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Electrophysiological characterization of transport across outer membrane channels from Gram-negative bacteria in their native environment

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Multi-drug-resistance in Gram-negative bacteria is often associated with low permeability of outer membrane. To investigate the role of membrane protein channels in the passage of antibiotics, we extract, purify, reconstitute them into artificial bilayer. Here we demonstrate that using a fusion of native outer membrane vesicles (OMV) facilitates channel reconstitution into bilayer and allows to characterize them in their native environment. Proteins from *E. coli* (OmpF, OmpC) were overexpressed from the host, and the corresponding OMVs were collected. Each OMV fusion revealed surprisingly only a few channel activities. The asymmetry of the OMV translates after fusion into bilayer with the LPS dominantly present at OMV addition side. Compared to conventional methods, channels fused from OMVs have similar conductance but broader distribution. The further addition of Enrofloxacin yielded higher association but lower dissociation rates attribute to the presence of LPS. We conclude using OMV is a robust approach for functional and structural studies of membrane channels in the native membrane.

Introduction

One identified bottleneck in the development of new antibiotics against Gram-negative bacteria is the low permeability across the cell wall. [1-2] Multi-drug resistant (MDR) bacterial pathogens, especially the 'ESKAPE' family or the so-called superbugs are responsible for two thirds of the serious infection issues in clinic worldwide. [3] As a response, European Union supported via the Innovative Medicine Initiative a series of private-public partnerships building a platform called "New Drugs for bad bugs" (www.ND4BB.eu) among the project named "Translocation" was devoted to investigate the low permeability issue. [4]

The envelope of Gram-negative bacteria allows selective permeation of nutrients while protecting from toxic substances. Gram-negative species contains an inner membrane and outer membrane with peptidoglycan layer sandwiched in the middle. The outer membrane layer is structural rigid due to the lipopolysaccharide (LPS) layer towards the environment. The LPS layer mainly protects the bacteria from certain chemical attack. Crystal structures showed the LPS has a docking position at some of the outer membrane proteins (i.e., see for OmpF [5]). Conventional patch clamp technique is not suitable to study bacteria on one aspect is these are too small, on the other aspect, the LPS prevents one from forming 'giga-seal' during the measurement. Moreover, artificial asymmetric membranes containing LPS to mimic the native environment are difficult to assemble, and in particular, free standing bilayer are only possible for very short LPS. [6-7]

One of the building blocks responsible for selectivity are channel-forming outer-membrane proteins facilitating the transport of hydrophilic molecules across the otherwise tight hydrophobic membrane. [8-9] In particular, the major outer membrane channels such as OmpF and OmpC from *E. coli* are part of the pathways of antimicrobial influx.[10-14] High-resolution crystal structure showed that OmpF is composed of three identical water-filled monomers. [15] Each monomer contains 16-antiparallel β-sheets that span in the membrane domain. There are 8 long loops connecting each pair of β-sheets (L1-L8), among them, L3 folds into almost the halfway of the channel lumen forming the narrowest constriction region (CR). The L3 loop is composed mainly of negatively charged amino acid groups inducing slightly cation selectivity in KCl at physiological condition.[16-19] OmpC is a similarly constructed membrane protein with smaller CR and expressed under extreme conditions by *E. coli* instead of OmpF.[20]

Recent advancements in proteomics provide quantitative numbers of the membrane proteins distribution in bacteria under various growth conditions.[21] Previously we characterize transport across *E. coli* channel at a single protein level reconstitution of single porin into artificial lipid bilayers. To gain information on the mode of permeation, the ion current in the absence and presence of small molecules was measured. [21-22] The contribution of the native environment such as LPS layer on the channel and the permeability for small molecules across the outer membrane is an open question.

