBac over PLL-g-PEG-NTA was achieved. Assuming that as many proteins can bind over the PLL-g-PEG-NTA adsorbed over the PEM, it would correspond to more than 1,200 proteins over a 800 nm diameter pore.

VI.3 Characterization of ptBLM formed over a PEM-filled pore

Next, the ptBLM formation over a PEM-filled nanopore was investigated.

VI.3.1 FRAP

FRAP experiments were carried out after ptBLM formation over the PEM sprayed chips, using 3% NBD-PC as a fluorophore. For these FRAP experiments, pore arrays of 800 nm in diameter were used (Table IV.2). After PEM spraying from the trans side of the chip, PLL-g-PEG-NTA was incubated on the cis side. Following, ptBLM was formed in the same way as described in chapter V, using His-NaChBac as anchor and 47% POPC, 50% POPS and 3% NBD-PC as lipids. The calculated diffusion coefficient of the ptBLM was 0.18 ± 0.04 µm² · s⁻¹ (Figure VI.3). This value is about 4 times lower than what has been observed for the same preparation on solid support but is still higher than what has been reported for POPS bilayers directly on PEM (0.08 ± 0.04 µm² · s⁻¹). The lower diffusion coefficient observed by Sugihara and al. has been related to the strong coupling between the charged lipids and the oppositely charged PEM. PLL-g-PEG can shield the charges of the underneath substrate. This shielding effect could explain why the ptBLM on the PEM has a higher diffusion coefficient than observed before. The reason why it is lower than in the case of the ptBLM on solid surface (0.64 ± 0.35 µm² · s⁻¹, see section V.4.1.1) could be that (1) PLL-g-PEG-NTA does not cover the entire surface and only partially shields the charges of the underlying PEM. (2) Other charges originating from the negatively charged NTA, not occupied by nickel ions, are susceptible to interact with the lipids and thus decrease their mobility.

**Figure VI.3:** FRAP experiment of a ptBLM formed over a PEM back-sprayed chip
(A) Picture right after bleaching and (B) 15 min after bleaching. The brighter dots are the pores. Note that the recovery occurs over many pores indicating a continuous bilayer.
FRAP experiments did not show any fluorescence inhomogeneity, which indicates that possible defects are small and regular and that no big defective areas occur.

**VI.3.2 EIS shows partial bilayer formation**

An EIS spectrum was recorded before and after ptBLM formation. In 92% of the experiments, an increase of impedance was observed between these two steps (Figure VI.4A), which is consistent with the formation of a lipid bilayer. In the other 8%, the resistance after ptBLM formation was lower than the starting resistance. This can be attributed to PEM dissolution or delamination during the process due to exposure to the detergent. Such experiments were not considered in the following analysis.

![Figure VI.4: EIS characterization of the ptBLM](image)

(A) EIS spectra showing an increase of resistance after ptBLM formation. Spectra before PEM spraying (blue), after spraying (red) and after ptBLM formation (black). At low frequency, the impedance can be approximated to be equal to the resistance (red circles). (B) Electrical equivalent circuit model and drawing of the ptBLM on a PEM-sprayed pore. (C) Electrical equivalent circuit model and drawing of the ptBLM containing defects. The interruption in the bilayer represents a defect area.

The ptBLM system can be modeled using the following circuit: $R_{\text{buffer}}(R_{\text{chip+PEM}}C_{\text{chip+PEM}})/(R_{\text{ptBLM}}C_{\text{ptBLM}})$ (Figure VI.4B). The impedance of a resistor is $Z = R$, the impedance of a capacitor is $Z = 1/j\omega C$, where $\omega = 2\pi f$, with $f$ being the frequency. The total impedance of the circuit presented in Figure VI.4B, $Z_{\text{eq}}$, can be represented by:

$$Z_{\text{eq}} = Z(R_{\text{chip+PEM}}C_{\text{chip+PEM}}) + Z(R_{\text{ptBLM}}C_{\text{ptBLM}})$$

Eq. VI.1
where

\[
\begin{align*}
Z_{(R_{\text{chip+PEM}}C_{\text{chip+PEM}})} &= \frac{R_{\text{chip+PEM}}}{1 + R_{\text{chip+PEM}} * j\omega C_{\text{chip+PEM}}} \\
Z_{(R_{\text{ptBLM}}C_{\text{ptBLM}})} &= \frac{R_{\text{ptBLM}}}{1 + R_{\text{ptBLM}} * j\omega C_{\text{ptBLM}}}
\end{align*}
\]

Eq. VI.2

At low frequency, the terms \( j\omega C \) are very low so that the approximation \( Z_{eq} = R_{\text{buffer}} + R_{\text{chip+PEM}} + R_{\text{ptBLM}} \) is justified (Figure VI.4A, red circles). Moreover, the resistance of the buffer can be neglected and thus, \( R_{\text{end}} \approx R_{\text{chip+PEM}} + R_{\text{ptBLM}} \), where \( R_{\text{end}} \) is the resistance found by EIS at the end of the ptBLM formation.

The resistance after ptBLM formation was in the best cases (160 MΩ) one order of magnitude lower than for the painted bilayer (1 GΩ). Based on this value, the assumption that the ptBLM is composed of an area with a perfect bilayer (\( \Theta \)), and some regions with defects with a total area of (1-\( \Theta \)) was made (Figure VI.4C). From the measured impedance, the percentage of the pore covered by a bilayer can be approximated, using the equations below (Eq. VI.3 and Eq. VI.4). \( G_{\text{bilayer}} \) represents the conductance of a lipid bilayer and is approximated to be 1 nS; \( G_{\text{buffer}} \) represents the conductance of the buffer and is equal to 179 µS, i.e. the inverse of the buffer resistance.

\[
R_{\text{end}} = R_{\text{chip+PEM}} + \frac{1}{\Theta G_{\text{bilayer}} + (1 - \Theta) G_{\text{buffer}}}
\]

Eq. VI.3

\[
\Theta = \frac{1 - (R_{\text{ptBLM}}) * G_{\text{buffer}}}{(R_{\text{ptBLM}})(G_{\text{bilayer}} - G_{\text{buffer}})}
\]

Eq. VI.4

The calculated ptBLM resistance (\( R_{\text{ptBLM}} = R_{\text{end}} - R_{\text{chip+PEM}} \)) and the estimated corresponding coverage obtained after ptBLM formation over a 800 nm pore are presented in Figure VI.5. It should be noted that the ptBLM resistance represents both, the resistance resulting from the area with a bilayer and the resistance from the defect areas (see Figure VI.4C). The coverage estimation is based on the following assumptions. (1) PEM resistance does not vary during ptBLM formation. (2) No leakage originated from the edges of the pore. (3) Ions passing through the defect areas diffuse through the entire PEM, without lateral resistance.
Chapter VI Formation of ptBLM suspended in a nanopore filled with a polyelectrolyte multilayer

