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Formation and electrical characterization of black lipid membranes in porous filter materials

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Black lipid membranes (BLMs) are planar lipid bilayers that can be formed in porous supports such as filters. A BLM as a biomimetic membrane has important applications in, for instance, biosensing and drug-permeability assays. In the present study, BLMs were formed in the pores of three different filters to investigate their ability to support the formation of a high-quality lipid bilayer. Poly(vinylidene fluoride) (PVDF), poly(acrylonitrile) (PAN), and polypropylene/polyethylene (PP/PE) filters were utilized as filter supports, and the bilayer formation was monitored *in situ* by capacitance measurements.

The maximum capacitance was obtained for PVDF ($5.34 \mu\text{F} \cdot \text{cm}^{-2}$) versus PP/PE and PAN (2.95 and $0.91 \mu\text{F} \cdot \text{cm}^{-2}$, respectively). Additionally, BLM leakage current measurements by wave deviation provided consistent results. Thickness and compactness of the bilayers were evaluated using pore forming protein (ClyA) insertion. The results show that PVDF with the highest porosity level was the most suitable support for BLM formation. The optimal thickness for the BLM in PVDF was 3–5 nm, where the most efficient protein insertion took place, in comparison with other filters.

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1 Introduction Black lipid membranes (BLMs) are the earliest biomimetic lipid bilayers and were developed by Mueller almost 50 years ago [1]. A BLM consists of two amphiphilic monolayers, of which the hydrophilic headgroups are in touch with the aqueous solution while the hydrophobic tails are shielded in the center of a bilayer sheet [2]. BLMs have different applications in nanotechnology, and are mostly used in biosensors, drug permeability assays, and as model membranes for membrane proteins function studies [2–4].

In the past decades, bilayer formation in a single-hole in a polymer film was developed into a routine procedure [5]. However, increasing attention is recently paid to BLM formation in porous materials due to the growing interest in fabrication of miniaturized devices, e.g., lab-on-chip systems, as well as reducing consumable materials and costs [6–9]. Also, ion channels have been successfully reconstituted using porous membranes [10]. Flaten et al. [11] used liposomal membranes in the pores and on the surface of a mixed cellulose-ester filter. The presence of a membrane structure in the pores was indicated by calcein

permeability assays and by electrical resistance measurements. Using this assembly, the authors were able to study the diffusion of 21 different drugs across the membranes. To study the formation of BLMs in a hydrated teflon filter, Phung et al. [12] formed a bilayer using phosphatidylcholine and cholesterol dissolved in n-octane by the painting method. The presence of a BLM was demonstrated by electrical impedance spectroscopy. They also showed an increase of the current due to adding the pore-formation agent gramicidin. In a separate study, BLM formation in different porous materials was considered; bilayer formation was observed in hydrophobic filters (teflon, nylon, polycarbonate, and silanized silver) whereas a BLM could not be formed in a hydrophilic filter (poly (vinylidene fluoride)) [2]. Apart from the above-mentioned experimental methods, the formation of BLMs can also be monitored by membrane capacitance measurements and analyzing the current-wave forms as a response to a triangular wave voltage. In addition, the quality of BLMs can be assessed using pore-forming protein channels. These protein channels can only be assembled functionally in densely packed BLMs with a 3–5 nm thickness, but they cannot form in disordered bilayers or in lipid mono- and multilayers [13].

The electrical properties of BLMs strongly depend on their quality. Therefore, electrical measurements are an excellent tool for BLM characterization. To study BLM electrical properties, an electrophysiology-setup can be utilized. An electrophysiology-setup consists of a voltage amplifier and a voltage waveform generator. This setup gives the possibility to apply a triangular wave voltage to examine the capacitive properties of BLMs [13]. In this research, the ability to use filter supports as potential porous materials for BLM formation was studied. Therefore, an electrophysiology-setup was used to indicate BLM formation in the filter pores in real-time. The stability and electrical characteristics of the formed BLMs in different filter types were studied. Furthermore, the *Escherichia coli* cytolysin A (ClyA) protein was inserted into the BLM to evaluate the thickness and quality of the bilayers.

