Lipid Membranes

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Monitoring and Quantifying the Passive Transport of Molecules Through Patch–Clamp Suspended Real and Model Cell Membranes**

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Abstract: *Transport of active molecules across biological membranes is a central issue for the success of many pharmaceutical strategies. Herein, we combine the patch–clamp principle with amperometric detection for monitoring fluxes of redox-tagged molecular species across a suspended membrane patched from a macrophage. Solvent- and protein-free lipid bilayers (DPhPC, DOPC, DOPG) patched from single-wall GUV have been thoroughly investigated and the corresponding fluxes measurements quantified. The quality of the patches and their proper sealing were successfully characterized by electrochemical impedance spectroscopy. This procedure appears versatile and perfectly adequate to allow the investigation of transport and quantification of the transport properties through direct measurement of the coefficients of partition and diffusion of the compound in the membrane, thus offering insight on such important biological and pharmacological issues.*

Transport of active molecules across biological membranes is a central issue for the success of many pharmaceutical strategies. However, despite this crucial importance, most approaches rely on the measurement of partition coefficients between water and hydrophobic solvent models. Furthermore, this thermodynamic method is by definition blind to any kinetics. Conversely, transport through artificial membranes is a mature field, but most of the methods involved cannot be transposed to real cellular membranes. It is the scope of this work to establish and test the concept of a new method for investigating passive transport of molecules through real biological membranes of any drug-targeted cells.

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To date, the most popular approach to form suspended planar lipid bilayers remains the "painting" technique.[1] Under these conditions, the resulting artificial membrane (typically from 50 to 500 μm in diameter) may, however, incorporate solvent molecules which may alter some of the membrane's properties. Solvent-free suspended lipid bilayers can be obtained using the Langmuir–Blodgett technique,[2] but the absence of a solvent–lipid annulus at the aperture edge destabilizes the artificial membrane. To overcome this drawback, nanometer-sized apertures have been used to paint stable suspended membranes over anodized porous alumina,[3] porous polycarbonate membranes,[4] and glass nanopores.[5] These configurations are notably useful for single ion-channel recordings after ion-channel proteins are inserted across the artificial membrane. Indeed, small apertures decrease capacitance changes and allow precise measurements of resistive variations when ions or molecules pass across the protein channels.[5] Nevertheless, they are not well adapted to electrochemical detection of molecular species crossing the membrane considering the drastic changes incurred by the nanovolume liquid entrapped between the lipid bilayer and an electrode due to the necessity of a Faradaic process.[6] On the other hand, the painting method cannot be adapted to investigations of real cell membranes.

In terms of membrane stability and electrochemical detection, the formation of lipid bilayers, suspended at glass pipettes with diameters in the range of 0.5 to 5 μm, by the tip–dip method[7] is a good compromise. However, bilayers obtained under such conditions are not always solvent-free and the access to the pipette tip is not easy, it being located under a lipid monolayer. Again, this method is not adapted for the suspension of real cell membranes.

Alternatively, the patch–clamp technique (that enables the study of single or multiple ion channels in cells)[8] allows sealing a membrane detached from real cells at the tip of a glass micropipette. This technique is commonly used for electrophysiological investigations in biology, particularly for deciphering vesicular exocytotic mechanisms or characterization of ionic channels.[8] However, to our knowledge, this approach has not been popular for investigating artificial membranes. One such investigation was reported in 1982 by Tank et al. who showed that an isolated patch of artificial membrane could be sealed onto a glass micropipette rim by ripping off a solvent-free proteoliposome in solution.[9] Furthermore, the inside-out patch mode was scarcely attempted with protein-free liposomes. Considering that proteins and cholesterol significantly increase the rigidity of cell membranes,[10] patching pure lipid liposomes remains

challenging and may explain the absence of many reports in that field. Besides, the characterization of artificial suspended bilayer membranes is traditionally performed indirectly by insertion of channel-active peptides such as alamethicin or gramicidin to establish the bilayer character of the membrane through their ability to promote ion exchange across the lipid bilayer.

In this work, we successfully used the "inside-out" patch–clamp configuration to prepare not only real suspended cell membranes, but also to form both solvent- and protein-free lipid bilayers suspended at micro-sized glass pipette tips. More importantly, combination of the patch–clamp method and amperometric detection of redox probes allowed the monitoring of molecular fluxes across these two types of membranes and offered quantitative insights on this important biological and pharmacological issue.

