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PERMEATION THROUGH NANOCHANNELS : A NOVEL SYSTEM FOR THE CHARACTERIZATION OF BIOLOGICAL CHANNELS

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ABSTRACT

Nanotechnology is expected to be one of the future key technologies. One approach to realize nano-objects is by self-assembly and involves an understanding of interaction on a molecular level. Numerous examples are found in nature as self-organization on a nanometer scale is a fundamental building principle of life. Here we discuss recent advances for characterization of natural nano channels using a microfluidic setup for high- throughput and parallelized processing, making biological channels accessible for true engineering applications. As an example we focus on the permeation of antibiotics through specific channels, a current problem in drug- screening technology. For that we miniaturize a classical artificial bilayer set-up, automating the formation of
planar membranes to host channels and combining this with microfluidics.

INTRODUCTION

An important aspect of nanotechnology, especially related to localized material transport in soft-matter engineering is fluidics on a molecular level, at which macroscopic equations like those of a Hagen-Poiseuille flow have to be replaced by models accounting for molecular interaction with the confinement [1-12]. Currently drilling nanometer sized holes in thin solid surfaces for use in an aqueous phase is possible by etching, lithography or focused ion beam milling. Another possibility, more interesting in several aspects is utilizing the many examples of nanopores found in nature, using a bottom-up approach in their incorporation into nanoscale systems. Cells are defined by a barrier separating an interior functional space from the outside – these two spaces need to be connected. To control the passage across the separating membrane nature has created a large number of membrane channels which act as highly selective gates for water soluble molecules. Exploiting the selectivity of such natural or bioengineered channels has promising applications for detecting molecules, characterizing molecular interaction, sequencing DNA, or observing peptide folding, and a potential to be utilized as selective gates in self-assembled nanoscale engineered systems [1-12].

A remarkable example for a highly sophisticated system of molecular harvesting and sieving with such selective components are E. coli bacteria. The whole bacterium is about 1 m in diameter and has an outer cell wall fairly permeable to smaller hydrophilic solutes [13,14]. The permeation across the outer membrane is controlled by membrane proteins called porins – a prominent example of these channels for passive diffusion is OmpF, present in about 100 000 copies [15]. The OmpF channel allows the passive passage of substances below about MW 400 Da [14, 16-18].

Nature optimized this channel for the uptake of nutrients, however other molecules otherwise undesirable for the bacterium also benefit from this hydrophilic pathway of entry [17]. For example it has been shown that for beta lactam antibiotics OmpF is the main entry pathway [18]. Currently we investigate the correlation between the chemical structure of antibiotics and their ability to permeate [18]. Understanding the permeation of different antibiotics might allow the synthesis of new molecules with optimized permeation.

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Under stress, e.g. in case of lack of nutrition, the pure diffusion process becomes too slow and the bacteria need to improve translocation of substrates, e.g. through specific binding. For these cases, nature has optimized specific membrane channels. The most extensively studied example of a specific porin is the maltooligosaccharide-specific channel Maltoporin from E. coli [19-22]. Maltoporin forms ion-conducting channels when reconstituted into lipid bilayers. The 3D structure of Maltoporin revealed that the monomer of Maltoporin of E. coli consists of an 18 stranded -barrel with short turns at the periplasmic side and large irregular loops at the outside of the cell [22]. Within this context it is also interesting to note that the bacteriophage Lambda, a virus, recognizes Maltoporin at the outer cell surface [23-25]. In absence of this membrane channel, phage Lambda does not infect the bacteria. Even minor mutations allow the bacteria to defend themselves against virus attacks; the virus itself can, in turn, mutate to restore binding ability, highlighting the flexibility of the recognition system. According to the high resolution X-ray structure the water filled channel is far too small to permit the translocation of the double strain DNA (about 20 Å) [26,27]. The infection mechanism thus must involve one of the following processes: Phage binding will cause a strong conformational change within the Maltoporin or, after binding the phage releases a separate DNA translocation machinery to bring its DNA across the hydrophobic membrane. To date none of these intermediate steps has been observed and the underlying process remains unclear. The higher time-resolution of a miniaturized instrument may for example allow for the detailed observation of such conformational changes and nanoscopic transport phenomena, potentially in combination with fluorescence microscopy. The Maltoporin channel also serves as a model system for the study of enhanced intra-channel translocation through binding in the channel, a still contentious process [28-31].