Outer membrane vesicles (OMV) are spherical vesicles naturally secreted by Gram-negative bacteria involved in their survival under stress conditions and regulating microbial interactions

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within communities.[23] Surprisingly OMVs may fuse with lipid liposomes just by mixing.[24] As they also contain outer membrane channel in their natural environment, we are tempted to use them for channel characterization.[25]

Within the current urgent need for novel antibiotics, the key questions concerning the contribution of the so-called porins to drug uptake are: why do some molecules permeate rapidly whereas others do not; which channels are involved; and what are the flux-limiting interactions between antibiotics and channels. Recently established methods such as whole cell uptake assays and mass spectrometry provide an answer to the total penetration, which is the relevant parameter for the survival of a cell.[26-27] However, whole cell measurements reflect the sum of many possible pathways (e.g., porins, degradation enzymes, efflux pumps, etc.) optimization of the chemical structure concerning permeation remains difficult while relying only on an integrated set of data.

Here we developed a direct approach to insert porins in their native environment including the LPS into a bilayer using OMV, with the goal to investigate to what extent the presence of LPS influences the flux of small molecules across porins. A further advantage is that such an OMV fusion approach is easy with respect to handling and may allow automatization.

Results

Inspired from the early formation of supported bilayer by fusing small unilamellar vesicles, outer membrane vesicles were added directly towards the artificial lipid bilayer (see Methods section) in 200 mM KCl, 20 mM MES and pH 6.0. Surprisingly only a few active channels were observed once fusion was obtained. In Figure 1, we show the result of typical OmpF insertion. Likewise, in patch-clamp, each vesicle fusion increases the conductance originating from an unknown number of porins. To distinguish single-channel conductance, we apply higher transmembrane voltages to induce gating as typically observed for OmpF or OmpC. The critical voltage necessary to observe channel gating for was below -125 mV at a negative voltage or above +150 mV at a positive voltage, comparable to previous observation with purified porin reconstituted into an artificial bilayer. At a transmembrane voltage of +150 mV, the change of the average current baseline due to OmpF monomeric gating is 39 pA ±5 pA corresponding to 1.1 ± 0.1nS for a trimeric OmpF. This observed conductance value is similar to purified OmpF reconstitution (800 pS at 200 mM KCl, pH 6.0),[19,22,29,30] however, the statistical distribution was significantly broader.