Table VI.1: End resistance, ptBLM resistance and bilayer coverage obtained from different preparations

<table>
<thead>
<tr>
<th>Protein</th>
<th>System</th>
<th>$R_{ptBLM}$ (MΩ)</th>
<th>$R_{end}$ (MΩ)</th>
<th>Coverage (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaChBac</td>
<td>standard</td>
<td>49 ± 38</td>
<td>73 ± 50</td>
<td>99.9494 ± 0.0940</td>
<td>8</td>
</tr>
<tr>
<td>KvAP</td>
<td>standard</td>
<td>41 ± 29</td>
<td>69 ± 34</td>
<td>99.9794 ± 0.0141</td>
<td>7</td>
</tr>
<tr>
<td>NaChBac</td>
<td>PLL-g-PEG-NTA inside</td>
<td>3 ± 2</td>
<td>26 ± 15</td>
<td>99.7672 ± 0.0933</td>
<td>4</td>
</tr>
<tr>
<td>NaChBac</td>
<td>photoresist</td>
<td>45 ± 42</td>
<td>62 ± 40</td>
<td>99.9806 ± 0.0124</td>
<td>3</td>
</tr>
<tr>
<td>KvAP</td>
<td>400 nm pore</td>
<td>25 ± 18</td>
<td>62 ± 19</td>
<td>99.9658 ± 0.0306</td>
<td>4</td>
</tr>
<tr>
<td>ASIC1a</td>
<td>standard</td>
<td>39 ± 27</td>
<td>63 ± 35</td>
<td>99.9756 ± 0.0246</td>
<td>3</td>
</tr>
</tbody>
</table>

$R_{ptBLM} = R_{end} - R_{chip} + R_{PEM}$. “System” indicates the protocol used during ptBLM formation. “Standard” corresponds to the system described in VI.3: PLL-g-PEG-NTA adsorption on a PEM-sprayed pore. “PLL-g-PEG-NTA inside” stands for the addition of PLL-g-PEG-NTA during PEM formation (VI.4.1). “photoresist” corresponds to the results obtained with the system described in VI.4.2. “400 nm” represents the results obtained with standard conditions on a PEM-sprayed 400 nm diameter pore (VI.4.3). “N” is the number of experiments.

Using NaChBac as an anchor protein, 7 out of 8 ptBLM formations yielded a coverage equal or higher than 99.9 % and the best obtained coverage was 99.9942 % (Figure VI.5A). The mean coverage was 99.9494 % ± 0.0940 (Table VI.1). This result is much higher than the coverage found by DPI on solid support (about 80 %, see chapter V). The low calculated coverage in the chapter V might have resulted from an underestimation of the surface covered by the protein. The mean ptBLM resistance was 49 ± 38 MΩ.

KvAP was also used for ptBLM formation together with 100 % POPC lipids. KvAP was solubilized by n-decyl-β-maltoside (DM) and was therefore bound over the PLL-g-PEG-NTA using DM containing buffer. However, lipid addition was carried out using DDM, as for NaChBac and ASIC1a. For KvAP, all experiments yielded a coverage higher than 99.9 % (Figure VI.5B), the best being 99.9934 %. The mean coverage was 99.9794 ± 0.0141 % and the mean ptBLM resistance 41 ± 29 MΩ (Table VI.1).

Using ASIC1a as an anchor for ptBLM formation, all experiments exhibited a lipid bilayer coverage higher than 99.9 %. The mean coverage was 99.9756 ± 0.0246 and the ptBLM resistance 39 ± 27 MΩ.

The high-standard deviations of the final and ptBLM resistances (Table VI.1) show that such complex bilayer preparations are not sufficiently reproducible.

The final resistance of the complete system was at least 1 order of magnitude lower than resistances of bilayers obtained by painting (see Figure IV.7).

Despite the low ptBLM resistance, the calculated defect areas are very small. Thus, small defects have very dramatic impact on the measured resistance. The fact that the model does not consider the lateral resistance of ion diffusion through the PEM could lead to an underestimation of the defect areas. For the NaChBac ptBLM, the average of defects is about 0.05 % of the total area ($2.5 \times 10^{-2}$ cm²). Considering the Hille equation (Eq. IV.2) and the thickness of the defect to be 4 nm,
this defect areas would correspond to a resistance of 53 MΩ. This value is very close to the average $R_{ptBLM}$ resistances observed and confirms the validity of the model used to calculate the coverage of the lipid bilayer.

Interestingly, ptBLM coverage did not correlate with PEM resistance (Figure VI.5C). If a low resistance of the PEM would result from the presence of big apertures in the PEM, the respective ptBLM coverage would be systematically much lower. This confirms the assumption that the whole pore is probably filled with PEM and that different PEM resistances arise from different PEM thicknesses.

Lipid composition (100 % POPC, 100 % DPhPC; 50 % POPS / 50 % POPC) as well as detergent removal method (Bio-beads) were varied for ptBLM formation using His-NaChBac or His-KvAP, without noticeable improvements (data not shown).

Based on these results, it can be concluded that single ion channel measurements are not possible using this PEM-supported ptBLM method. Variations of the system and its parameters were tested in order to elucidate if an increase of resistance of the PEM-supported ptBLM can be achieved.

**Figure VI.5:** Resistance of the ptBLM and estimation of the pore coverage
(A) Resistance (black) and coverage (red) obtained for different experiments using His-NaChBac as an anchor for bilayer formation. Each pair of a red and a black column represents one experiment. (B) Same representation for the ptBLM using His-KvAP as an anchor. (C) Resistance of the bilayer and coverage as function of PEM resistance using His-KvAP (blue points) or His-NaChBac (green points) as an anchor. Each point represents one experiment.
VI.4 Improvement of the sealing

The coverage approximation in the previous section (VI.3.2) was calculated with the assumption that the edges of the bilayer did not leak. In the presented system, the PLL-g-PEG-NTA adsorbs to the whole chip surface and not only to the PEM in the pore and the sealing over the edges of the pore might not be perfect (Figure VI.6A, arrows). Two strategies were developed to counteract this drawback and to form the ptBLM only over the PEM (VI.4.1 and VI.4.2).