A typical classical set-up for the characterization of channel forming proteins is based on conductance measurements and shown in **figure 1**. The measurement cell consists of two chambers in insulating half-cells separated by a hole (typically less than 0.1 mm diameter) through a thin Polytetrafluoroethylene film sandwiched between the half-cells. Prior to each measurement the hole has to be pretreated to render it lipophilic by coating it with a >1 µl hexadecane/hexane (1:100 v:v) droplet. After

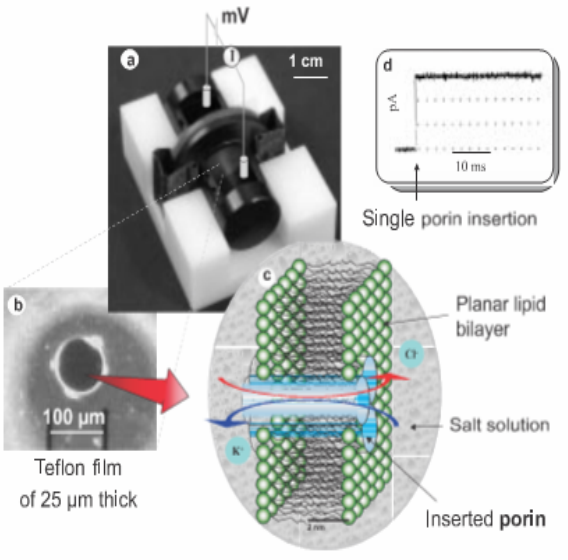

Figure. 1. Classical planar lipid bilayer measurement setup. A planar bilayer is formed across an aperture separating two aqueous solutions. A single porin spontaneously inserts, allowing a constant ionic current. (a) Measurement cell, currently the volume of each cell can be less than 250 µl, (b) view of the Teflon aperture under the microscope, (c) scheme of the bilayer with an incorporated porin and (d) typical current recording of a single porin insertion (adopted from Reference [5]).

allowing for hexane evaporation, each chamber is filled with buffer (for example, 1 M KCl). Solvent-free black lipid bilayers then may be formed according to the classical Montal-Mueller technique [20] by spreading lipids in hexane/chloroform (9:1) across the aqueous buffer. For sake of stability we use 1,2-Diphytanoyl-sn-Glycero-3-Phosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL). This lipid is in the fluid phase over the entire time- and potentially temperature-range of measurement and thus a good matrix for channel forming proteins. After 20 min allowing for evaporation, the buffer level is lowered below the hole level and risen again. Typically after the first or second trial a stable unilamellar membrane is formed. In order to insert single porin trimers within a reasonable time, but to avoid insertion of multiple proteins, a careful balance between the concentration of the protein solution, detergent concentration and buffer volume has to be found. One single porin trimer has to find the membrane and to insert while all others must be inactivated, e.g. by precipitation. Purified OmpF from a stock of 1 mg/ml in 1% n-Octylpolyoxyethylene (octyl-POE) (Alexis, Lausen, Switzerland) was diluted 10^2-10^5 times depending on exact measurement conditions in a buffer also containing 1% octyl-POE. From our own experience in our laboratory the insertion and membrane stability was optimal if smallest amounts (less than 1µl) of protein solution were injected into the chamber.

Membrane current was measured through homemade or commercially available (World Precision Instruments, Sarasota, FL) Ag/AgCl electrodes. One electrode was used as ground and the other connected to the headstage of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), allowing the application of adjustable potentials (typically, 100 mV) across the membrane. The ion current was digitized by an Axon Digidata 1440A digitizer and, controlled by Clampex 10.0 software and analysed by Clampfit 10.0 software (all from Molecular Devices,Sunnyvale, CA). A typical recording shows in **figure 2** the insertion of a single OmpF trimer into the membrane visible by a jump in conductance. Titration with ampicillin causes channel blocking depending on the concentration, corresponding to binding and penetration of the antibiotic molecule through the channel.