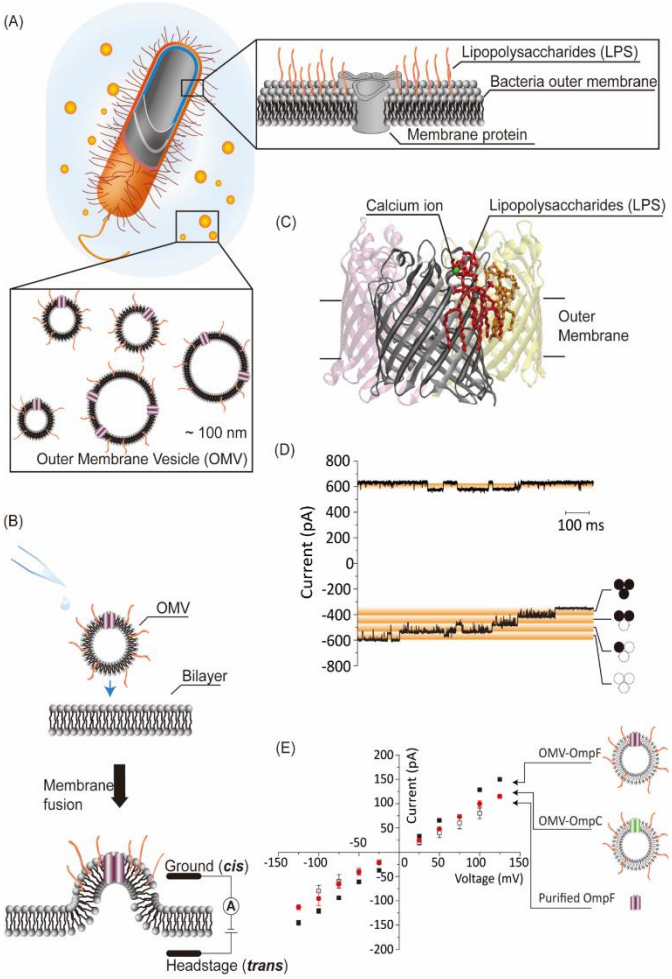

Figure 1 (A) Outer membrane structure of Gram-negative bacteria, composed of a phospholipids inner leaflet, lipopolysaccharides (LPS) outer leaflet as well as of membrane proteins. The bacilli secrete outer membrane vesicles (OMV) whose diameters are around 100 nm. (B) OMV vesicles are added directly to the planar artificial lipid bilayer, forming BLM-OMV system. Fusion and protein activity is followed by applying external voltages. The ion current steps correspond to open porin insertion. (C) Crystal structure of LPS binding with OmpC homolog (5FVN) with each monomer in a different color. Calcium (green) binding site suggests the involvement of divalent cation. Two LPS segments are plotted in red and orange. (D) Typical OmpF ion current during OMV fusion, the applied voltage is ±150 mV, and the shown ion trace correspond to three trimeric OmpF (E) I-V response of OmpF and OmpC from OMV fusion. 200 mM KCl, 20 mM MES, pH 6.0 was used throughout. At least 3 individual experiments were performed.

To investigate whether this is a general feature of OMV reconstitution and not specific to OmpF, we then overexpressed OmpC, an OmpF homolog, in the same strain and fused the secreted OMVs to the planar lipid bilayer. The induced channel activity confirmed the trend for OmpF. The typical ion current versus applied voltage is plotted in Figure 1 (E) and compared to purified OmpF. In the traditional purification – reconstitution protocol we obtain at 200 mM KCl and pH 6.0 for OmpF 800 pS and OmpC 600 pS, while the fusion of OMV showed conductance of OmpF 1100 ± 100 pS and for OmpC 840 ± 45 pS.[19] Typically only 1-3 active channels were observed per OMV fusion event number of experiments: n>20), both with OmpF and OmpC containing vesicles.

To elucidate possible stabilization effect of LPS in presence of divalent ions, we added 5 mM EDTA and incubated the vesicles

at 4 °C overnight. Within the experimental error, we did not see a significant change.

In the second series of measurements, we added Enrofloxacin (250µM), a model antibiotic having the advantage of being well characterized.[30] We first fused OMV containing OmpF to the lipid membrane, and Enrofloxacin was added to the same side as OMV addition. In agreement with previous single channel recording, interaction spikes were observed from the negative polarity transmembrane voltage. A statistical analysis of the events provides the association rate (k_{on}=12000 $s^{-1}M^{-1}$) and dissociation rate (k_{off} =270 s^{-1}) of Enrofloxacin association to the channel at -50 mV. There is no interaction been observed when positive voltage is applied. To compare the difference between the OmpF-OMV and purified OmpF, we made similar measurements with purified OmpF and found substantially higher on-rate for the same concentration of Enrofloxacin, statistical analysis revealed k_{on} = 72000 $s^{-1}M^{-1}$ and k_{off} = 500 s^{-1}, similar values were also obtained earlier with OmpF-WT.[7,11,30] Such asymmetry in the measured interaction kinetics hints towards the barrier offered by the LPS for the transport of substrate molecule across the outer membrane of Gram-negative bacteria and also demonstrates the LPS facing towards the addition of substrate side. The on-rate of Enrofloxacin drops by almost 6 times in the presence of LPS and slowed down the translocation of the substrate molecule by almost 50%, see Figure 2 (B and C) for rate kinetics comparison.

According to previous studies using traditional purified protein reconstitution approach [31-32], the extracellular side of the channel from OMV fusion is the same as the sample addition side, which is identical to the mechanism as the purified protein reconstitution, as illustrated in Figure 1(C). Since the channel orientation from OMV fusion is the same as with the conventional reconstitution method, we conclude that both the LPS and channel extracellular side face the cis side, which is the OMV addition side.