VI.4.1 Simultaneous addition of PLL-g-PEG-NTA and PEM

The first strategy consisted in including the PLL-g-PEG-NTA directly into the PEM. Three to six of the first bilayers of the PEM were constructed by incubation instead of spraying, using both PLL-g-PEG-NTA and PAH for the positive polyelectrolyte deposition and PSS for the negative. The further 22 to 25 bilayers were sprayed following the standard procedure. The coverage decreased to 99.8 % ± 0.1 % (Table VI.1) and the resistance to 3 ± 2 MΩ. Assumingly, the membrane protein density is lower in comparison with the classical protocol, which could explain the lower ptBLM coverage. Silanization is known to improve free-standing bilayer sealing by increasing the hydrophobicity of the supporting material. However, no clear improvement has been observed when this modified protocol was used on silanized chips (data not shown).

VI.4.2 Surface protection by a photoresist

A further strategy, which avoids the decrease in protein density is to protect the area around the pores by a material that can be removed after PLL-g-PEG-NTA addition without affecting neither the PEM nor the PLL-g-PEG-NTA (Figure VI.6B). A technique using a photoresist has been developed by Falconnet and al. to pattern different PLL-g-PEG copolymers. Photoresist removal by an organic solvent did not affect PLL-g-PEG. This method was adapted to the silicon chips used as a support for ptBLM formation. After chemical vapor deposition of chromium on the trans side, a positive photoresist was spin-coated to the cis side of the chip (Figure VI.6C). Positive photoresist is degraded after exposure to UV and development. The chromium protects the photoresist from UV and thus, only the photoresist over the pores would be removed after UV illumination and development (Figure VI.6D). The use of these modified chips for the formation of ptBLM with NaChBac yielded a coverage of 99.9806 % ± 0.0124 % (Table VI.1). These results represent an improvement (standard condition using this ion channel led to 99.9494 % ± 0.0940 % coverage), but the resistance is still lower than for painted bilayers, indicating that there is still room for further improvement of the method.
Chapter VI Formation of ptBLM suspended in a nanopore filled with a polyelectrolyte multilayer

Figure VI.6: Photoresist protection of the chip
(A) Schematic representation of the problem to be addressed. (B) Photoresist strategy to improve the sealing: The chip is protected by a photoresist and sprayed with PEM. After PLL-g-PEG-NTA addition, the chip is dipped in an organic solvent. The photoresist is removed and only the PLL-g-PEG-NTA on top of the pore remains. (C) Protocol used for photoresist deposition. (1) Chromium is deposited on the trans side of the chip. (2) Photoresist is deposited on the cis side. (3) Upon UV exposure from the trans side, followed by development, only the photoresist inside of the pore is removed. The rest of the photoresist was protected from UV exposure by the chromium. (D) SEM picture of a 800 nm pore array after photoresist protection. The pores remained open.

VI.4.3 ptBLM formation over smaller pores
In a further experiment, the impact of pore size was investigated. Formation of ptBLM on a single pore chip of 400 nm in diameter decreased the resistance and the coverage, in the contrary to what have been expected (Table VI.1). This result could be explained by a different topology of the PEM in the pore of 400 nm in diameter. A systematic AFM study to observe a possible correlation between the PEM topology and the achieved resistance could confirm this hypothesis.

All the electrochemical results described above confirm the presence of lipid bilayers around the immobilized proteins. These bilayers have defects, calculated to occupy at the most 0.05 % of the pore area. Despite their small size, their effect on bilayer resistance is very high and could not be extenuated by the various tested strategies.
In conclusion, the achieved ptBLM resistance remains one order of magnitude lower than the resistance of the painted bilayer, making such a system unsuitable for single ion channel recording. The results support the hypothesis that bilayer patches are formed around the tethered protein and several reasons could explain the low resistance. (1) The protein density might be too low to allow complete bilayer coverage. Bilayer patches might form around the immobilized protein molecules but do not grow far enough to form a single bilayer. (2) Detergent removal might be incomplete. Detergent molecules present in the bilayer would act as open pores. (3) The roughness of the PEM layers might impose a problem. The bilayer patches could be not able to fuse together because they are not exactly on the same level. The bilayer is approximately 4 nm thick, and differences of couple of nanometers might be an obstacle to the formation of homogeneous bilayers. (4) Open ion channels would drastically decrease membrane resistance. Hence the spectrum is acquired at voltage conditions were ion channels are supposed to be closed. However, the presence of open ion channels cannot be excluded. (5) A proportion of the bound ion channels could be in a nonnative conformation. In such cases, formation of a tight bilayer around these proteins would be impossible.

VI.5  Ion channel activity on a PEM-sprayed pore

For this part of the thesis work, the system described in Figure IV.7 was used but the volume of compartments was only 200 µL. Furthermore, two Ag/AgCl electrodes were used for EIS and voltage-clamp.

VI.5.1 NaChBac activity

After formation of the PEM-supported ptBLM with NaChBac as an anchor, a voltage-clamp of -100 mV followed by a voltage-clamp of +100 mV was applied. NaChBac is expected to be open at +100 mV, and closed at -100 mV.

As described before, no single ion channel activity could be recorded because the resistance after bilayer formation is too low. Consequently, for voltages of +100 mV, the current remained in the nanoampere range instead of the ideal picoampere range and the noise was higher.

A good way to estimate whether open ion channels are present in the ptBLM is to add a specific inhibitor. During recording, nimodipine was added to the cis side of the system at a concentration of 100 µM. Out of 8 experiments, a current decrease was observed 5 times. The highest decrease (1.5 nA) is presented in the Figure VI.7. The reported conductance of NaChBac is 12 pS, but the conductance previously found in our group was 120 pS (see chapter IV). 1.5 nA corresponds to the closing of 1 250 ion channels considering the first conductance and 125 ion channels considering the second value.
The other experiments exhibited a decrease of only $158 \pm 68 \text{ pA}$, which corresponds to 13, respectively 80 ion channels closing. The fact that the decrease was not observed in all experiments, even when the calculated lipid coverage was similar, confirms that the observed effect is not related to simple adsorption of the nimodipine to the lipids or to the PEM. The observed differences in inhibition could be explained by several factors. (1) The amount of NaChBac bound over the pore may vary. (2) Different amount of active protein can be bound over the pore. (3) Degradation of the frozen ion channel during storage could cause variability in the measurements. The trace of Figure VI.7 was obtained three months after protein purification, and the other experiments were performed after 5 months and later. From the literature, nimodipine has an IC$_{50}$ of 1 µM for NaChBac$^{230}$. At 100 µM, most of the active ion channels should be inhibited.