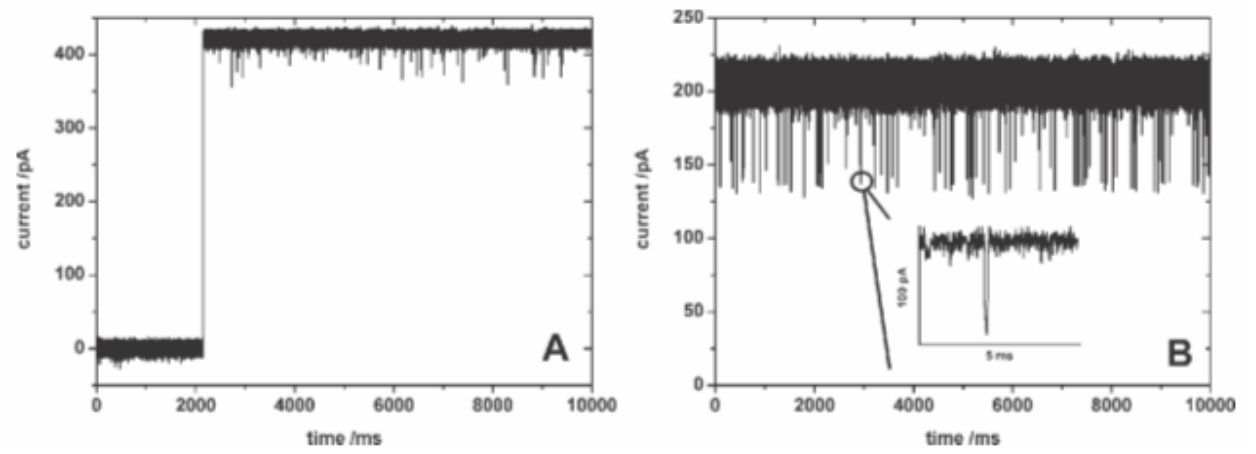

Figure 2. Typical recording of the ion current through OmpF. A: Current recording with +100 mV applied voltage in 1 M KCl, 10 mM TRIS, pH 7, room temperature, showing the sealed bilayer and the single step increase in conductance corresponding to the insertion of an OmpF channel (approximately 4.2 nS). B: Addition of 5 mM ampicillin creates strong ion fluctuations in the channel conductance measured at +50 mV applied voltage. Zooming in with a higher time resolution (B, inset) shows clearly isolated closures of single monomers. These closures correspond to the penetration of Ampicillin molecules into the channel, hindering the ions passing through. Note that the blocking is in the range of ms which is remarkable long for diffusion. Raising the concentration increases the number of blocking events.

AUTOMATED SYSTEM FOR MEMBRANE-CHANNEL ANALYSIS

The above described set-up has a few drawbacks. The most important are a limited time and/or current resolution due to the large capacitance of >100pF caused by the lipid bilayer spanning the Teflon hole, and the instability of the single bilayer with respect to mechanical and electric interference, making the measurements time- and skill-intensive. A straightforward first improvement for both problems is to reduce the hole size. Our own experience shows that significantly reducing the hole-size often results in a hydrophobic blob blocking the aperture – with smaller hole sizes also diminishing resolution gains are rapidly reached partly due to the overall macroscopic geometry and capacitance of the measurement system [32]. Other attempted methods of noise reduction include e.g. the emulation of low-noise patch-clamp recording by forming an artificial bilayer on the tip of a patch pipette [33,34], mostly abandoned because of complicated usage and appearance of artefacts [35]. An early semi-automated approach was based on etching a micrometer sized hole into a silicone-wafer and sealing this aperture with a giant vesicle [36]. Other recently suggested solutions for the automated formation of a solvent-containing BLM were proposed by Malmstadt et al. by solvent extraction through the walls of a microfluidic channel [37,38], and by Funakoshi et al. by contacting two lipid monolayers in an intersection of lipophilic and aqueous phases [39]. These methods yield a micro-bilayer inside a microfluidic channel perpendicular to the channel wall, which cannot be used for electrolyte exchange and substrate titration, cancelling two of the main advantages of the artificial bilayer system. Suzuki et al. developed a bilayer formed on a silicon insulator substrate by pressure-thinning a lipid plug [40], primarily for optical observations. Also, due to the insulated silicon substrate used there is a large shunt capacitance across the membrane increasing current noise. While a similar system has been realized on a polymeric substrate by Sandison et al. [41], in both cases the bilayer is only accessible by a microfluidic system on one side, imperatively needing a trench design for organic solvent removal. Sandison et al. [39] also proposed a system fully accessible from both sides, with controlled delivery of proteins possible [40], where the bilayers formed across a large Polydimethylsiloxane (PDMS) aperture by spontaneous thinning of lipid plugs. However, the size-limitations of the aperture results in increased noise and the lack of reproducibility prohibits automation of this setup.

