Chip platforms with synthetic lipid bilayers for electrophysiological analyses of pore proteins and extracellular vesicles

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Abstract In this paper, we present microfluidic chip platforms to enable electrophysiological measurements of nanometer-sized extracellular vesicles. The basis of the chip platform is the realization of a synthetic free-standing lipid bilayer spanned within a microfabricated aperture. To allow ion channel current measurements, the background current noise level should be reduced to a minimum. This can be realized by coating microfabricated apertures from silicon, silicon oxide, or silicon nitride with PTFE or Parylene. Three promising chip platform designs are presented. Electrophysiological measurements conducted with these microfluidic systems show gating events of membrane proteins fused into synthetic lipid bilayers.

Keywords Synthetic lipid bilayer · Electrophysiological analysis · Extracellular vesicles · Parylene · Microfabrication

1 Introduction

Synthetic lipid bilayers are an attractive medium to investigate properties of biological cell membrane parts and its components. The membrane of biological cells plays a crucial role in various functions supporting the survival of the cell, a cell population, or even the whole organism [1]. To protect the cell from its environment and to ensure nutrient uptake, a complex system of regulated passive and active pore proteins are incorporated into the bilayer membrane. For example, the global struggle with antibiotic resistant bacteria is strongly related to biological membranes [2, 3]. Pore proteins and efflux-pumps located in the membrane of bacteria are responsible for the translocation of nutrients and blocking of harmful substances [4, 5]. The large variety of pore proteins, the regulation of the open and close stages of the pores, the heterogeneity of bacteria populations, and the short doubling times of bacteria make it very challenging to inhibit the growth of these fast adapting populations. Cell membranes also play an important role in cell-to-cell communication. Extracellular vesicles...
Electrophysiological methods are a tool that can be applied to investigate the functionality of membrane components. Determination of charged particle transport through the membrane helps to further understand cell proliferation and disease development. Electrophysiological measurements recording the current flow of electrolyte or other charged particle translocation over the membrane gives insight about the opening and closing stages of pore proteins.

For mammalian cells (typically between 8 μm and 50 μm in diameter), ion channel current measurements can be conducted using a patch-clamp system [8, 9]. Here, a part of the membrane is patched with a pipette tip filled with an electrolyte solution and clamped with a set of electrodes to observe changes in the current. The diameter of the patched area is in the single micrometer range. By fixing the sample and utilizing micromanipulators, the patch can be realized.

Patch-clamping is quite challenging for much smaller biological samples such as bacteria, extracellular vesicles (EVs), or cell organelles. Other methods are required to investigate the electrophysical properties of these small particles.

One of the key challenges is to realize a controlled environment in which membrane proteins or patches of native bacterial lipids containing the proteins can be investigated. A solution that enables such investigations is by creating synthetic bilayer lipid membranes (BLM) and functionalize them by fusing biological particles (purified proteins, outer-membrane-vesicles (OMVs), or extracellular vesicles) into the BLM [10–12].

There are various microfabricated chip-based methods to realize synthetic lipid bilayer membranes. One option is to construct the bilayer lipids on a solid support [13, 14]. Also, tethered or cushioned BLMs were investigated [15, 16]. Such BLMs, placed on an indium tin oxide electrode covered by a polyaniline and poly acrylic acid film are very suitable to for atomic force microscopy investigations [17]. For electrophysiological membrane investigations, free-standing lipid bilayers are the most promising solution [18].

In this work, microfabricated chip platforms for the study of EVs and membrane pore proteins are discussed.

2 Aperture shape and material selection

The main advantage of microfluidic devices with free-standing BLMs is that the membrane is accessible from both sides. This is not the case for lipid bilayer systems that are on one side supported by a solid. Free-standing BLM platforms allow the use and integration of sensors on both sides of the membrane. They also allow measurements that require asymmetric buffer solutions (electrolyte or pH difference across the membrane). In addition, in this setup it is easy to replace or adjust the buffer solution at each side of the BLM.

The shape of the aperture and selected materials are important parameters for realizing a stable and thin membrane. The membrane should be free of lipid clogs and should not contain an excess of solvent. The presence of solvents such as n-decane and n-hexane in the hydrophobic region of the BLM does stabilize the membrane but unfortunately, also makes the membrane thick. In addition, solvents also negatively act on the functionality or even denature the pore proteins under investigation [19]. High-quality lipid bilayers spanned within an aperture should have a thickness of about twice the length of the selected phospholipid and should be free of excess solvent. For the commonly used synthetic pure lipid DPhPC (1,2-dihytyanoyl-sn-glycerol-3-phospho-choline) the thickness of the bilayer at 20°C is approximately 3.6 nm [20] and the permittivity of the hydrophobic chain approximately 2.2 [21]. Assuming that a lipid bilayer acts as a parallel plate capacitor, a rough estimation of the capacitance of a good quality 100 μm diameter DPhPC bilayer membrane amounts to 42 pF according to:

\[ C_{\text{mem}} = \varepsilon \varepsilon_0 \frac{A}{d} \]  

where \( A \) is the area of the membrane and \( d \) its thickness. A method to determine the quality or thickness is by applying a triangular voltage over the membrane and record the responding current trace.