In conclusion, partial inhibition of NaChBac activity by an inhibitor could be observed but the effect was not reproducible. Hence, to draw reliable conclusions, one would need results from several voltage-clamp experiments on NaChBac ptBLM with different nimodipine concentrations, in order to determine the IC$_{50}$. Ideally, addition of the inhibitor should be performed using a flow system, to exclude the possibility that the observed change is a result of a perturbation of the lipid bilayer.

**Figure VI.7:** Nimodipine addition to NaChBac ptBLM is followed by a current decrease
(A) The current trace was recorded at a clamped voltage of +100 mV. At time 1, current increases. This increase could be due to ion channels opening. At time 2, 0.5 % Ethanol (EtOH) is added to the solution as a control. At time 3, EtOH concentration is increased to 1 %. At time 4, the solution is exchanged to come back to 0 % EtOH. At time 5, nimodipine dissolved in EtOH is added to the cis side. The final concentration is 100 µM and 1 % EtOH. It is followed by a decrease of current of about 1.5 nA. The peaks at time 2, 3, 4 and 5 correspond to pipetting inside of the chamber. (B) Chemical structure of nimodipine.
VI.5.2 KvAP activity and the need of a microfluidic system

Upon switch of polarity, some current patterns were observed for ptBLM with KvAP. However, a clear conclusion could not be drawn, because no inhibitor was available for this system. Another way to distinguish KvAP activity and unspecific noise would be to compare the current in the presence or in the absence of KCl. However, this implies the exchange of the solutions from the PMMA measurement cells, which might damage the ptBLM and would make it difficult to compare the current traces before and after the exchange. Therefore, the silicon chip was integrated into the microfluidic system after PEM spraying.

VI.5.3 Chip integration into a microfluidic system

The microfluidic system consisted of two PDMS slabs containing 500 µm wide and 250 µm high channels and a space for the silicon chip (Figure VI.8A). The two slabs are pressed against each other, on one side with a glass slide, and the other side with a PMMA plate, containing screw openings (Figure VI.8B). The system is connected to a system pump with nanoliter precision via tubings made of polyether ether ketone (PEEK) (Figure VI.8D). The liquid is pumped through the microfluidic system by applying a sub-pressure in order to avoid leakage. Further precautions consisted of PDMS treatment with helium plasma and the use of silicon paste.

The volume between the entrance of the system and the chip is about 20 µL, which is one order of magnitude lower than in the previous PMMA measurement cell. It is important to be able to work with small volumes of membrane proteins because they are often difficult to purify in sufficient quantities. Two electrodes of Ag/AgCl (Figure VI.8C) were screwed into the PMMA plate, allowing EIS and voltage-clamp measurements.

PtBLM formation was carried out in the microfluidic system using NaChBac, KvAP or ASIC1a as anchor (Table VI.2). ASIC1a is not included in the table because only two experiments have been performed. The best of these two experiments gave a bilayer resistance of 157 MΩ and a coverage of 99.970%.

For NaChBac and KvAP proteins, the ptBLM resistance decreased by 50%. This resistance decrease could have several explanations, such as (1) very small remaining leakage between the chip and the PDMS, (2) a loss of material due to the hydrophobicity of the PDMS or (3) a damage of the bilayer by the shear force in the channel due to the liquid flow.

Table VI.2: End resistance, ptBLM resistance and bilayer coverage obtained with the microfluidic system

<table>
<thead>
<tr>
<th>Protein</th>
<th>System</th>
<th>$R_{\text{ptBLM}}$ (MΩ)</th>
<th>$R_{\text{end}}$ (MΩ)</th>
<th>Coverage (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaChBac</td>
<td>microfluidic</td>
<td>21 ± 15</td>
<td>57 ± 28</td>
<td>99.9548 ± 0.0372</td>
<td>8</td>
</tr>
<tr>
<td>KvAP</td>
<td>microfluidic</td>
<td>21 ± 18</td>
<td>53 ± 27</td>
<td>99.9556 ± 0.0278</td>
<td>9</td>
</tr>
</tbody>
</table>

$R_{\text{ptBLM}} = R_{\text{end}} - R_{\text{chip}} + \text{PEM}$.
Even if the resistance was lower in the microfluidic system, the coverage stayed within the same range and the microfluidic system was still used.

Addition of nimodipine to a NaChBac ptBLM resulted in a 150 pA current decrease, confirming that the observed decrease in section VI.5.1 was not an artifact due to pipetting of the inhibitor solution.

**Figure VI.8: Experimental setup of the microfluidic device**
(A) PDMS slabs with the channels and the chip area. (B) Assembled PDMS slabs with a silicon chip in the middle. On top of the PDMS slabs is a PMMA plate with openings for screws. At the bottom is a glass slide. (C) Ag/AgCl pellets soldered to a wire and glued to screws. (D) Assembled device in the Faraday cage.

**VI.5.4 KvAP activity measurement in the microfluidic system**
Overall, similar current patterns were observed in 5 out of 16 ptBLM experiments using ptBLM with KvAP as an anchor. A typical signal trace is presented in Figure VI.9. In a KCl containing buffer, an increase of current was observed after switching the voltage to +200 mV. This could be related to opening of KvAP ion channels. The high capacitance of the silicon chip (around 700 pF) causes a high capacitive current at each voltage change. Hence, the first milliseconds of the measurement are predominantly capacitive currents and it is difficult to estimate the exact current resulting from the possible ion channel opening. In the example presented in Figure VI.9, the increase of current after voltage change to +200 mV would be at least 460 pA, but likely more. At a clamping voltage of -200 mV only capacitive current was measured. The different current behavior observed at +200 mV and -200 mV clearly indicates voltage-dependency. When the buffer was changed to NaCl, only little
increase was recorded at +200 mV. The fact that the observed trace is KCl-specific indicated a possible recording of KvAP activity. Moreover, the exchange from KCl to NaCl buffer was done using the pumping system and a flow of -0.3 µL · s⁻¹. This slow flow likely did not disturb the ptBLM. After changing back to KCl buffer, signals similar as before were observed.

Considering the conductance of KvAP of 170 pS, the observed signal would correspond to about 14 ion channels, but it has to be kept in mind that the current was most probably underestimated, because of the capacitive current in the first milliseconds. In previous experiments, similar patterns, but with amplitude up to 1 nA had been observed at an applied voltage of +100 mV. This current corresponds to the activity of about 60 ion channels.

Recently, silicon nitride chips coated with a layer of SU-8 photoresist were developed, which decreased their capacitance by one order of magnitude. In future experiments, the use of such chips could avoid the capacitive current.