We combined the advantages of some of the above approaches resolving the time-resolution problem and keeping the original advantages of the free-standing artificial bilayer, by furnishing a microfluidic system for high resolution current recordings, with flow access from both sides of the bilayer and suitable for optical microscopy. We followed a recent approach by Nanion Technologies (Munich, Germany) who offered glass chips with a micro meter sized holes designed for automatic patch-clamp, the so-called Port-a-Patch [42-46]. Based on their commercial system for cell patch-clamping, we have combined the low-noise measurement characteristics of bilayers formed in patch-pipettes, with a novel and simple way of forming small area planar bilayers by giant liposome adsorption. The technique relies on using suction and lipid adsorption to patch a giant unilamellar vesicle onto a glass aperture which subsequently ruptures forming a planar lipid bilayer free-standing over the aperture, stabilized its portions resting on the glass support [46]. The technique has been shown to allow very low noise measurements of alamethicin conductance states [43] and requires very low sample volumes for which two electrolyte drops of several µl placed on the two sides of the glass aperture can be used. We combined this system with a microfluidic flow. As schematically shown in **Figure 3** this allows to have controlled volumes in the µl range, limits evaporation and contamination from the outside [46].

The adsorption and subsequent bursting of giant unilamellar vesicles allows a quick and automatic formation of a planar lipid bilayer across a glass chip. Water-soluble peptides can readily be introduced into the bilayer by simple addition to the buffer flow, and their reconstitution into the bilayer may be followed with time resolution of tens of microseconds on the pA current scale. The reconstitution of a single membrane protein is a more complex scenario. Our protocol for micellar insertion of membrane proteins into traditional

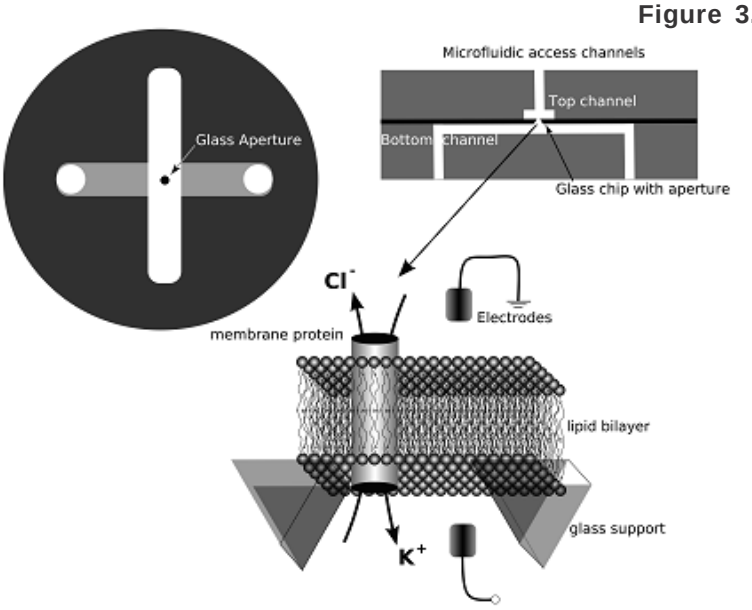

Figure 3. Schematic representation of the miniaturized free standing bilayer bilayer setup. A glass slide (diameter approximately 40 mm) with a micrometer-sized conical aperture is pressed between two polydimethylsiloxane (PDMS) layers. The lipid bilayer is formed by flushing giant unilamellar vesicles (GUVs) through the upper microfluidic channel and applying suction through the lower channel causing the GUVs to be pulled over the hole, adsorb to the glass and burst. Channel conductance is measured after incorporation by two electrodes inserted in the upper- and lower channel respectively.

planar lipid membranes involves detergent-solubilized proteins. In the case of a partially glass supported membrane addition of even small quantities of detergent (down to 1 x 10^-7 mol%) leads to immediate lysis of the membrane.

