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A Simple Method for the Reconstitution of Membrane Proteins into Giant Unilamellar Vesicles

Armelle Varnier · Frédérique Kermarrec · Iulia Blesneac · Christophe Moreau · Lavinia Liguori · Jean Luc Lenormand · Nathalie Picollet-D'hahan

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Abstract A simple method for the reconstitution of membrane protein from submicron proteoliposomes into giant unilamellar vesicles (GUVs) is presented here: This method does not require detergents, fusion peptides or a dehydration step of the membrane protein solution. In a first step, GUVs of lipids were formed by electroformation, purified and concentrated; and in a second step, the concentrated GUV solution was added to a small volume of vesicles or proteoliposomes. Material transfer from submicron vesicles and proteoliposomes to GUVs occurred spontaneously and was characterized with fluorescent microscopy and patch-clamp recordings. As a functional test, the voltage-dependent, anion-selective channel protein was reconstituted into GUVs, and its electrophysiological activity was monitored with the patch clamp. This method is versatile since it is independent of the presence of the protein, as demonstrated by the fusion of fluorescently labeled submicron vesicles and proteoliposomes with GUVs.

Keywords Biomembranes · Membrane protein · Membrane channel · Giant unilamellar vesicle · Proteoliposome · Fusion · Lipid

A. Varnier · F. Kermarrec · N. Picollet-D'hahan (✉)
CEA, DSV, iRTSV, Biopuces, 17 rue des Martyrs, 38054 Grenoble Cedex 9, France
e-mail: Nathalie.picollet-dhahan@cea.fr

I. Blesneac · C. Moreau
IBS (CEA, CNRS, UJF), Protéines Membranaires, 41 rue Jules Horowitz, 38027 Grenoble, France

L. Liguori · J. L. Lenormand
HumProTher Laboratory, TheREx, TIMC-IMAG, CNRS UMR5525, UJF, UFR de Médecine, 38706 La Tronche Cedex, France

Introduction

Giant unilamellar vesicles (GUVs) and proteo-GUVs are models for biological membranes, with a size in the same range as that of cells (10–100 μm). In contrast to small unilamellar vesicles (SUVs, 30–50 nm) and large unilamellar vesicles (LUVs, 100–200 nm), GUVs can be studied with optical microscopic techniques such as phase contrast, differential interference contrast and fluorescence microscopy (Bagatolli 2006; Menger and Keiper 1998; Girard et al. 2004). In addition to imaging methods, the quantitative study of dynamics in membranes can be achieved with methods such as fluorescence recovery after photobleaching (FRAP) (Tareste et al. 2008), fluorescence correlation spectroscopy (FCS) (Kahya et al. 2003) and scanning fluorescence correlation spectroscopy (SFCS) (Ruan et al. 2004). GUVs can also be micromanipulated with pipettes (Sun et al. 2009) or with patch-clamp electrodes (Wunder and Colombini 1991), and recently flow-cytometric techniques were applied to these biomimetic systems (Lamblet et al. 2008). Different applications of GUVs have been reported, such as electrophysiological recordings of ion channels (Wunder and Colombini 1991; Regueiro et al. 1996; Kreir et al. 2008), studies of membrane–protein interactions (Ruan et al. 2004; Lee et al. 2008; Tamba and Yamazaki 2005; Riske and Döbereiner 2003), DNA–membrane interactions (Angelova and Tsoneva 1999), lipid domain characteristics (Kahya et al. 2003; Bagatolli and Gratton 2000), actin-based motile processes (Delatour et al. 2008) and membrane morphological changes induced by local pH gradient (Khalifat et al. 2008). GUVs have also been used as biomimetic containers that were fused by micromanipulation in order to study single-molecule enzyme activity (Hsin and Yeung 2007).

This reflects a large interest in the use of proteo-GUVs in biological issues that contrasts with a limited number of publications in this field. This could be explained by the difficulties in forming proteo-GUVs. Different methods have been proposed that require more or less complex procedures or specific conditions in order to preserve proteins from denaturation (Girard et al. 2004; Kahya et al. 2001; Doeven et al. 2005; Montes et al. 2007).