![Figure VI.9: Measurement of KvAP activity](image)

Results of a voltage-clamp experiment on a KvAP ptBLM. (A) In KCl buffer, increase of current of ≈ 460 pA was observed after switching the voltage to +200 mV. After switching the voltage to -200 mV, only a capacitive current was observed. (B) In NaCl buffer, almost no increase of current was observed.

VI.5.5 ASIC1a activity

ASIC1a is known to be activated by pH changes. Several attempts to observe the activity of ASIC1a in the microfluidic system by switching from a buffer of pH 7.4 to pH 6.5 were performed without success. In order to be able to record the activity of ASIC1a, the change of pH has to occur fast.
Bolshakov et al. showed that changes of pH within several seconds did not induce any measurable ion channel activity. This observation could probably be explained by the low unitary conductance of ASIC1a (30 pS). At an applied voltage of +100 mV, the activity of one ASIC1a ion channel would correspond to a peak of about 3 pA, which is lower than the noise of the system. For a pH drop, synchronous activation of several ASIC1a ion channels would occur, resulting in a macroscopic current. However, when applying a pH gradient, ASIC1a channels will open asynchronously, generating current peaks in the range of picoampere, which is below the detection limit. Subsequently, each channel switches to the desensitized form. In such scenario, no macroscopic current can be detected.

In order to be able to detect ASIC1a activity, a fast buffer exchange should be achieved using the microfluidic device.

VI.6 Conclusion and outlook

A successful formation of ptBLM over a PEM-sprayed nanopore was proven by independent methods such as QCM, DPI and FRAP. To our knowledge, this is the first time that formation of a ptBLM, supported over a polymer-filled pore, has been demonstrated. ptBLM formation was achieved using three different ion channels: NaChBac, KvAP and ASIC1a. Furthermore, ptBLM was integrated into a microfluidic system. Electrochemical measurements revealed the existence of small defects in the formed ptBLM. These defects resulted in a rather low resistance of the ptBLM. The resistance reached a maximum of 160 MΩ, which is approximately one order of magnitude lower than the value of a painted bilayer. The attempts to improve this resistance were not successful. Nevertheless, it remained possible to record the activity of an ensemble of NaChBac and KvAP ion channel molecules, proving the functionality of the proteins in the PEM-supported ptBLM.

The concept of ptBLM formation over a pore is a big advantage over the existing solid supported ptBLM because both sides of the ion channel are accessible, allowing addition of inhibitors or effectors.

Considering the insufficiently high ptBLM resistance and its low reproducibility, electrochemical detection may not be a suitable detection method. Nevertheless, ptBLM has a big potential for activity measurements of membrane transport proteins using optical detection methods. Moreover, ptBLM integrated in a microfluidic system is suitable for microscopy studies. However, it remains to be proven if in such a system the signal resulting from protein activity would be sufficiently high to surpass the background signal resulting from diffusion of ions and molecules through the defect areas. Even though the PEM remained stable for several days, a decrease of resistance between the beginning and the end of the experiment was observed in about 8% of the experiments. This is most likely due to a dissolution or delamination of the PEM during the process. It remains to be elucidated...
to what extent PEM dissolution or delamination happens during the successful experiments. Moreover, the stability of the PEM did not allow a reuse of the chips. In conclusion, the successfully established ptBLM method was tested on a new support in the following chapter.
Chapter VII ptBLM formation on pH-sensitive polymer brushes grafted on pore walls

Note: Part of this chapter was published in the article: Smart polymer brush nanostructures guide the self-assembly of pore-spanning lipid bilayers with integrated membrane proteins, G. W. de Groot, S. Demarche, (equally contributed) M. G. Santonicola, L. Tiefenauer, G. J. Vancso, *Nanoscale*, 2014, 6, 2228-2237. Reproduced with permission from the Royal Society of Chemistry.

The principle was developed by S. Demarche, W. de Groot and G. Santonicola. W. de Groot functionalized the pores, performed the AFM and FTIR experiments. S. Demarche set up the lipid bilayer and the ptBLM formation protocol. S. Demarche and W. de Groot carried out the ptBLM experiments.

It has been shown in previous studies that nanopores can be functionalized with smart polymers. For example, pH-sensitive polymers were covalently attached to nanopore walls by localized surface chemistry. These smart polymers allowed a modulation of the pore diameter in function of the pH222, 284. In this chapter, the use of covalently immobilized polymers in a nanopore as a support for ptBLM formation was investigated. Covalently immobilized polymers are more robust than sprayed polyelectrolytes (Chapter VI).

VII.1 Principle

Recently, a method to graft a pH-sensitive polymer to the cylindrical walls of a nanopore was developed222, 285 (Figure VII.2A). The functionalization was performed on silicon chips containing a single pore or pore arrays of 200 nm in diameter. The surface of the nanopore wall was activated by a surface-initiated atom transfer radical polymerization (SI-ATRP) initiator ((3-(2-bromoisobutyryl)propyl)dimethylchlorosilane), which was deposited by vapor phase deposition (Figure VII.1-1).

![Figure VII.1: Reaction scheme of functionalization of silicon with PMMA-NTA](image)

(1) Deposition of a surface-initiated atom transfer radical polymerization (SI-ATRP) initiator (((3-(2-bromoisobutyryl)propyl)dimethylchlorosilane)) on the silicon surface by vapor phase deposition. (2) Grafting of poly(methacrylic acid) (PMAA) by SI-ATRP of sodium methacrylate. (3) Conversion of the carboxylic acid groups of PMAA into active esters by immersion in N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). (4) Reaction of the esters with aminobutyl-NTA.
Following, the pH-sensitive poly(methacrylic acid) (PMAA) polymer was grafted by SI-ATRP\textsuperscript{286} (Figure VII.1-2).

Successful functionalization of the silicon surface by PMAA was proven using Fourier transform infrared spectroscopy (FTIR) (Figure VII.2D).

The presence of the polymer in the pore was proven using AFM (Figure VII.2C) and EIS. The pKa of the PMAA brushes is calculated to be 6.5, and the pores were proven to be closed at a pH higher than 6.5 and open at a lower pH (Figure VII.2C).

In this study, the pores were further functionalized with NTA groups by conversion of the carboxylic acid groups of PMAA into active esters by immersion in N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS)\textsuperscript{287} (Figure VII.1-3), followed by reaction with aminobutyl-NTA\textsuperscript{288} (Figure VII.1-4, see III.2.5 for more details). FTIR confirmed the presence of the NTA group after this last reaction step (Figure VII.2D).

Subsequent experiments addressed the question if the pores functionalized with PMAA-NTA could be used as a support for ptBLM formation.

