Handling of artificial membranes using electrowetting-actuated droplets on a microfluidic device combined with integrated pA-measurements

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Artificial membranes, as a controllable environment, are an essential tool to study membrane proteins. Electrophysiology provides information about the ion transport mechanism across a membrane at the single-protein level. Unfortunately, high-throughput studies and screening are not accessible to electrophysiology because it is a set of not automated and technically delicate methods. Therefore, it is necessary to automate and parallelize electrophysiology measurement in artificial membranes. Here, we present a first step toward this goal: the fabrication and characterization of a microfluidic device integrating electrophysiology measurements and the handling of an artificial membrane which includes its formation, its displacement and the separation of its leaflets using electrowetting actuation of sub- μ L droplets. To validate this device, we recorded the insertion of a model porin, α -hemolysin. © 2012 American Institute of Physics. [doi:[10.1063/1.3665719](https://doi.org/10.1063/1.3665719)]

I. INTRODUCTION

The cell membrane is a lipid bilayer and because of its structure and hydrophobicity, it is not permeable to big or charged particles. Consequently, membrane proteins such as channels or porins are essential for the traffic of such compounds between the intra and extracellular space as well as between cell compartments. It is possible to study these proteins at the single-molecule scale thanks to electrophysiological techniques such as patch clamp: a fragment of cell membrane is isolated at the extremity of a glass pipet and exposed to different solutions. Ionic currents flowing through this membrane are recorded via Ag/AgCl electrodes placed on each side of the membrane and connected to an amplifier. This powerful technique leads to deep and accurate characterizations of ion transport through proteins. However, as natural biological membranes are used as protein support, it is difficult to determine if the effect of any drug applied to the protein studied is direct or not, and this kind of study is limited to the proteins for which a specific inhibitor is known to avoid any mis-interpretation of the results. Consequently, artificial membranes play a key role in fundamental and technological research¹ by raising the number of proteins which can be studied and by facilitating the data analysis, as they provide a user-controlled environment. Traditional techniques, based on the pioneer work of Montal and Mueller² on artificial planar membrane formation, consist in forming a lipid bilayer by deep-coating over a micro-aperture in a hydrophobic sheet separating two perfusable compartments containing Ag/AgCl electrodes. More recently, some miniaturizations for membrane formation setup have been developed. These devices are using microfluidics techniques and the principle is to approach two water-in-oil interfaces covered by a phospholipid monolayer and to bring these interfaces into contact to form a lipid bilayer. Two methods are possible: using continuous microfluidics based on micro-channels³ and using digital microfluidics based on droplets.^{4,5} The traditional issue of the low availability of biological reagents could

find an answer in droplets where sub-microliter volumes are used. The system based on droplets, called DIB (for Droplet Interface Bilayers), has already shown significant realizations in the area of electrophysiology: *in vitro* transcription within droplets,⁶ high-throughput,^{4,7} formation of asymmetric bilayer⁸ for example. Main results on DIB are collected in a recent review.⁹ One of the next challenges for DIB-based research is the full integration within a microsystem to automate and parallelize electrophysiology measurements. In order to automate DIB formation, droplets can be moved using different techniques such as wettability or thermal gradient, surface acoustic wave and electrowetting. Electrowetting enables to move, split, and merge droplets with the possibility of integration within a micro-system.^{10,11} As a first step, an electrowetting-based formation of membrane has been reported.¹² In this work, two droplets are deposited on measurement microelectrodes at an initial distance of hundreds of microns. After deposition, the microsystem is top closed with a ITO (Indium Tin Oxide)/Cytop substrate. Droplets are approached by electrowetting and a membrane is formed in the contact area of droplets. Based on the same physical phenomena, we designed an open microfluidic device that allows to have a full access to droplets from above. This device enables one not only to build membranes but also to handle them meaning to move them and to separate their leaflets. These functions of handling are a significative step toward building a large and dynamical network of membranes. The possibility of building, moving, and splitting membranes enables to sequentially bring different chemicals to an already existing DIB through a second permeabilized bilayer. This microfluidic device also integrates electrophysiological measurements using micro-fabricated Ag/AgCl electrodes. This paper presents a description of these new membrane handling capabilities, as well as a characterization of the microsystem properties and a validation of its use with a transmembrane protein, α -hemolysin.