2 Materials and methods 1,2-Diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) powder was purchased from Avanti Polar Lipids (Alabaster, AL). Spectroscopic grade pentane (purity $\geq 99\%$) and hexadecane purity 99% were obtained from Chem-Lab (Zedelgem, Belgium) and Sigma-Aldrich (Steinheim, Germany), respectively. Tris-buffered saline (TBS) pH 7.4 consisting of 15 mM Tris-HCl from Sigma (St. Louis, MO) (purity $\geq 99.9\%$) and 150 mM NaCl from Sigma (purity $\geq 99\%$) was used as the electrically conductive buffer to study the electrical conductivity characteristics of the BLM. β -Dodecyl maltoside (DDM) from GLYCON Biochemicals GmbH (Luckenwalde, Germany) and polyacrylamide gels (30%) from AppliChem (Darmstadt, Germany) were used for ClyA purification. Technical ethanol (purity 95%) used for

washing was purchased from Chem-Lab. Different filters, made of poly (vinylidene fluoride) (PVDF) (Durapore, Carrigtwohill, Ireland), poly (acrylonitrile) (PAN) (Ultra, Oceanside, CA), polypropylene/polyethylene (PP/PE) (Freudenberg, Weinheim, Germany) were used as the support materials for BLM formation. During the experiments, the filters were covered with a thin PTFE film (Goodfellow, Huntingdon, UK) with a 180 μm aperture to reduce the surface area of the filters.

2.1 Filter characterization

2.1.1 Contact angle measurements To measure hydrophobicity and hydrophilicity of the filters, water contact angles were measured at room temperature with a Kruss device (DSA 10-MK2, Hamburg, Germany). Contact angle measurements were performed by the sessile drop method [14]. For each filter type, the contact angle was measured at four different spots and averaged.

2.1.2 Morphological study using SEM To visualize the morphology of the filter membranes, a scanning electron microscope (SEM) (RAITH GmbH, Dortmund, Germany) was used. SEM images were taken in high vacuum conditions at 10 kV at room temperature. All samples were coated with a thin gold layer (5–10 nm) to enhance their conductivity using a gold sputter coater (Edwards S150A, Grand Island, NY).

2.1.3 Porosity measurement To measure the specific surface area of each filter, a surface area analyzer (NOVA 2200e, Quantachrome, FL) was used based on the adsorption of nitrogen gas and the Brunauer–Emmett–Teller (BET) theory. Surface area was analyzed for PAN at 200 °C. The porosity of the filters was calculated by Eq. (1) according to NOVA 2200e manual,

$$\text{Porosity} = \frac{P_V}{\frac{1}{d} + P_V} \times 100 \quad (1)$$

where P_V is the total pore volume per unit mass of filter ($\text{cm}^3 \cdot \text{g}^{-1}$) obtained by NovaWin software (version 11.03) and d is the mass density of the filter ($\text{g} \cdot \text{cm}^{-3}$).

2.2 BLM formation For preparing the bilayer, each filter was covered with a teflon sheet (20 μm thickness, aperture with a 180 μm diameter) to decrease the surface area. Teflon was mounted in between the two compartments of the setup, as shown in Fig. 1. Each compartment had a 500 μL columnar chamber with a circular opening at its central wall to make a half cell. A complete cell was assembled by connecting both half cells using two screws and a rubber O-ring seal in between both compartments to prevent liquid leakage. All electrical measurements through the BLMs were performed with Ag/AgCl electrodes, which were fixed in a specified hole in each half-cell compartment. In all the experiments the liquid DPhPC was used because of its ideal bilayer forming properties such as its fluid lamellar

Figure 1 The BLM formation setup consists of two vertical compartments, in between which the teflon sheet and the filter were placed.

phase across a wide temperature range (-120 to $+120^{\circ}\text{C}$) [15, 16] and its resistance to oxidation [13].