We have established several methods of protein insertion, broadly relying on the extraction of the detergent responsible for the membrane rupture. During detergent extraction the amphiphilic environment of a lipid bilayer has to be available to the membrane proteins to reconstitute into (similar to micellar insertion into the traditional bilayer). Several methods exist for detergent removal, ranging from simple dilution [47-50] through dialysis [51-56], gel filtration [57-60], ultracentrifugation [61,62], and ultrafiltration [63] to selective precipitation of detergent [64]. However, such procedures require optimization of several experimental parameters for each specific protein and prolong the total time that the membrane protein is exposed to a hydrophilic environment usually leading to precipitation. A recently more widely used method for the removal of low cmc detergents is the selective adsorption of the detergent on hydrophobic resins [65-67], usually Bio-Beads®, ideally suited for our purposes. As detergent extraction, by any of these methods, is not possible in situ after the adsorbed bilayer has been established, we have either endeavoured to insert the protein into the giant unilamellar vesicle (GUV) before adsorption [45,68], or into small unilamellar vesicles [69-72] which were subsequently fused to the adsorbed established bilayer [73-75]. Both of these approaches have advantages and disadvantages. Direct insertion of proteins into the GUVs may be challenging: the protocol needs to be optimized for individual proteins due to the relative instability of GUVs; while different buffers can enhance membrane stability and binding to the glass substrate in the presence of very small amounts of detergent, removing as much detergent as possible is crucial – but too long detergent extraction by dialysis or the application of Bio-Beads® may result in the dissolution of the GUVs. In addition, as the proteins are inserted into the membrane prior to the start of the measurement, the technique is less suitable for the characterization of new channels, as the channel conductance signatures should be known to distinguish proteins from current leaks. On the other hand, the average number of proteins in each GUV can be efficiently optimized by the initial protein concentration, and once fusion has occurred no more proteins insert – also, industrially it is easier to aliquot a standard solution of proteo-GUVs for fusion, making this approach ideal for the parallel-processing of the

same, well-characterized membrane channel on multiple bilayers.

Insertion of proteins into SUVs and their subsequent fusion is relatively easy, standard protocols exist for protein reconstitution. SUVs are also significantly more stable than GUVs, making longer detergent-extraction procedures feasible. As the protein-containing vesicle fuses with the already established and well-sealing membrane, this method can be used for the characterization of new channels with relative ease. While GUVs to efficiently adsorb onto glass surfaces need to be neutrally or positively charged, the SUVs used for fusion, choosing the GUV composition appropriately, may be of nearly any lipid composition – including typical bacterial lipids. The negative aspect of vesicle fusion is that it is difficult to control the number of proteins inserting into the bilayer, as usually several vesicles fuse in rapid succession – buffer perfusion and limited inhibition of vesicle fusion may resolve these issues.

For the direct protein reconstitution into OmpF, GUVs were prepared by electroformation [76] using the protocol in [46] in an indium tin oxide (ITO) coated glass chamber. Lipid solution (5 mg/ml DPhPC + 5% stearylamine and 5% cholesterol dissolved in a mixture of chloroform and methanol) was deposited on the ITO-coated glass surface. After evaporation of the solvent sucrose solution isoosmolar to the saline buffer to be used for later measurements was applied between the ITO slides, and an AC voltage used to swell GUVs. After successful swelling, the vesicles were used directly for the reconstitution of the proteins. Purified OmpF (original concentration 1 mg/ml) in a solution of 1% octyl-POE and 150 mM KCl was reconstituted into GUVs by adding to 250 l of the GUV solution resulting in a final concentration of OmpF of around 10 nM and a final concentration of detergent of around 0.001%. The mixture of GUVs and protein was agitated for 20 minutes at room temperature, followed by the addition of SM2 Bio-Beads® (Bio-Rad Laboratories, Hercules, CA) at 100 mg/ml in the GUVs solution. The mixture was agitated with the Bio-Beads® for 1 hour at room temperature and incubated overnight at 4°C to remove the detergent. Bio-Beads were extracted with gentle centrifugation and the protein containing GUVs used immediately. When kept at 4°C, storage of the proteoliposomes was possible for 2-3 days. The fusion of the obtained GUVs to the glass substrate can be improved by addition of divalent cations into the solution or lowering the pH.