II. EXPERIMENTAL SECTION

A. Microfluidic device

The device is based on a borosilicate glass plate ($20 \times 20 \text{ mm}^2$, 2 mm thick) where different levels of electrodes, insulator, and surface treatments are performed in clean room using standard micro-fabrication techniques. The Figure 1 represents a top view (left) and a cross section (right) of the device. First, a linear path of twelve aluminum electrodes ($800 \times 800 \mu\text{m}^2$, 100 nm thick, and separated by $30 \mu\text{m}$) is engraved in the substrate using a patterned protection with a photosensitive resist, glass etching with a CHF_3 plasma and aluminum evaporation under an electron beam. These electrodes are devoted to electrowetting actuation of droplets. They are covered with a $2 \mu\text{m}$ thick layer of evaporated parylen C to ensure electrical insulation necessary for electrowetting between droplets and bottom electrodes. On this insulating layer, gold micro-wires, $50 \mu\text{m}$ wide and 100 nm thick, are deposited in the parylen using similar procedures. Each of the measurement electrodes consists in two microwires connected on both of their extremities. These wires have two functions: maintain a controlled potential for electrowetting and enable electrophysiological measurements between droplets. On the top of the

FIG. 1. Top view (left) and cross section of the microfluidic device. Only the two center electrowetting electrodes (out of twelve) are represented in this cross section.

device, a 30 nm thick layer of amorphous fluoropolymer AF1601 (1 wt. % in FC40) is spin-coated to make the surface hydrophobic except at the extremity of each gold wire where squares are opened in the hydrophobic layer by lift-off. On these squares, two drops of Ag/AgCl mixture (45%–55%, engineered conductive materials) are manually deposited using a 50 μm diameter wire under microscope to form two micro-pads ($200 \times 200 \mu\text{m}^2$).

B. Integrated electrical measurements within droplets: Principle, interfacial resistance and accuracy

Inspired from electrophysiology techniques, the Ag/AgCl mixture is used to create a bridge between electronic conduction within gold micro-wire and ionic conduction in the electrolyte (Eq. (1)). On the positive voltage pad, the anionic oxidation consumes silver metal atoms (Ag) and on the negative pad, the cationic reduction consumes silver salt.

The microsystem is electrically reversible by construction and the current flow can be reversed by only changing the polarity of the voltage. To measure a charge transfer within a protein using this integrated measurement, we need to characterize the electrical behavior, in DC terms, of the gold-Ag/AgCl-electrolyte interface. A DC voltage between 4 and 20 mV is applied to gold micro-wires while Ag/AgCl pads are connected through a 150 mM KCl droplet. The flowing current is measured using a 1 k Ω resistance (Figure 2, on the left). Typical results are plotted on the right of Figure 2. From current data, we can estimate a resistance of 27 k Ω in this experiment. This resistance is the combination in series of three resistors: two $R_{\text{Ag/AgCl}}$ corresponding to the Ag/AgCl pads and the third one R_{KCl} to the KCl electrolyte. One can estimate the last value $R_{\text{KCl}} = \frac{l_{\text{KCl}}}{\sigma_{\text{KCl}} \times A_{\text{KCl}}} \approx 10 \text{ k}\Omega$ with $l_{\text{KCl}} = 800 \mu\text{m}$ (distance between pads), $A_{\text{KCl}} = 200 \mu\text{m}^2$ (pads area), and $\sigma_{\text{KCl}} = 2 \text{ S/m}^{13}$. The value of the Ag/AgCl pads resistance $R_{\text{Ag/AgCl}}$ is then in the range of 10 k Ω in this experiment. This value strongly depends on the microsystem from tens of k Ω to several hundreds of k Ω corresponding to a minimum conductance larger than 1 μS . In any case, this value is very large compared to a single protein conductance which varies from 0.5 pS (gramicidin A (Ref. 14)) to 500 pS (Nuclear pore complex¹⁵) while channels and porins usually have a conductance of hundreds of pS (Refs. 14, 16, and 17) at physiological salt concentration.