Detection of ferrocenemethanol ($FcCH_2OH$) passive transport across a biological cell membrane was first investigated. Detection of transported molecules was performed by amperometry using a carbon-fiber microelectrode positioned in front of the membrane. Indeed, the small double-layer capacitance of the microelectrode allows a suitable time response (millisecond or less) for monitoring the passage of redox probes.[11] Thus, a patch–clamp micropipette (see Supporting Information) containing a ferrocenemethanol solution could be moved, at a rate of $1\,\mu m\,s^{-1}$, in front of a carbon fiber ultra-microelectrode (UME; ca. 10 μm diameter) whose potential poised at $+400$ mV vs Ag/AgCl corresponded to the plateau of the redox probe electrochemical wave. Without any patched membrane, a steady-state current for $FcCH_2OH$ oxidation was observed (32.5 ± 4.2 pA; $n=3$) as soon as the pipette was in the vicinity of the electrode (electrode/membrane distance <1 μm) reflecting the spontaneous steady-state diffusion of $FcCH_2OH$ through the open micropipette tip. When the same type of pipette was first used to excise a patch of a RAW264.7 macrophage membrane ("inside-out" configuration, see Supporting Information) the $FcCH_2OH$ outflow drastically decreased (2.3 ± 1.2 pA; $n=2$), showing that $FcCH_2OH$ could cross the cell membrane at a much lower rate because of the barrier preventing its diffusional leakage (Figure 1). Additionally, 23 s after the beginning of the experiment, increasing the electrode–micropipette tip distance resulted in both cases into a drastic current decay as a result of the decrease of the collection and widening of the thin-layer cell width.[12]

This demonstrated the method validity and performance. However, to investigate the quantitative aspects of the method it was suitable to focus onto model (artificial) membranes since they are best characterized. For this reason we investigated membrane patches excised from pure phospholipids giant unilamellar vesicles (GUVs) prepared by electroformation (Supporting Information).[13] 1,2-diphytanoyl-sn-glycero-3-phosphocholine lipid (DPhPC, see Supporting Information) is one of the most commonly used lipids for electro-physiologically significant measurements as well as for experiments involving peptide–lipid interactions.[14] Indeed, choline head-groups are among the most abundant ones encountered in mammalian cell membranes,[15]

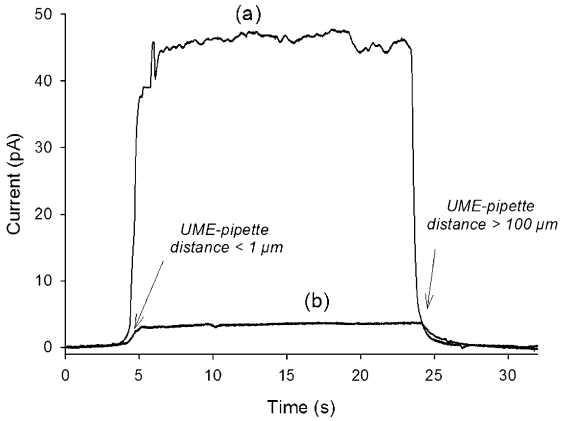

Figure 1. Amperometric recording of the ferrocenemethanol redox probe outflow from a patch micropipette to the surface of a carbon fiber ultra-microelectrode (UME; $C=1$ mmol L^{-1} in PBS; $E=+400$ mV vs. Ag/AgCl): without any membrane at the tip of the micropipette (a); with a patch of macrophage membrane in "inside-out" configuration (b).

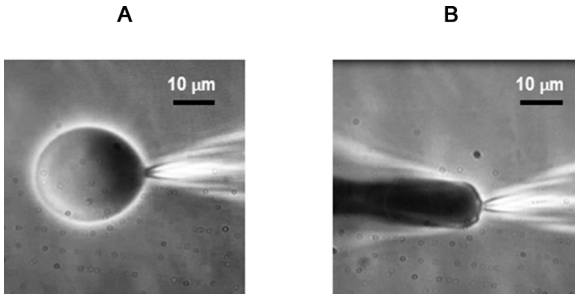

Figure 2. A) Phase-contrast microscopy images showing the formation of a giga seal between the pipette and a GUV; B) typical amperometric configuration showing a carbon-fiber microelectrode (left) positioned in the vicinity of the artificial patched membrane detached from the GUV in (A) and held by the micropipette (right).

and DPhPC exhibits high mechanical and chemical stability thus providing relevant model bilayers.

The tip of the patch micropipette was pressed against the GUV allowing the formation of a giga seal (Figure 2 A). The micropipette was then quickly withdrawn from the vesicle, ripping off the sealed patch of lipid bilayer attached to the micropipette (see details in Supporting Information). The success of the procedure was about 90% as checked by recording typical current responses (Supporting Information).[8,16]

The same procedure was performed on another phospholipid with choline head-groups (dioleoylphosphatidylcholine; DOPC) as well as on DOPG (dioleoylphosphatidylglycerol; DOPG) (See Supporting Information).