**Figure VII.2: Nanopore functionalization with PMMA-NTA**

(A) Schematic representation of the nanopore grafted with pH-sensitive polymers. At pH below 6.5 the PMAA polymer collapses and the pore is open. At pH higher than 6.5 the polymer swells and closes the pore. (B) Schematic representation of ptBLM formation on the pore functionalized with PMAA-NTA. (C) AFM pictures of a nanopore functionalized with PMAA at pH 4 and pH 8. At pH 4, the pore is open, whereas it is closed at pH 8. (D) FTIR of a PMAA brush grafted to a nonporous support before (black) and after (red) NTA-functionalization. (C) and (D) measurements have been carried out by W. de Groot.
VII.2 Membrane protein immobilization on poly(methacrylic acid)-nitrilotriacetic acid functionalized surfaces

A nonporous silicon surface was functionalized with PMAA. PMAA was further derivatized with NTA on one half of the area. The whole silicon surface was then incubated with a nickel solution, followed by incubation with a His-GFP solution. Confocal microscopy showed an increase of fluorescence only within the area that was functionalized with NTA. This result proves that immobilization of the His-GFP to the NTA group occurred. EDTA competes with the oligohistidine-tag for nickel ions. After EDTA incubation, fluorescence intensity decreased of about 92 %, confirming the specific immobilization of the His-tagged protein to the NTA group (Figure VII.3C). Immobilization was also achieved using the His-mCherry-NaChBac membrane protein (Figure VII.3B).

VII.3 Formation of ptBLM on the PMAA-NTA polymer brushes

Bilayer formation over pores functionalized with PMAA-NTA was investigated using electrochemical (EIS) or optical (FRAP) methods. ptBLM was first formed as outlined in Chapter V by His-tagged protein immobilization, addition of micelles composed of lipids and detergent and subsequent detergent depletion. However, all the ptBLM formed in this way exhibited resistance below 30 MΩ. Therefore, in this chapter, the bilayer was formed using a different protocol, namely the direct rupture of positively charged liposomes (POEPC) to the negatively charged PMAA polymer (Figure VII.2B).

VII.3.1 Optical proof of bilayer formation

VII.3.1.1 Lipid bilayer formation in the absence of proteins

Rupture of POEPC liposomes was first monitored by FRAP on a 200 nm pore array (Table IV.2) using NBD-PC as fluorophore. A coefficient of diffusion of $0.27 \pm 0.14 \mu m^2 \cdot s^{-1}$ was calculated (Figure VII.4), which is in agreement with the presence of a lipid bilayer. Recently, formation of a lipid bilayer over zwitterionic polymer brushes was established. The coefficient of diffusion of such bilayers was around $1 \mu m^2 \cdot s^{-1}$, which is similar to values obtained for bilayers on glass supports. The concept presented here is different, since the PMAA is negatively charged at pH 7.4, and the support is porous. The electrostatic interaction between the polymers and the lipid head groups could be one reason for the lower coefficient of diffusion. Another explanation could be the roughness of the PMAA due to the presence of pores, mechanically hindering the mobility of the lipids.
VII.3.1 Lipid bilayer formation in the presence of proteins

Bilayer formation by direct rupture of liposomes was carried out after immobilization of His-NaChBac to the NTA group of the PMAA polymer brushes (Figure VII.2B and Figure VII.4). The coefficient of diffusion of such ptBLM depended on protein concentration. For protein concentration of 25 µg · mL⁻¹, the coefficient of diffusion (D) was 0.22 ± 0.07 µm² · s⁻¹, and for concentration of 250 µg · mL⁻¹ it was 0.06 ± 0.03 µm² · s⁻¹. As expected, the presence of proteins decreased the coefficient of diffusion (see discussion in section V.4.1.1). It is likely that the incubation with a higher protein concentration resulted in a higher amount of protein bound to the surface. Consequently, more anchorage points for the lipids were present, and the resulting bilayer had a lower fluidity. However, both coefficients are consistent with the presence of a lipid bilayer.
Chapter VII ptBLM formation on pH-sensitive polymer brushes grafted on pore walls

Figure VII.4: Optical proof of bilayer formation on a chip functionalized with PMAA-NTA
Pores functionalized with PMAA-NTA after incubation with positively charged liposomes composed of 97 % POEPC and 3 % NBD-PC. (A) Confocal image of the pore array after the formation of bilayer. Pores are visible as brighter areas. (B) FRAP experiment on the pore array. Picture (1) was taken directly after bleaching and (2) 9 minutes after bleaching. Pores are not visible anymore because a lower image resolution was used. (C) Table of calculated diffusion coefficients for various protein concentrations (protein concentrations during incubation are indicated).

Confocal images acquired after immobilization of His-mCherry-NaChBac and rupture of liposomes composed of 97 % POEPC and 3 % TopFluor-PC also confirmed the presence of both, proteins and lipids, on the nanopores (Figure VII.5).

Figure VII.5: Both proteins and lipids are present on the chip after ptBLM formation
Sequential confocal pictures of the pore array after ptBLM formation with His-mCherry-NaChBac and liposomes composed of 97 % POEPC and 3 % TopFluor-PC. (A) Detections from excitation at 488 nm and emission at 503 nm – 523 nm. Lipid molecules labeled with TopFluor-PC are detected. (B) Detection from excitation at 543 nm and imaging at 550 nm – 650 nm. The protein His-mCherry-NaChBac is detected.
The PMAA-NTA brushes have the potential to serve as a support for lipid bilayer formation in the presence or in the absence of membrane proteins. The fact that a bilayer is formed in the absence of proteins is a tremendous advantage over PLL-g-PEG-NTA, because the surface density of proteins can be varied without hampering bilayer formation.

VII.3.2 Electrochemical monitoring of lipid bilayer formation

Rupture of POEPC liposomes was then investigated by EIS on a single pore of 200 nm in diameter (Table IV.2) and in the absence of proteins. NTA groups were loaded with nickel, rinsed and incubated with the liposomes overnight. EIS spectrum was recorded after extensive rinsing. In most of the cases, the total resistance increased. Since both the pH and ionic strength were maintained constant, it can be assumed that the resistance change was due to the lipid addition. As the lipids were proven to be fluidic (VII.3.1), it can be concluded that a lipid bilayer was formed. In the EIS spectra, the impedance value at the lowest measured frequency is approximated as being equal to the resistance of the PMAA-NTA pore plus the resistance of the bilayer (see section VI.3.2). The coverage was calculated using the equation defined in chapter VI (Eq. VI.4). The resistance of the bilayer was equal to or higher than 90 MΩ in 29 % of the cases. The average calculated coverage was 99.9753 % ± 0.0410 % and the highest coverage was 99.9966 % (Figure VII.6). These values are in the same range than those calculated for the ptBLM over a PEM-sprayed pore of 800 nm in diameter (chapter VI).