To accelerate the BLM formation in filter pores, the filter surface was pretreated with a small drop of hexadecane dissolved in pentane (10% v/v). Pre-treating the filters with hexadecane forms an annulus around the rim of the pores, so it enables the polymeric materials to display an appropriate hydrophobic interface with bilayer. In the next step, each half cell cavity was filled with $500\ \mu\text{L}$ TBS buffer. As soon as the diffusion of electrolytes was detected by measuring conductivity between electrodes, the BLM was formed using the painting method (Fig. 2).

In this method $3.5\ \mu\text{L}$ of DPhPC in pentane ($10\ \text{mg} \cdot \text{mL}^{-1}$) was injected under the teflon opening. Right after adsorption of

Figure 2 Schematic drawing of the painting method, with addition of lipid solution at the bottom of the pores.

the lipid solution into the filter pores, the self-assembly process of the lipid bilayer started spontaneously.

2.3 Electrical recordings To study the electrical properties of the resultant filter-supported planar bilayers, a patch-clamp amplifier (Axopatch 200B, Axon Instruments, Foster City, CA) with a recording band width of $100\ \text{kHz}$ was used to measure ionic currents with Ag/AgCl electrodes [13]. The analog voltage signal generated by the amplifier was converted into digital representations by a 16-bit digitizer (Digidata 1320 A/D converter, Axon Instruments) with a dynamic input range of $\pm 10\ \text{V}$. This results in an amplitude resolution of $0.305\ \text{pA}$ per bit.

Furthermore, all electrical recordings were done in a Faraday enclosure to isolate the current measurements from external radiative electrical noise. The Faraday box had a common ground with the patch-clamp amplifier.

2.3.1 BLM capacitance The quality of the lipid bilayer can be evaluated by measuring its capacitance and leakage current value. Measuring the bilayer capacitance is possible by using a voltage waveform generator, which inputs a triangular-wave voltage (frequency $20\ \text{Hz}$, amplitude $100\ \text{mV}$) into the amplifier. Since for a capacitor the current (I) is proportional to the rate of change of the voltage (dV/dt), a square-wave current output is expected for the bilayers (Fig. 3).

The peak amplitude of this output signal is proportional to the bilayer capacitance (I_C). The input waveform was calibrated by a MCB-1U bilayer model cell (Molecular Devices, Sunnyvale, CA), so $100\ \text{pA}$ corresponds to $100\ \text{pF}$. If the quality of the bilayer is not ideal, leakage current

Figure 3 A typical BLM capacitance measurement scheme. Triangular-wave voltage as an input to the BLM capacitor creates a square-wave current (gray solid line in I_m vs. time plot). Capacitance can be evaluated by the peak magnitude (I_C). Leakage current of the bilayer (I_R) can be determined by deviation of the current wave in the leaky signals from the ideal bilayer (red solid line in I_m vs. time plot).

occurs, which is observed as a current wave deviation from the ideal square-wave output (I_R) [13].

2.4 Nanopore-forming protein test

2.4.1 Preparation of the ClyA protein The thickness and quality of the bilayer were evaluated using a nanopore-forming protein. *E. coli* recombinant cytolysin A (ClyA) was chosen due to its low intrinsic noise forming in the bilayer recordings. ClyA protein monomer was purified using affinity chromatography and oligomerized by adding 5% DDM. Then by running blue native polyacrylamide gel electrophoresis using 4–20% polyacrylamide gels, the bands corresponding to oligomeric ClyA were excised from the gel. Afterwards, the bands were placed in TBS buffer pH 7.4 and stored at 4 °C.

2.4.2 Protein-insertion measurements A range of 0.1–10 ng of oligomeric ClyA was added to cis chamber after many successful forming bilayers for each filter. Henceforth, the bilayer reconstituted with ClyA nanopore, simultaneously. This allows the ClyA to get inserted into the bilayer perfectly. The pores formed by ClyA induces an increase in the recording current. The rate of the current change depends on the applied potential, salt concentration of the buffer [17] and the number of pores. It is essential to emphasize that, without a compact and high quality bilayer (a monolayer or multilayer), no ClyA protein can be assembled in the formed membrane.