The material specifications of the microfluidic system are crucial to construct stable BLMs. The system should be compatible with the aquatic based electrolyte and hydrophobic lipophilic nature of the lipid chains. The lipids should only form a (bi)layer over the aperture, and should not stick at the walls of the channels or reservoirs. Among other materials suitable for the fabrication of the aperture, PTFE is the most used material for constructing BLMs. PTFE is hydrophobic and after pre-treatment with n-hexadecane, also lipophilic [22].

There are different methods to construct free-standing BLMs in a micro-aperture. Painting [23], monolayer folding [10, 24], tip-dip [25], pseudo painting/air bubble bursting [26], and inkjet printing [27] are the most common methods. A graphical overview about the painting, monolayer folding, and tip-dip procedure is depicted in [28, 29]. For microfluidic devices with apertures surrounded by a channel structure, the painting and monolayer folding techniques are the most compatible. When there is more space available around the aperture, like in the 3d-printed...
reservoir system presented in [30], the pseudo painting/air bubble bursting method is highly suitable. With this method, stable BLMs can be realized within a few seconds, containing almost no protein hindering solvent (Fig. 1).

3 Microfluidic aperture system designs

In [31–33], the stability of free-standing BLMs painted in a microfabricated tapered shaped silicon-nitride aperture was investigated. Both sides of the silicon-nitride layer and supporting silicon were covered with silicon-dioxide. The apertures have a diameter of 20–30 µm and a thickness of 240 nm. Compared to straight etched apertures, the tapered shape (obtained by isotropic etching) has a smaller aspect ratio (diameter versus side wall length) and therefore, a smaller annulus disc and less stress around the lipid bilayer. It also requires less solvent in the hydrophobic part of the membrane because the annulus is less thick. This makes the tapered aperture better suited for pore protein investigations. With this system membranes were created with a lifetime of 45 h for plain BLMs, and a lifetime of 15 h for BLMs with fused gramicidin pore proteins.

Further iterations of the tapered aperture design were made to reduce the background current noise ([33]; Fig. 2a). This was achieved by adding an insulation layer on top of the silicon layer (not on top of the tapered silicon-nitride). A layer of PTFE, silicon-dioxide, and a combination of the two have been tested. The averaged peak-to-peak current noise was reduced from 5–10 pA for uncoated devices, to 3–5 pA and 1–2 pA for devices coated with PTFE or silicon-dioxide and PTFE, respectively. In order to show the performance of the system, the determination of small amplitude and fast gating events, the peak-to-peak current noise is given instead of the rms current noise. With this device single-channel current events (ions from the
buffer solution migrate through the pore protein by applying a DV voltage over the BLM of fast, less than 0.5 ms, gating alamethicin were successfully recorded (Fig. 2b).

An attractive alternative for insulating PTFE coatings is parylene [30, 34, 35]. In Fig. 3a, a schematic of a microfabricated system including a 10 µm thick parylene film with 45 µm diameter apertures is depicted. This sheet was bonded between mechanically structured PMMA substrates [34]. The functionality of the microfluidic device was tested by recording the ion channel activity of OprM fused with a DPhPC bilayer (Fig. 3b).

The advantage of parylene is that it is hydrophobic and lipophilic, and compared to PTFE does not require pre-treatment steps with solvents before constructing a free-standing membrane. Another advantage of parylene is that the deposition process yields a conformal coating of the microfabricated aperture. In Fig. 4a, a silicon-nitride aperture (thickness 500 nm) conformal coated with 10 µm parylene-C is depicted [30, 36, 37]. These small robust apertures placed in a 3d-printed holder (Fig. 4b,c) with fluid reservoirs and Ag/AgCl electrode pairs are designed to be operated by an automated pipetting robot (9 mm pitch shown in Fig. 4b). Combining standardized high throughput screening technology with functionalized synthetic membrane systems will have practical advantages for membrane translocation investigations.

To demonstrate the performance of a 90 µm diameter aperture coated with Parylene-C, EVs overexpressing the membrane protein OmpF where introduced in one of the fluid reservoirs. In Fig. 5, the successful incorporation of EVs is shown by the occurring current events due to ion transport through open OmpF pores.

4 Conclusions

In conclusion, there are various methods to realize functionalized free-standing synthetic lipid bilayer systems. Microfabricated aperture designs are because of their geometry accuracy, aspect ratio, easy accessibility from both membrane sides, and fabrication reproducibility very suitable for membrane translocation investigations. The material and shape of the aperture play an important role in realizing stable and thin BLMs. Microfluidic based aperture systems show a promising basis to be further developed towards high-throughput automated membrane translocation platforms for extracellular vesicles investigations.

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