VII.3.2.1 Influence of lipid composition on lipid bilayer formation

In order to avoid the presence of too many charges in the lipid bilayer, it was investigated what ratio of POEPC is required for liposome rupture. It was found that liposomes formed of 20 % POPC and 80 % POEPC retained the ability to rupture, whereas a higher content of POPC hindered bilayer formation. Incubation of liposomes with higher content of POPC showed, either no increase in resistance, or an increase below 30 MΩ, which can be attributed to a low coverage of the bilayer. This observation clearly indicates the limitation of using charged polymers and lipids to form lipid bilayers. Positively charged lipids do not occur naturally and consequently, such a lipid bilayer is very different from a natural cell membrane. The positive charges might change the function of the membrane proteins. However, it has been proven by Kunze and al. that a lipid transfer between a charged supported bilayer and oppositely charged vesicles is possible, and simulation experiments revealed that neutral lipids can also be exchanged by this technique. Thus, formation of a lipid bilayer using liposomes composed of 80 % POEPC and 20 % POPC, followed by incubation with vesicles composed of negative and neutral lipids could lead to the formation of a lipid bilayer that is more similar to natural lipid bilayers.
VII.3.2.2 Influence of other parameters on lipid bilayer formation

In a further series of experiments, the influence of temperature and liposome size on the formation of lipid bilayer was investigated. The temperature during incubation with liposomes was increased from room temperature to 37 °C or to 55 °C. This did not yield any improvement in final resistance and thus did not improve direct rupture of liposomes. It is an advantage to obtain most efficient lipid bilayer formation at room temperature, as temperature should be maintained as close as possible to the temperature the protein is usually exposed to, which is mostly below 37 °C. This is important for stability and functionality of the fragile membrane proteins.

In a next step, liposomes of diameter of 200 nm and 400 nm were tested. A resistance of the bilayer above 90 MΩ was achieved more frequently when using liposomes of 50 nm in diameter than when using liposomes of larger diameter. No statistics are presented here because the experiments were not performed in sufficient number of replicates.

Figure VII.6: Resistance and calculated coverage of a POEPC bilayer on a single pore of 200 nm in diameter, functionalized with PMAA-NTA

(A) Resistance (black) and calculated coverage (red) of a bilayer formed on a pore functionalized with PMAA-NTA after rupture of positively charged liposomes containing 100 % POEPC. Each pair of a red and a black column represents one experiment. (B) Distribution of different coverages obtained after the fusion of POEPC liposomes (in %). (C) Distribution of different resistances (in MΩ) obtained after the fusion of POEPC liposomes.
VII.3.2.3 Formation of lipid bilayer in the presence of immobilized membrane proteins

Similar to the FRAP experiments (see VII.3.1.2), a bilayer was formed after the ion channel His-NaChBac was immobilized to the pore functionalized with PMAA-NTA. After the incubation with the membrane protein, the buffer was replaced with the liposome solution without rinsing. Resistances of over 90 MΩ were achieved, which is in agreement with lipid bilayer formation over the PMAA-NTA brushes after protein immobilization. A direct passage from the solution containing the detergent to the liposome solution, which is free of detergent, may denature the protein. However, the solution free of detergent contains lipids and it is possible that the addition of lipids around the protein is faster than the removal of detergent and that the protein does not denature.

To further simplify the process and to reduce the risk of protein denaturation during immobilization, it was investigated whether a lipid bilayer can be formed by direct proteoliposomes rupture. For these experiments, KvAP proteoliposomes were used. They consisted of 100% POEPC lipids and the ion channel in a ratio of protein and lipid of 1/200 (w/w). In a previous experiment, recording of this ion channel activity proved that the protein was successfully integrated into the proteoliposomes (IV.2.2). In two out of four experiments, the resistance of the lipid bilayer was higher than 90 MΩ. After rupture of the proteoliposomes, it can be expected that the His-tagged membrane protein molecules will bind the NTA groups underneath and will adopt a uniform orientation.

This hypothesis could be tested by an experiment in which proteoliposomes that contain a His-tagged membrane protein would be ruptured over pores functionalized with PMAA-NTA. Subsequent addition of fluorophore-labeled antibodies against the His-tag would designate proteins that did not bind to NTA groups and might therefore be in opposite orientation. An absence of the fluorescence, originating from the antibodies, would indicate that the protein molecules integrated into the bilayer are bound by the His-tag to the NTA groups of the surface, and hence adopt a defined orientation.

VII.3.2.4 Reuse of the functionalized chips

The possible reuse of chips, which were functionalized with PMAA-NTA, was tested. After each bilayer formation, the chip was cleaned with 100% EtOH. When protein was immobilized during the experiment, the chip was cleaned with 100% EtOH, pepsin (HI 7073L, HANNA instruments Inc., USA) and 20 mM EDTA. EIS spectra obtained using the same chip during subsequent experiments are shown in Figure VII.7. The spectra show that a bilayer with resistance of 100 MΩ was obtained even after seventh use of the same chip. Moreover, several of the experiments in the series involved immobilization of proteins. The option of reusing the chips represents an important advantage of covalently attached polymers.
**Figure VII.7: Reuse of a nanopore chip functionalized with PMAA-NTA**

Spectra at the beginning (dashed line) and end (full line) of the experiment. Spectra were obtained using the same PMAA-NTA-functionalized chip for three subsequent experiments. The chip was used four times before being used for these three experiments (Exp. 5 to 7). His-NaChBac was immobilized on the chip in experiment 5. Experiments 6 and 7 did not involve protein immobilization.

**VII.3.2.1 Measurement of the current**

Measurement of current resulting from an activity of a single ion channel or of an ensemble of ion channels was not possible using this preparation method. Even for resistances of more than 90 MΩ, the current trace was not stable under voltage-clamp conditions (Figure VII.8). This phenomenon was also observed for a POPS bilayer formed over a positively charged PEM when the resistance was below 200 MΩ (data not shown).

However, these fluctuations of current were not observed when a voltage-clamp was applied to chips functionalized with PMAA-NTA, without the addition of lipids. This finding indicated that the fluctuations are caused by the charged lipids. Calculation of the coverage indicates that the bilayer contains small defects, which are likely present as gaps in the bilayer. Upon application of the voltage, these lipid pores could develop a certain dynamic and thus cause erratic current jumps as recorded in Figure VII.8.