3 Results and discussion

3.1 Filter properties Table 1 contains the relevant parameters (water contact angle, porosity, pore diameter and thickness) characterizing the used filters (PVDF, PAN, and PP/PE). The contact angle measurements allow to quantify the hydrophobic or hydrophilic nature of the surfaces.

Hydrophobic materials form contact angles with water between 90 and 180°, while hydrophilic materials create contact angles smaller than 90°. PAN filter showed a contact angle as low as 51.0° indicating its hydrophilic nature as compared to hydrophobic PP/PE, which displayed a contact angle as high as 126.3° (see Fig. 4 and Table 1). The contact angle of water with the PVDF filter could not be accurately determined because of the high water permeability of this filter and hence no droplet could be formed [2]. Despite this inherent difficulty, Moustafa et al. [18] reported a contact angle of a PVDF filter of 69.8°.

Table 1 Properties of filter supports.

filter support type	PVDF	PAN	PP/PE
contact angle with water/°	69.8 [18]	51.0	126.3
porosity (%)	70 ^a	3	38.2 [19]
pore diameter/ μm^a	0.45	0.01–0.1	30
thickness/ μm	100	150	180

^aData provided by the manufacturer.

Figure 4 Contact angle measurement. A droplet of water on (A) PP/PE filter (126.3°) and (B) PAN (51.0°).

The structure and morphology of all the used filters were studied by SEM. Gold-coated scanning electron micrographs of the filters are represented in Fig. 5.

The microscale analysis of the PVDF surface confirmed its sponge-like porous structure (Fig. 5A), with an average pore diameter of $\sim 0.45 \mu\text{m}$. The PP/PE filter shows a fibrous network structure with a pore diameter of $30 \mu\text{m}$ (Fig. 5B). The PAN filter displays a fine ball-shape texture with an average pore diameter of less than $0.1 \mu\text{m}$ (Fig. 5C). This special ball-shape texture makes it an unsuitable support for BLM formation, which can be also seen from the electrical data discussed in the following section.

3.2 Electrical analysis To determine the formation and quality of the BLM in the porous materials, the electrical properties were monitored by electrophysiological techniques. Electrical measurements were carried out to display (mainly) the increasing value of the capacitance upon the formation of the BLMs. As shown in Fig. 6(I.A–I.C), upon the injection of the lipid solution in PVDF, PAN, and PP/PE filters, a clear and significant increase in capacitance can be observed. For instance, in PVDF filter (Fig. 6(I.A)) a rapid (within seconds) formation of a lipid bilayer is indicated. After 60 s the input triangular wave was turned off, which is reflected as the value of the current at 0 pA. On the other hand, to evaluate whether the filter pores are blocked with oil (hexadecane) remnants or solvent, a zapping test with a high voltage electrical shock (1.3 V for around 0.5 ms) was applied. The zapping test breaks the bilayer, resulting in a current increase, whereas in the case that the pores are blocked, the current would remain at zero after zapping. In Fig. 6(I.A), the BLM zapped at 65 s and current increased subsequently. This confirms the existence of BLMs in the PVDF filter. In the PP/PE filter (Fig. 6(I.B)), a stepwise increase in capacitance was detectable during the measurement. The capacitance increased much more at around 180–200 s until the majority of the bilayers were formed in the pores. The formation of the most compact bilayers led to an equivalent capacitance as high as 750 pF ($2.95 \mu\text{F} \cdot \text{cm}^{-2}$) (1 pA corresponds to 1 pF). As indicated in Fig. 6(I.C), the relative capacitance signal for BLMs created in PAN filter pores is depicted within a longer period. In this filter, BLM formation in the pores was indicated with an initial increase in capacitance value at around 100 pF ($0.39 \mu\text{F} \cdot \text{cm}^{-2}$) and it enhanced until 232 pF ($0.91 \mu\text{F} \cdot \text{cm}^{-2}$) in 30 min.

According to the results in Table 2, the highest capacitance value was obtained for the PVDF filter

Figure 5 Scanning electron microscopy images showing the morphology of filters: (A) PVDF, (B) PP/PE, and (C) PAN.