The patch success rate of pure DOPC vesicles was only 15% (Figure 3 A). This poorer success compared to DPhPC may stem from weaker lateral mechanical stability arising from the absence of methyl groups on the hydrophobic backbone. In accordance, Figure 3 A shows that patch success increased with the amount of cholesterol which improves the mechanical stability of cell membranes.

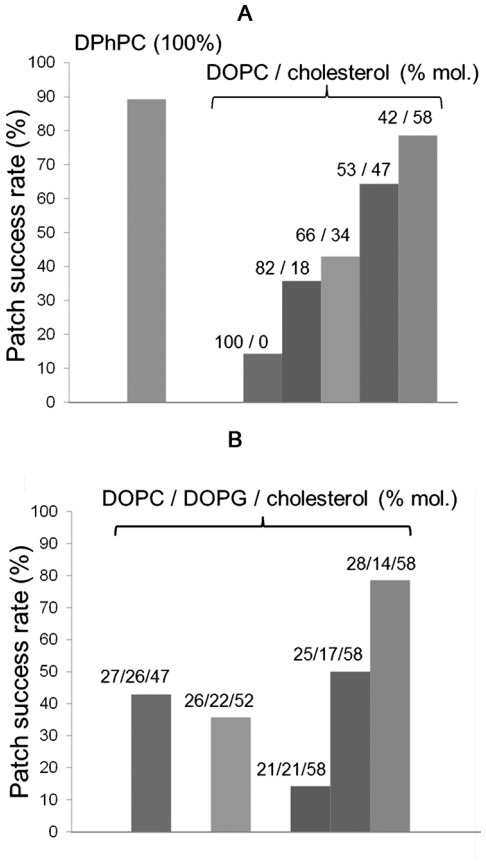

Figure 3. A) Patch success rates obtained for pure DPhPC vesicles or from DOPC vesicles containing increasing amounts of cholesterol; B) Patch success rates obtained from vesicles composed of various DOPC/DOPG/cholesterol mixtures. Success rates were determined using series of 20 trials for each configuration.

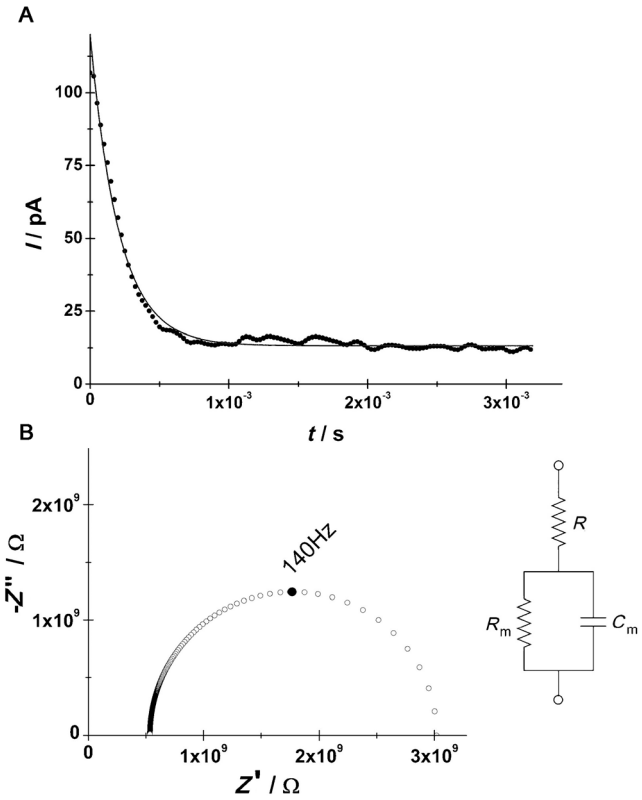

Figure 4. A) Current response to a 20 mV potential step (●) applied with two Ag/AgCl electrodes on each side of the membrane; fit with a single exponential decay function (solid line). B) Impedance diagram provided by applying a fast Fourier transform to the regression curve in (A) and corresponding circuit.