For the SUV fusion with established bilayers either of two general fusion principles were used – fusion of charged vesicles to an oppositely charged bilayer [77-78], and fusion mediated by the binding of Phosphoethanolamine headgroups to divalent ions [79]. Small Unilamellar Vesicles (SUVs) were correspondingly produced from the following lipids and lipid mixtures: DPhPC + 10 mol% 1, 2-Diphytanoyl-sn-glycero-3-Phosphoethanolamine (DPhPE), POPC + 10 mol% DPhPE, POPC + 10 mol% 1, 2-Diphytanoyl-sn-glycero-3-Phospho-L-Serine (DPhPS) and E. coli polar lipid extract (composed of 67% Phosphatidylethanolamine, 23.2% Phosphatidylglycerol and 9.8% Cardiolipin) (all lipids from Avanti Polar Lipids, Alabaster, AL). PC and PE headgroups are neutral, PS and PG headgroups are negatively charged at the pH used.

Liposomes were prepared by drying a lipid solution in chloroform with a stream of nitrogen. The films were left under vacuum for a minimum of 4 hours in order to remove all organic solvent. The resulting dried lipid films were rehydrated using an aqueous buffer of 150 mM KCl, 10 mM HEPES (pH 7.0), to a final total lipid concentration of 10 mM, and the mixture was vortexed to produce large multilamellar vesicles (MLV). The MLVs were subjected to sonication in a sonication bath to produce SUVs. The size distribution was continuously checked by dynamic light scattering from samples removed. After 60-75 minutes of sonication (depending on type of lipid), the size distribution reached a plateau, further sonication not resulting in significant size decrease. It was found that lipid mixtures containing a significant proportion of phosphatidylcholine lipids do not readily form SUVs by sonication alone, but give the same size distribution if prior to sonication are subjected to the following cycle ten times: freezing the vesicle suspension in liquid nitrogen and thawing the sample in a water bath at 40ºC. The vesicles so obtained had a polydisperse size distribution with over 95% of liposomes between 50 nm and 120 nm, uniform across different types of lipid.

OmpF proteoliposomes were assembled by direct incorporation into pre-formed SUVs. A specific volume of 1 mg/ml OmpF stock solution in 1% octyl-POE was added to 250 µl of liposome suspension, and incubated at room temperature with continuous agitation for 20 minutes. The total volume of mixture assured a concentration of octyl-POE lower than the value of its CMC (0.23%). It was found that adding 5 µl of the OmpF stock, corresponding to 0.166 µM concentration of functional OmpF trimer in the final solution (or approximately 60000 lipid molecules per OmpF) resulted on average in 1-2 trimers being incorporated per liposome. The octyl-POE detergent was removed from the proteoliposome solution by one of two methods. The detergent was generally adsorbed onto SM2 Bio-Beads® (Bio-Rad Laboratories, Hercules, CA), at a concentration of 0.4 g of Bio-Beads® / ml. The suspension was agitated at room temperature for 3 hours, after which the Bio-Beads were extracted, and exchanged with the same quantity of fresh Bio-Beads®, followed by an additonal 3 hours of agitation and final extraction. The Bio-Beads® were extracted by gentle centrifugation at 1000 g for 30 seconds, the supernatant kept and the pelleted Bio-Beads® discarded.

For control measurements the detergent was also extracted using dialysis, with 1 ml liposome suspension placed in a partially permeable membrane (Medicell, Liverpool, UK), with a molecular weight cutoff of 12-14000 Da (pore size cca. 2.5 Å), and dialysed at room temperature in 1 l of 150 mM KCl pH 7.0 bathing solution for 4 hours, after which the bathing solution was exchanged and the liposome suspension dialysed for an additonal 4 hours. The dialysed samples gave similar results as the samples subjected to Bio-Beads®. The fusion was achieved either in the presence of Ca^{2+} and Mg^{2+} ions, in case of SUVs containing PE lipids in conjunction with adsorbed bilayers consisting of DPhPC + 10mol% DPhPE, or in the case of SUV containing negatively charged lipids by charge-charge interaction with adsorbed bilayers consisting of DPhPC + 10 mol% stearylamine. In either case, a concentration gradient of the order of 200 mOsmol/kg was applied across the lipid membrane to facilitate vesicle fusion. A typical recording of continuous SUV fusion onto a bilayer can be seen on **figure 4**. Continuous single channel recordings may be achieved by using less active Mg^{2+} ions for fusion, or adding EDTA to extract Ca^{2+} ions