($5.34 \mu\text{F} \cdot \text{cm}^{-2}$), while the capacitance values were smaller for PP/PE and PAN filters (2.95 and $0.91 \mu\text{F} \cdot \text{cm}^{-2}$, respectively). These results indicate that a larger number of BLMs were formed in PVDF pores, which makes it an advantageous BLM support as compared to PP/PE and to PAN. This observation contradicts the earlier findings by Phung et al. [2], who reported the inability of PVDF filters to support bilayer formation. This inconsistency can be explained by the additional hexadecane pre-treatment step performed in this work, as described in Section 2.2.

Figure 6(II.A–II.C) shows the time-dependent square-wave current for BLMs in PVDF, PP/PE, and PAN filters, respectively. The leakage current of the formed bilayers in

the filters was evaluated by measuring the current wave deviation from the ideal square-wave [13]. The bilayer leakage current can be influenced by a variety of parameters, for instance, pore size [13], thickness, porosity, tortuosity, and filter material [2]. Taking all these parameters into account, the leakage current value of the formed bilayers in the PVDF filter was found to be within the range $0.63\text{--}0.95 \mu\text{A} \cdot \text{cm}^{-2}$. The corresponding values for PP/PE and PAN are $0.03\text{--}0.33$ and $0.04\text{--}0.14 \mu\text{A} \cdot \text{cm}^{-2}$, respectively (see Table 2), which are considerably lower than the PVDF value.

In addition to the above-mentioned parameters, the capacitance can also be considered another important factor for leakage current analysis of BLM. In the results presented in Table 3, the leakage current of the bilayer in the PVDF filter increased with an increase in capacitance. This effect reflects that increasing the number of formed bilayers in the filter is directly related to a decrease in the resultant BLM quality.

3.3 Protein insertion The inserted ClyA pores with their preferable orientation in a compact lipid bilayer can be detected based on a slight asymmetrical variation of the measured current–voltage relationship. Upon addition of a ClyA solution with a concentration of $0.1\text{--}10 \text{ ng}$ to the cis chamber containing a bilayer, a stepwise increase in the current value was observed. This step-by-step current increase indicates the insertion of ClyA nanopores into the perfectly formed single bilayers in the PVDF pores (Fig. 7A). Each step is supposed to be an insertion event resulting in the formation of a single channel in the bilayer, which corresponds to the open conductance of a single ClyA channel during a time span. According to previous studies by Soskine et al. [20], a single ClyA nanopore inserted to a compact bilayer has a wide distribution of open conductance of 2.03 nS at $+35 \text{ mV}$, while it is 1.79 nS at -35 mV .

In our experiments the average current increase for a single step was 170 pA at $+100 \text{ mV}$. Therefore, the calculated distribution of conductance related to a single ClyA nanopore was about 1.71 nS . Furthermore, ClyA nanopores were also inserted successfully into the bilayers formed into the PAN filter (Fig. 7B). The average current rise of a single step for ClyA insertion was 193 pA at -100 mV . Thus, the calculated conductance distribution was 1.9 nS , which is in nice agreement with the data of Ref. [20]. Unlike PVDF and PAN filters, such multiple protein ClyA insertions were not observed for the formed bilayer in the PP/PE filter (Fig. 5C). Changes in the current value for the PP/PE filter showed just a single pore insertion in the formed BLMs in the filter pores. In addition, the current behavior of the nanopore was not stable in time. The absence of nanopore formation indicates that the ClyA protein nanopores were not successfully inserted in the formed bilayers in PP/PE filter, which can be explained by the existence of imperfect, low quality single or multiple bilayers.

Figure 6 Real time electrical measurement. (I) Increasing capacitance by applying painting method and the formation of BLM in filters: (A) PVDF filter, (B) PP/PE filter, and (C) PAN filter. Zapping the bilayer after a while was used to ensure that the high capacitance is due to BLMs in the pores. If the capacitance value were obtained due to oil or solvent remnants, it would not break by zapping. (II) Square-wave current for BLMs. The wave-shape deviation from the ideal square wave indicates the leakage current of BLM.