Dioleoylphosphatidylglycerol (DOPG) was of interest since it bears a negatively charged head-group. However, in contrast with DPhPC and DOPC, it was impossible to patch pure DOPG vesicles, presumably because of the electrostatic repulsions between the negative DOPG heads and the negatively charged surface of the glass patch–pipette.[17] Importantly, successful patch-rates were achieved from vesicles made from various DOPG/DOPC/cholesterol mixtures (Figure 3B). Starting with a cholesterol proportion of 58%, the patch success rates increased with the ratio DOPC/DOPG, the best result (ca. 80% success) being obtained for a ratio of 2:1. Conversely, approximately 50% of cholesterol was optimum for vesicles having the same amount of DOPC and DOPG but the average success rate was lower, ranging between 40 and 45% (Figure 3B).

The quantitative validation of the method was thus performed with DPhPC. First, the suspended lipid bilayers were characterized by electrochemical impedance (Figure 4). The evaluation of the capacitance was made by comparison to blank experiments (i.e. without membrane) involving only the resistance of the system (electrolyte plus capillary of about 1.5 μm in diameter) and the stray experimental capacitances (electronics, wires, …). The patch–clamp measuring instrumentation could then eliminate these parasitic contributions (about 5 MΩ and 1 pF, respectively) by using the blank response as a feedback signal in subsequent experiments involving patched membranes. Thus, the current responses monitored for a potential step reflected only the capacitance C_m and the resistance R_m of the membrane patches. Figure 4A validates this methodology by showing that the current response involves a single exponential decay. The Fourier transform of the signal (Figure 4B) in presence of the membrane shows a single time constant indicating that the system behaves as a capacitor in parallel with a resistor as shown by the electrical equivalent circuit presented in Figure 4B.[18,19] From the time constant (= 1/140 Hz) and the amplitude of the capacitive loop, the membrane resistance R_m and the membrane capacitance were calculated (a few GΩ and 0.2 ± 0.1 pF) in agreement with the expectations for such a membrane.[20]

To explore passive transport across suspended DPhPC membranes patches, we investigated a series of redox probes so that their fluxes across the membrane could be monitored by amperometry as performed above for the macrophage membrane-patch. This was achieved by patching a pure DPhPC vesicle with a micropipette previously filled with the electroactive substance. Then, the pipette was brought into close proximity (< 1 μm) of the carbon-fiber microelectrode, whose potential was set at a value located on the plateau of the probe redox wave. This ensured a precise and accurate

detection, in real time, of the substrate flux crossing the lipid membrane (Figure 2B) similarly to the electrochemical monitoring of single-cell secretion.[21] This amperometric method was tested towards representative redox molecules, such as ferrocenemethanol, hydroquinone, benzoquinone, $[Fe(CN)_6]^{3-}$, and $[Ru(NH_3)_6]^{3+}$ (Supporting Information). Figure 5A summarizes the fluxes percentages detected for these various redox probes in the presence of suspended DPhPC lipid membranes relative to their values in the absence of a membrane patch. As expected, electrically uncharged molecules fluxes were detected, being smaller than in the absence of the membrane thus reflecting the reduced transport kinetics. Conversely, no flux could be detected for charged probes in agreement with other measurements at single cells.[22] Benzoquinone was found to cross the DPhPC lipid membrane more efficiently than hydroquinone (8.2 vs. 0.6%) in agreement with its higher lipophilicity. Figure 5B shows a typical current/time response obtained for a probe able to cross the suspended membrane placed sequentially at two distances between the 10 μm carbon fiber electrode and the patched pipette. In each case, a stationary current regime was rapidly achieved when the distance between the electrode and the pipette was less than 5 μm owing to the establishment of a steady-state diffusion regime with a collection efficiency nearly quantitative.[23] Conversely, the current decreased sharply upon increasing the pipette–electrode gap thickness reflecting the decreasing collection efficiency.[24] Such current changes were clearly not observed for charged molecules unable to cross the membrane (Figure 5C). Nevertheless, in all cases, a huge current was detected when the membrane was ruptured by applying a high potential to it (±500/±1000 mV range) used as a control after each experiment (see (3) in Figure 5B and C).

The redox probe detection at the microelectrode was investigated as a function of the gap size μ, both experimentally and theoretically, to access important physicochemical and physiological parameters related to the diffusion coefficient of the compound in the membrane (D_m) as well as its partition coefficient between the membrane and the solution (κ). The inverse of the detected current was found to increase linearly with μ (see Supporting Information). This linear variation was in full agreement with the relationship given in Equation (1) (see Supporting Information for the derivation

$$\frac{1}{i} = \left(\frac{1}{nFD\pi R_m^2 C^b}\right)\mu + \left(\frac{(R_m \sin\theta) + (Dl/KD_m)}{nFD\pi R_m^2 C^b}\right) \quad (1)$$

of this relationship; D, D_m and κ are defined in the text; see Supporting Information for the definition of all other parameters). The slope and the intercept of the curve allowed the determination of the ferrocenemethanol diffusion coefficient in solution ($5.52 \pm 0.86 \times 10^{-6}$ cm^{2}s^{-1}) and of the product κD_m ($1.63 \pm 0.14 \times 10^{-9}$ cm^{2}s^{-1}), respectively. The redox probe diffusion coefficient value was thus found in good agreement with reported ones in the literature.[25] This validates therefore the theoretical model used to derive Equation (1).