The dehydration/rehydration method (Criado and Keller 1987) was successful at producing giant proteoliposomes that could be used for patch-clamp recordings. This method produces a heterogeneous population of proteoliposomes with a large proportion of multilamellar vesicles. It was further improved by applying an alternating current electric field during the rehydration step (Girard et al. 2004), on the basis of the technique of electroformation of lipid GUVs (Angelova and Dimitrov 1986) that produces a more homogeneous population of GUVs. The main drawback of the dehydration/rehydration method is the requirement for precise control of the partial dehydration step: A low rate of dehydration of proteoliposomes under a controlled humidity atmosphere is required in order to avoid protein denaturation. Furthermore, the electroformation step precluded ionic strength over 5 mM for the initial proteoliposome buffer and for the rehydration buffer (Girard et al. 2004). Improvements were brought with the addition of sucrose during the dehydration step, preserving the activity of the proteins (Doeven et al. 2005), and with a new protocol of electroformation that allowed the use of physiological ionic strength buffers (Montes et al. 2007; Pott et al. 2008). Other methods insert membrane proteins into preformed lipid GUVs. Fusion of submicron proteoliposomes with GUVs can be induced by positively charged lipids and fusion peptides (Kahya et al. 2001). This method is relatively straightforward, but the main drawback is that the resulting proteo-GUVs contain foreign molecules, namely, positively charged lipids and fusion peptides. Reconstitution of membrane proteins directly into GUVs by adding detergent-solubilized proteins to giant vesicles has also been reported (Kreir et al. 2008; Battle et al. 2009). However, the method required careful detergent removal with Biobeads and the protocol must be adjusted for each membrane protein since the nature and the concentration of the detergent have to be optimized for the stability and solubility of the proteins.

In this study, we present an innovative method for transferring membrane proteins from small proteoliposomes to GUVs. A solution of concentrated GUVs was incubated with small proteoliposomes, containing the human voltage-dependentm anion-selective channel (VDAC) (Colombini 1989; Liguori et al. 2008). This mitochondrial protein was reconstituted into GUVs, and its electrophysiological activity was tested with the patch-clamp technique. The method presented here does not require detergents, fusion peptides or a dehydration step of the membrane protein solution. The protocol is simple, can be achieved in less than 24 h and can be easily integrated in automated processes as it requires only pipetting of various solutions.

Materials and Methods

Preparation of Recombinant Proteoliposomes Containing VDAC

Human VDAC1 cDNA was cloned into the pIVEX 2.4b NdeI plasmid (Roche Diagnostics, Indianapolis, IN). Recombinant VDAC1 was synthesized in vitro using the RTS 500HY system (Rapid Translation System, Roche Diagnostics). Proteoliposomes containing VDAC1 were produced as described (Liguori et al. 2008). Briefly, synthetic liposomes (1,2-dioleoyl-*sn*-glycero-3-phosphocholine [DOPC]: 1,2-dilinoleoyl-*sn*-glycero-3-phosphoethanolamine [DOPE]: 1,2-dimyristoyl-*sn*-glycero-3-phosphate [DMPA]:cholesterol; 40:20:20:20 volume ratio) were added to the RTS reaction mixture at 1:1 v/v ratio and incubated for 48 h (1 ml final volume) at 26°C in the Proteomaster (Roche Diagnostics). Synthetic liposomes were prepared following the classical evaporation/sonication system, filtered through a 0.22-μm filter and added to the in vitro reaction mixture at a final concentration of 5 mg/ml. After proteoliposome production, the mixtures were centrifuged at 13,000 × *g* for 20 min at 4°C and the supernatant was discarded. The pellet was resuspended in 1 ml Tris-Cl 50 mM (pH 7.2). Recombinant VDAC1 proteoliposomes were purified by loading the resulting solution onto a three-step discontinuous sucrose gradient (60, 25 and 10%) prepared in Tris-Cl 50 mM (pH 7.2). After ultracentrifugation (1 h at 200,000 × *g* at 4°C), fractions were collected and then analyzed by Western blotting using an anti-his antibody (monoclonal anti-His HRP-conjugated antibody; Sigma-Aldrich, St. Louis, MO) to verify the presence of VDAC and silver staining (data not shown). The protein concentration of purified VDAC proteoliposomes ranged 0.7–1 mg/ml. Proteoliposomes (100–120 nm diameter), diluted in TRIS-HCl 50 mM (pH 7.5) to a final concentration of 150 μg/ml, were used throughout this study. As control, purified empty proteoliposomes obtained from an in vitro reaction in the presence of a control vector which did not contain VDAC cDNA were used.