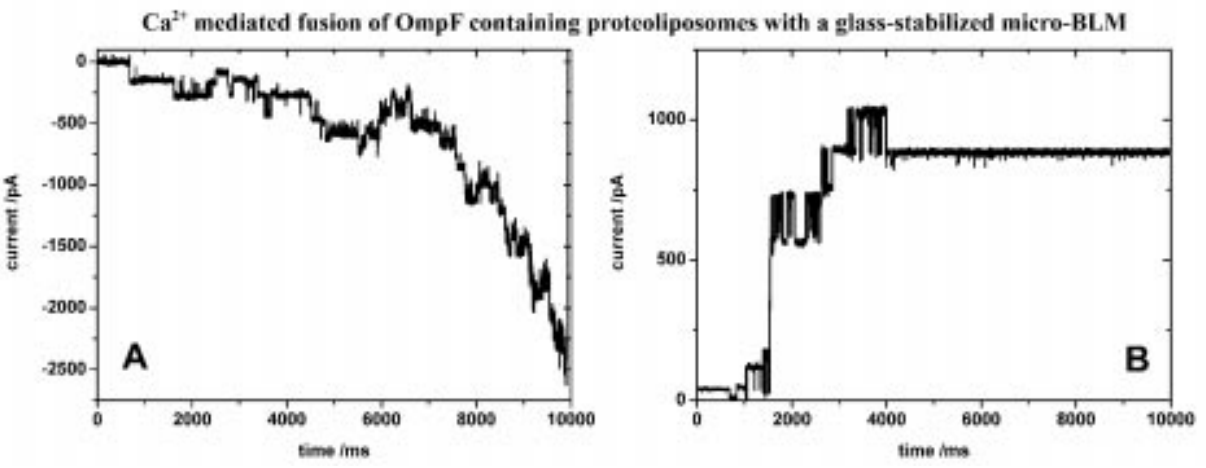

Figure 4. Step-wise increasing conductance through a micro-bilayer produced by the fusion of OmpF containing proteoliposomes to the planar membrane, measured in 150mM KCl (single OmpF conductance corresponding to 0.9 nS). A: Uncontrolled continuous fusion of negatively charged proteo-SUVs (E. coli polar lipid extract) to a positively charged micro-BLM (DPhPC + 10% stearylamine), at -150mV applied voltage. B: Ca^{2+} mediated fusion of proteo-SUVs (E. coli polar lipid extract) to a micro-BLM (DPhPC + 10% DPhPE), measured at +150 mV. 5mM $CaCl_2$ causes rapid fusion events, here modulated and stopped after the insertion of 7 proteins by rapid perfusion and addition of surplus of the chelating agent EDTA.

responsible for binding, in conjunction with rapid perfusion. Between these two methods we are, at least in principle, able to successfully insert nearly any membrane channel that can be reconstituted into proteoliposomes.

CONCLUSION

In general the miniaturisation and integration of electrophysiological setups into microfluidic systems [80,81] imply the following advantages: fast and precise fluid perfusion with low sample and buffer consumption, miniaturisation of the system as a whole to decrease noise and electromagnetic interference problems, and the option of a simultaneous optical observation of analyte translocation in the near future. The capacitance of the bilayer as well as the support holding the bilayer is kept small, due to the 170 micrometer thick isolating glass that separates the two electrolytes which is surrounded by highly insulating PDMS ($4x10^{13}$ Ohm-meter); large porous Ag/AgCl electrodes are integrated into the system closest to the microfluidic channels keeping the access resistance small and reducing noise.

We have shown the realization of a planar lipid bilayer with an over 100 G-Ohm resistance on an approximately 1 micrometer glass aperture clamped between two microfluidic channels. The system enables rapid solution exchange on both sides of the bilayer, and complete external control of the electrophysiological measurement. The stability of bilayer is significantly superior to traditionally built bilayers [82], especially against perturbation by electrical- as well as mechanical forces, and electrophysiological measurement of protein channels can be recorded. This concept can be readily upgrade to fully automate the formation of the bilayer, so that the solvent-free " BLM" can be produced with minimal operator intervention. By building a more complex microfluidic inlet, with several branching channels, solvent exchange and measurements could be fully automated too. Micro-fabrication allows easy and cheap set-up of many parallel experiments on one or on different chips aiming towards minitaturized high-throughput screening.

Overall, we demonstrate a novel engineering solution for the massively parallel characterization of membrane channels in a self-assembled quasi-natural environment by electrophysiological and potentially optical methods. Currently the technique is being explored for antibiotic-screening, however it is likely to be expanded into several other areas.

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