In conclusion, the inside-out patch–clamp technique was successfully implemented to prepare living cell patches as well as solvent- and protein-free lipid bilayer membranes by excision from a living macrophage membrane or from giant unilamellar vesicles. Importantly, this original preparation of suspended lipid membranes was successful not only with usual lipids, such as DPhPC, but also with others (DOPC, DOPG) reputed to be more difficult to yield stable film over glass micropores of large radii. Although in these cases cholesterol needed to be added to improve the patches stability. Note that

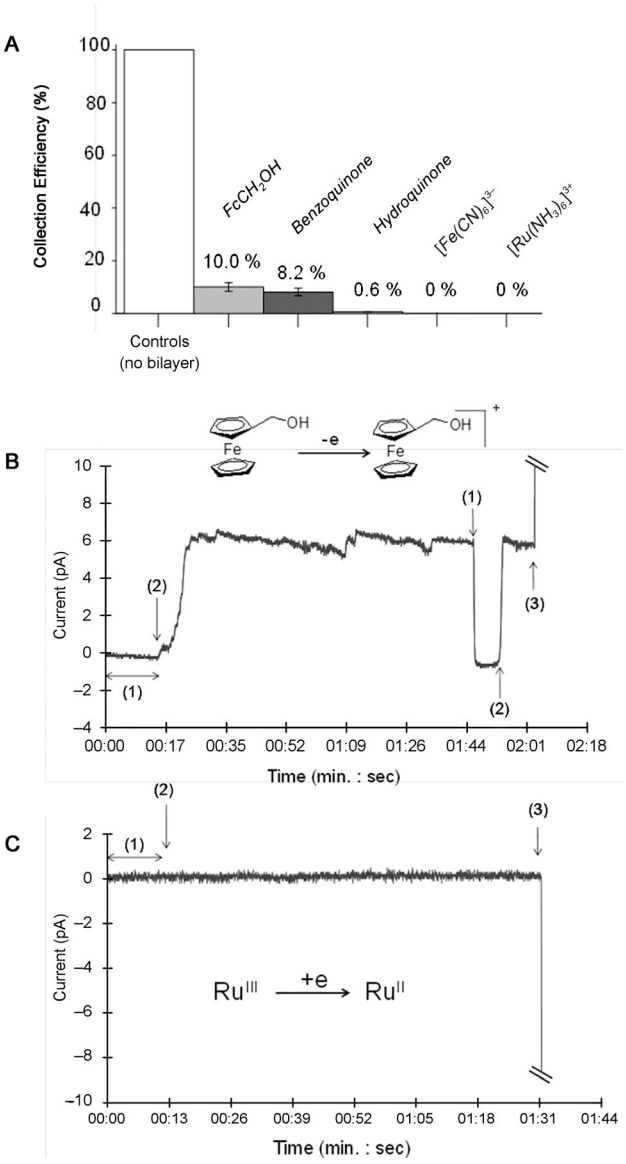

Figure 5. A) Relative amperometric fluxes detected for various electroactive substrates (1 mM each inside the patch micropipette) diffusing out the micropipette in the absence (taken as reference, 100%) and the presence of a pure DPhPC suspended membrane (each bin represents the average value obtained for 20 experiments); controls have been done for each experiment with the same pipette. B) and C) typical current/time response obtained for ferrocenemethanol and $[Ru(NH_3)_6]^{3+}$ as a function of the electrode/membrane distance d ($d > 100$ μm (1); $d < 1$ μm (2)). (3) indicates the moment of membrane rupture obtained by applying a potential step within the ±500/±1000 mV range. In (B) and (C) the arrows indicate the moment at which the gap distance is varied.

this is not a limitation for biologically oriented applications because cell membranes contain large proportions of cholesterol. The patch quality and proper sealing were successfully characterized by electrochemical impedance spectroscopy. Combining this method with amperometric detection provided an efficient means for quantifying in real time the passive transport of redox probes across these micrometer-sized suspended membranes. Therefore, this patch–clamp excision procedure appears sufficiently versatile to allow the investigation of transport and the quantification of the transport properties through direct measurement of the coefficients of partition and diffusion of the probe in model and real cell membranes.

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