For lower current measurements (below nA), a patch clamp amplifier (RK300, Biologic corporation) is used. This amplifier enables us to measure sub-pA current with a bandwidth of 10 kHz. To limit electromagnetism environment, the microfluidic device is placed in a faraday cage and, in this configuration, raw data of current has a resolution of $\pm 3 \text{ pA}$ (data of pA-current resolution measurement are shown on Figure 6 on the left).

FIG. 2. On the left, outline of resistance measurement setup. On the right, the current is measured as a function of the applied voltage. From the linear fit of voltage-current data, a 27 k Ω resistance is measured.

FIG. 3. 350 nL DPhPC droplets in hexadecan are moved by electrowetting. The droplet on the right is moved to approach the droplet on the left under a voltage $U_{ew} = 20$ V. Between each image, from A to D, the right droplet is translated from an electrode to its neighbor on the left. The displacement is $800\ \mu\text{m}$, the distance between centers of electrodes. The droplets diameter is $800\ \mu\text{m}$. On image E, a membrane is formed.

III. MEMBRANE HANDLING AND CHARACTERIZATION

A. Droplet motion, membrane formation and displacement, leaflets separation

A suspension of large uni-lamellar vesicles (LUV) is prepared by extrusion of a 2 mg/mL solution of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC, Avanti Polar Lipids) in KCl 150 mM through a polycarbonate membrane having pores of 100 nm diameter. Two 350 nL drops of this suspension are deposited on the microfluidic device immersed in a bath of hexadecan using a micropipette. A stabilization time of 10 min is necessary to form a dense lipid monolayer at the water/hexadecan interface to prevent droplets to merge after bringing them into contact.⁴

Droplets can be moved using electrowetting actuation under an AC voltage $U_{ew}(t) = U_{ew} \sin(2\pi ft)$ where the frequency f is 3 kHz . The necessary voltage to move a droplet is in the range of tens of volts. They travel linearly on the 12 electrode path by applying a voltage on electrodes and can be approached as shown on Figure 3. After approaching droplets in a close position as in Figure 3(d), droplets relax deformations due to displacement by draining the hexadecan film between interfaces during tens of seconds¹⁸ and after, a steady state is reached. A membrane is formed within the contact area between droplets (Figure 3(e)). The membrane is stable for hours (typically 4 h) before droplets merge.

Once the membrane is formed, one can handle it by electrowetting. Two ways of handling are possible: the membrane displacement and the separation of phospholipid leaflets. The top of Figure 4 represents an experiment of membrane displacement and the bottom, the corresponding stages of voltage sequence. One of the droplets involved in the membrane is pulled onto the neighbor electrode and, the other one being free (no voltage is applied to its electrode), it follows the first one. However, if the second droplet is maintained on its electrode by application of an electrical potential, the membrane leaflets separate while the droplets move apart. Figure 5 shows such a separation (top) and the corresponding voltage sequence (bottom).

FIG. 4. A membrane is formed between droplets and this membrane is moved by electrowetting on the top part of the figure. The voltage sequence to move the membrane is represented on the bottom. U_{ew} is 14 V .

FIG. 5. The membrane leaflets are separated by electrowetting actuation of droplets on the top of the figure. The voltage sequence of this splitting is represented on the bottom. U_{ew} is 20 V. Dotted lines remind the previous droplet position.