In a different set of experiment, we reconstituted OMVs into Giant unilamellar vesicles (GUVs) (see supplement) with a typical final diameter from 10 to 20 µm and can be used in a patch-clamp setup. Under the microscope, the vesicles appeared spherical and were subsequently patched in the Port-a-Patch tool. Both OmpF and OmpC activities could be obtained when analyzing the channel gating conductance then normalized to single trimeric porin with similar behavior as observed in a planar membrane (Figure S3).

Conclusions

We suggested a simple approach to study membrane proteins in their native environment. Fusion of OMV with planar lipid bilayer allows single or few porin insertions. The reason for such an unexpected low porin number might be an intrinsic selection for single channel reconstitution. Other condition resulted in 2-D crystals, which in all likeliness are not readily able to fuse with a lipid bilayer.[25] Single channel traces revealed a somewhat lower conductance and with a broad standard deviation compared to purified and reconstituted single membrane protein. We compared single channel translocation data of OmpF-OMV with purified OmpF-WT and found a significant shift in the rate kinetics. Such kinetic analysis hinted us with orientation and fusion of OMV with LPS facing the side of substrate addition. We expect that this approach is also valid for many other porins.

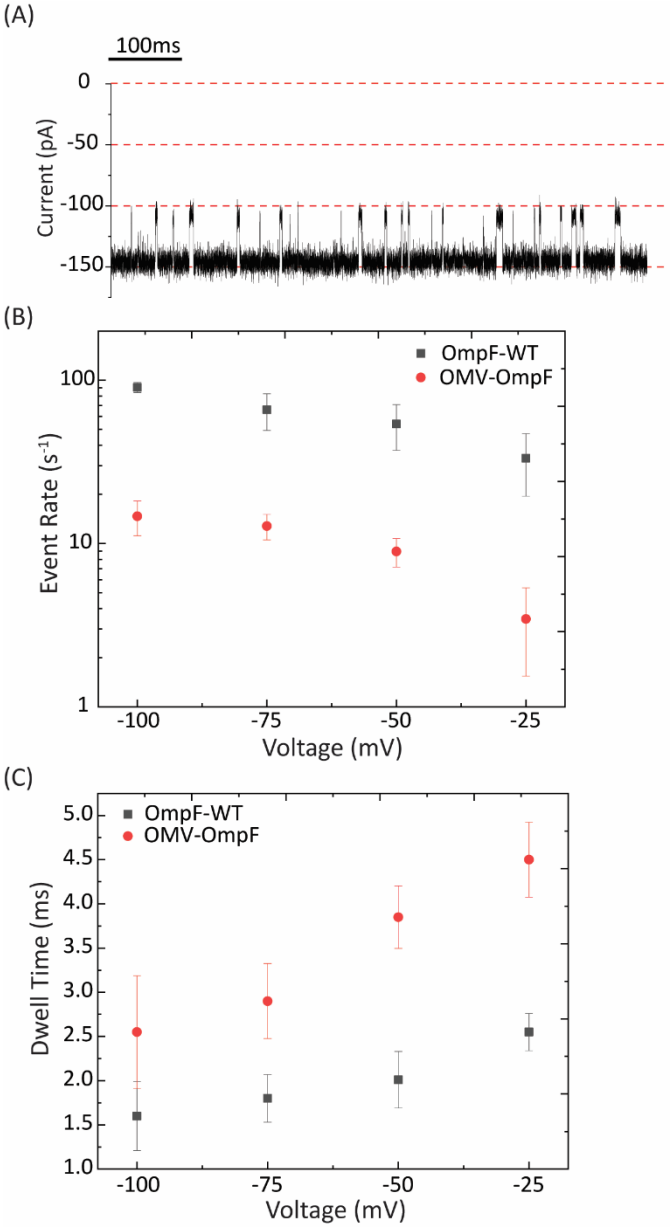

Figure 2 (A) Single molecule characterization of OmpF in OMV fused in the presence of 0.25 mM Enrofloxacin (200mM KCl + 20mM MES at pH 6) applied potential -100mV. (B & C) are the statistical analysis of the traces measured at different applied voltage for both OMV-OmpF and OmpF-WT.

Conflicts of interest

There are no conflicts to declare.

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