As for the ptBLM described in chapter VI, such a ptBLM appears to be suitable for optical detection of ion channel activity rather than for electrochemical detection.
VII.4 Conclusion and outlook

EIS and FRAP experiments have proven formation of a ptBLM on a pore functionalized with PMAA-NTA. The ptBLM was formed by immobilization of the membrane protein followed by direct rupture of liposomes. The bilayer was fluidic and exhibited a resistance of up to 142 MΩ. Further characterization of the system remains to be carried out. (1) It should be confirmed that the lipid bilayer truly forms around the immobilized protein. (2) The surface density of the immobilized membrane protein should be determined. (3) The possibility to form the lipid bilayer with lipid compositions closer to the one occurring in living cells should be investigated. (4) The functionality of the membrane protein should be confirmed.

Despite the above-mentioned issues that remain to be investigated, it has been demonstrated that this method represents several advantages over a PEM-supported ptBLM. (1) The functionalized pore can be reused without losing the polymer brushes. (2) The PMAA-NTA brushes are stable during the experiment because they are covalently linked to the wall of the nanopore. (3) PMAA is pH-responsive. At pH below 6.5 the polymers collapse and the nanopores are open, while they swell at higher pH and close the nanopores. It is thus theoretically possible to form a ptBLM over closed PMAA-NTA nanopores, then open the pores and measure the activity of ion channels. (4) Protein density can be varied in a wide range without impairing the formation of the lipid bilayer.

A possible combination of the ptBLM system, presented in the chapter VI, and the system presented in this chapter can also be considered. In such an experiment, the membrane protein would be immobilized on PMAA-NTA brushes, a lipid bilayer would be formed around the immobilized protein.
molecules and the defects would be filled by direct rupture of liposomes composed of 20 % POPC and 80 % POEPC.

In conclusion, the work presented in this chapter represents a good starting point for further investigation and optimization of forming ptBLM over a nanopore functionalized with covalently linked polymers.
Chapter VIII  Summary and outlook

Artificial bilayers are important tools for investigating the activity and properties of membrane proteins. Compared to the patch-clamp method, where the natural membranes of living cells are used, assays employing artificial bilayers have the advantage that the composition of lipids and the density of the reconstituted proteins can be controlled. However, the integration of the membrane proteins remains the limiting steps in the development of such bioanalytical assays. To develop a versatile assay for membrane transport proteins, a high number of protein molecules in the membrane is required. Therefore, the focus of this thesis was on obtaining artificial bilayers with a high density of proteins.

First, proteoliposomes were fused to a painted bilayer, using either a salt gradient or the nystatin-ergosterol method. The nystatin-ergosterol method was proven to be successful for ion channels, which could not be integrated in their active form using only a salt gradient. However, the fusion rate remained rather low, probably because of the presence of lipid plugs. By using well defined pores in silicon chips, the occurrence of lipid plugs was drastically reduced and fusion rate was increased. Consequently, it was possible to measure ion channel activity in such reconstituted membranes. However, only few copies of a functional membrane protein could be integrated into the lipid bilayer by this method.

Therefore, a method yielding a higher protein density was developed. The nitrilotriacetic acid-derivatized poly(L-lysine)-g-poly(ethylene glycol) polymer was used for the immobilization of membrane proteins at a high density on solid support. Subsequently, a protein-tethered lipid bilayer was formed with either a voltage-gated (His-NaChBac) or a ligand-gated (ASIC1a) ion channel. First results of a ligand-binding study by dual polarization interferometry indicated that the ligand-gated ion channel embedded in the bilayer was functional. However, the calculated dissociation constant was higher than previously published and further investigations are needed.

To combine the advantages of the protein-tethered bilayers with the advantages of the free-standing bilayers, protein-tethered bilayers were formed on a nanopore filled with a polyelectrolyte multilayer. Protein-tethered bilayers with functional ion channels were prepared and the sensing chip was integrated into a microfluidic device. Electrochemical characterizations indicated little reproducibility and a relatively low resistance of the lipid bilayer. This was probably caused by defects and by an improper seal of the bilayer on the chip. Despite these complications, ion channel activity was recorded electrochemically using a single pore of 800 nm in diameter.

In another series of experiments, protein-tethered bilayers were formed on a supporting layer of polymer brushes, derivatized with nitrilotriacetic acid and covalently grafted on the nanopore wall.
Further characterization of the system remains to be carried out. This supporting material is more stable than the polyelectrolyte multilayer. However, it is as well less versatile as it requires the presence of chemically active groups on the surface to be used for bilayer formation.

In summary, a variety of protein-tethered bilayers was developed in this thesis, using polymers covalently linked to or adsorbed on solid or porous supports. First results indicated that the proteins embedded in such bilayers are functional. In general, these procedures are applicable for any kind of membrane transport protein containing a His-tag. The choice of one system over another depends on the specific requirements of the functional assay, for which the bilayer is being developed.

Functional assays should fulfill several criteria in order to be widely used.

1. **Versatility of the method.** Protein-tethered bilayers are ideal to vary the lipid composition and the protein density. Moreover, buffer conditions can be varied on the two sides of the bilayer.

2. **Cost-efficiency.** In the presented assays, the silicon chip is the most expensive part of the device. However, these chips are solid and can be reused; thus, the cost of the system remains reasonable.

3. **Automation.** The sensing chip was successfully integrated into a microfluidic system, which may allow adequate automation and multiplexing.

4. **Reproducibility.** The low reproducibility of the presented protein-tethered bilayers remains a major drawback, which limits possible applications. The presented methods yield bilayers of relatively low resistance. Moreover, reproducibility of this resistance was limited. It is not clear yet if a higher resistance can at all be achieved for protein-tethered lipid bilayers formed on a polymer-filled pore. It was shown that the low resistance resulted from very small defects. These defects could be inherent to the preparation method.

It is likely that upon changing the supporting material used for the protein-tethered bilayer formation, the electrochemical detection could still be performed. The size of the pore should be decreased to only some nanometers in diameter in order to obtain one molecule of membrane protein per pore and a sufficiently high resistance. Thus, instead of aiming to achieve a high protein density over one pore, pore arrays with a single protein molecule per pore could be used for measurements.

Moreover, such protein-tethered lipid bilayers could be used for optical assays, for which the resistance is not of pivotal importance. Instead, it is important that the signal resulting from protein activity is sufficiently high to surpass the background signal, which results from diffusion of ions and molecules through the defect areas. This approach remains to be investigated.
Future work should focus on optimizing one of the approaches presented in this work so that it becomes sufficiently reproducible, versatile, cost-effective and sensitive for bioanalytical applications in drug discovery and elsewhere.
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