3.4 Effect of filter properties The obtained capacitance values in different filters show a direct relation with the filter porosity. These results can be explained based on the following equation:

$$C = \frac{\epsilon_r \times \epsilon_0 \times A}{d} \quad (2)$$

where C is the capacitance, ϵ_r is the relative permittivity of the medium, ϵ_0 is the permittivity of vacuum ($\epsilon_0 = 8.854 \times 10^{-12} \text{ F.m}^{-1}$), A is the surface area of the membrane, and d is the thickness of the membrane. The larger the porosity, the more surface available for forming BLMs and thus the larger the capacitance. This is consistent with our results, which show that the PVDF filter with the highest porosity,

shows the highest capacitance values, as compared to PP/PE and PAN filters.

Also, the stability of the formed BLM is directly related to both the size and shape of the filter pores. Filters with a smaller pore size provide BLMs with smaller surface area. It is worth mentioning that smaller BLMs are much more resistant to mechanical shock and pressure variations [13]. However, there is an experimental optimal pore size for porous materials using for BLM formation, because very small pores can get blocked with oil or solvent remnants easily. Our results indicate that more stable BLMs were formed in PVDF in comparison with PP/PE and PAN. This fact can confirm that PVDF has an optimal pore diameter (0.45 μm) and pore shape versus PP/PE (pore diameter 30 μm) and PAN (pore diameter of 0.01–0.1 μm).

Table 2 Values of capacitance and leakage current for the BLM formation in filters.

type	max. capacitance/ $\mu\text{F} \cdot \text{cm}^{-2}$	leakage current/ $\mu\text{A} \cdot \text{cm}^{-2}$
PVDF	5.34 ± 0.05	0.63–0.95
PP/PE	2.95 ± 0.04	0.03–0.33
PAN	0.91 ± 0.04	0.04–0.14

Table 3 Correlation between the leakage current and the capacitance in PVDF filter.

capacitance/ $\mu\text{F} \cdot \text{cm}^{-2}$	leakage current/ $\mu\text{A} \cdot \text{cm}^{-2}$
1.57	0.63
3.54	0.79
5.34	0.95

Figure 7 Pore-forming protein insertion through BLMs in (A) PVDF, (B) PAN, and (C) PP/PE filters at ± 100 mV.

4 Conclusions In this study, BLMs were formed as model cell membranes using different porous polymeric materials (PVDF, PAN, and PP/PE) as BLM filter support. Before the formation of the lipid bilayers, the filters were pre-treated with hexadecane, which, unlike earlier trials in literature [2], turned out to be beneficial for bilayer formation in hydrophilic filters (PVDF and PAN).

The BLM formation process in filters and their related characteristics were studied using two approaches: capacitance measurements and protein insertion. In the first approach, for all three filter types the capacitance increased significantly by thinning the bilayer in the filter pores upon BLM formation. In addition, the deviation of current wave shape (I_R) as a response to input triangular-wave voltage from a compact bilayer was used as an important factor for the quality of the bilayers. A larger capacitance corresponds to more numerous BLMs formed in filter pores. By increasing the number of formed bilayers in the filters, the I_R also increased gradually. This increased I_R is directly related to the decreased resultant quality of the BLMs in the filter pores.

In the second approach, the thickness and compactness of the formed BLMs in different filters were evaluated by ClyA protein insertion. ClyA protein has a high tendency to reconstitute in compact and high quality lipid bilayers with a thickness of 3–5 nm. This behavior of ClyA nanopores confirms the existence of a high-quality lipid bilayer in different supports. Adding ClyA protein causes a step-by-step current increase for the BLMs in the PVDF and PAN filters, which confirmed the existence of higher quality and more compact BLMs in comparison with the PP/PE filter.

In summary, we showed a successful strategy to form stable lipid bilayers on a porous hydrophilic polymeric support by adding oil (hexadecane), while the quality and stability of the formed bilayer was evaluated using complementary approaches. Using oil, the formation of a high-quality BLM was facilitated and its stability in the filters was increased. Furthermore, forming bilayers in filter supports helps to reduce consumable materials and costs, which gives flexibility to use novel cheap materials to fabricate miniaturized membrane in biosystems such as lab-on-a-chip devices.

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