B. Membrane characterization

To characterize the formation of the membrane, we used integrated electrical measurements. For low voltage, typically below a few hundreds of mV to prevent electroporation of the membrane,¹⁹ the electrical characteristic of the membrane is a dielectric and a membrane separating two saline solutions has the behavior of a capacitor. To measure the bilayer capacitance, a low frequency voltage ramp (50 mHz) is applied between the droplets via the Ag/AgCl pads and the corresponding current is recorded through the patch clamp amplifier. The Figure 6, on the left, shows the raw data of current recorded while applying a voltage ramp of 50 mV amplitude and of $2 \times 50 \text{ mV}/10 \text{ s} = 10 \text{ mV/s}$ slope. The current I shows symmetric steps corresponding to the charge and discharge of a capacitor under linear voltage and values of these steps are plotted as a function of the voltage ramp slope $\frac{dV}{dt}$ (Figure 6 on the right). The capacitance C is calculated using the proportionality between the current and voltage ramp slope in a capacitive system $I = C \frac{dV}{dt}$. A capacitance of 500 pF is found and, thanks to an optical estimation of the membrane area to $0.08 \pm 0.008 \text{ mm}^2$, a specific capacitance of $0.6 \pm 0.1 \mu\text{F}/\text{cm}^2$ is obtained. This value is in good agreement with reported values for supported²⁰ and suspended DPhPC bilayers.^{3,21–23} Moreover, the specific capacitance of a DPhPC bilayer can also be calculated using the physical properties of DPhPC bilayer: a thickness of $d = 3.5 \text{ nm}$ (Ref. 24) and a dielectric constant of $\epsilon_{\text{DPhPC}} = 2.2$.²⁵ This calculation leads to a specific capacitance of

$$\frac{\epsilon_0 \epsilon_{\text{DPhPC}}}{d} = 0.55 \mu\text{F}/\text{cm}^2$$

also in good agreement with our measurements.

We observed a slight slope of the current plateaus (Figure 6, left), which reveals a resistance of 70 G Ω rather than a fully capacitive behavior. This resistance includes the combination in parallel of the resistance due to unperfected insulation in the set up (300 G Ω) and the

FIG. 6. Raw data of current are presented on the left for a slope voltage of 10 mV/s. Current I is plotted as a function of voltage slope on the right. The behavior is linear.

FIG. 7. Current registered during α -hemolysin insertion within the membrane. The current shows steps of 15 pA and each step is the signature of a single protein insertion within the membrane.

intrinsic resistance of the membrane R_m . This value can be calculated using combination in parallel which gives a value of $R_m = 91 \text{ G}\Omega$ in good agreement with reported values for DPhPC bilayer.²⁵ The combination resistance is far larger than the resistance of the pipet seal in a classical patch clamp experiment (typically $1 \text{ G}\Omega$ (Ref. 26)) and than the one reported for a similar micro-device ($2 \text{ G}\Omega$ (Ref. 12)). The difference with the last value could be explained by a better electrical insulation in our microfluidic device due to the absence of ITO cover plate bridging droplets.

C. Functionality of the microsystem with transmembrane protein

Concerning the electrophysiological capabilities of the device, we needed to validate that the membrane is functional, meaning that transmembrane proteins can insert and be active within this membrane, and that we can measure this activity with the integrated electrodes. Therefore, we used α -hemolysin, a standard transmembrane protein known for inserting spontaneously into lipid bilayers, forming a pore through which ions can cross the membrane following an electric field.⁴ Two 350 nL droplets of DPhPC LUV suspension (MES 100 mM, pH 6.5, and NaCl 150 mM) containing $5 \mu\text{g/mL}$ of α -hemolysin are deposited on the device and brought into contact to form a membrane. A 50 mV DC voltage is applied between the droplets via the gold electrodes and the ionic current flowing through the membrane is measured and plotted Figure 7. On this figure, we can measure current steps of 15 pA. Each step corresponds to the insertion of a single protein within the membrane.⁴ From these steps, one can calculate a conductance of 300 pS for a single α -hemolysin in agreement, after a correction due to different ion concentrations, with reported values in planar bilayer²⁷ and in DIB system.⁴

IV. CONCLUSION

We presented here a micro-device for membrane handling and integrated electrophysiology measurement in artificial membranes. We can approach droplets to form a lipid bilayer, move them together while they are involved in a membrane and separate them to split the membrane. This gives us a large degree of freedom in the design of dynamical membrane networks. We electrically characterized the artificial membrane formed using this device and obtained results consistent with literature data and estimation. The currents flowing through a protein inserted in

the membrane can be measured. The next challenge is to parallelize experiments by coupling several of such devices to miniaturized amplifiers.²⁸ Together with the small amount of protein needed for such experiment, thanks to the sub- μL volume of the droplets, this would open the way to high-throughput electrophysiology studies and to electrophysiological drug screening, knowing that membrane proteins are both an important therapeutic target and the pathway for drugs to penetrate the cells.

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