GUV Formation

GUVs were produced by electroformation according to Angelova and Dimitrov (1986), with the following modifications.

Soybean polar extract (Avanti, Birmingham, AL) 90 mol%, cholesterol (Sigma-Aldrich) 10 mol% and the fluorescently labeled lipid *N*-(teramethylrhodamine-6-thiocarbamoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (TRITC-DHPE) (Invitrogen, Carlsbad, CA) <0.5 mol% were mixed in hexane solution to a final concentration of 5 mg/ml.

Lipid samples (20 µl) were deposited with a Hamilton syringe on four platinum wires (Fisher Scientific, Fair Lawn, NJ; 99.9% purity, 0.5 mm diameter and 10 cm length). The solvent was removed by evaporation for 2 h at 25°C. Two pairs of wires spaced by 2 mm were placed in two 0.5-ml microtubes and connected to a pulse generator. An AC electric field was applied for only a few seconds with a voltage of 0.4 mV peak to peak at 10 Hz frequency, while 0.5 ml of a buffer solution (5 mM HEPES [pH 7.4], 200 mM sucrose) was added to each tube. The voltage was then adjusted to 3 V ptp at 10 Hz, and electroswelling lasted 2 h. At the end of the electroformation, the electrodes were removed, leaving the GUV solution ready for use. GUVs were quite delicate, and their time stability was 2 days.

Purification, Concentration of GUVs and Fusion with Submicron Vesicles

Figure 1 shows a schematic overview of the preparation of proteo-GUVs. After electroformation was performed, the solution contained a mixture of GUVs, small-sized vesicles and lipid aggregates. Since small vesicles could interact and stick to the patch-clamp pipettes, preventing seal formation required for electrophysiological recording, vesicles <1 µm in diameter were discarded. In addition, small vesicles could contribute to the background signal during optical characterization of GUVs.

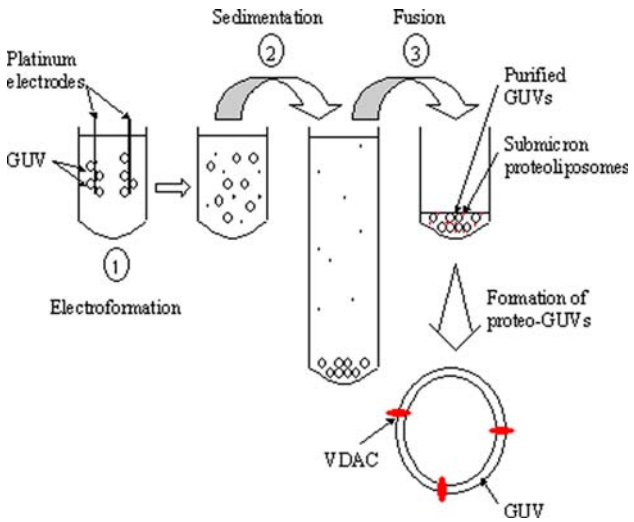

Fig. 1 General procedure for proteo-GUV formation from lipids and submicron proteoliposomes. In step 1, GUVs are formed by electroformation in a buffer solution with sucrose. In step 2, the GUVs are purified and increased in concentration by sedimentation in a buffer solution containing glucose. Step 3 consists of transferring the pelleted GUVs to the solution of submicron-sized proteoliposomes and incubation at 4°C, resulting in the formation of proteo-GUVs

Simultaneous purification and concentration of GUVs were performed by adding 1 ml of the GUV solution to the top of a 10-ml Falcon tube containing 10 ml of 5 mM HEPES (pH 7.2) and 200 mM glucose. The density difference of the buffer solutions inside and outside the GUVs induced sedimentation of the largest vesicles. The bottom solution (around 0.5–1 ml) was carefully pipetted and a second sedimentation was performed on a new column (10–11 ml) in order to complete the purification of GUVs.

Purified and concentrated GUVs were harvested at the bottom of the second sedimentation column. Spontaneous fusion of submicron vesicles, containing or not containing proteins, with GUVs was achieved by adding 50 µl of GUVs to 1–4 µl submicron vesicles. The mixture was handled cautiously and placed overnight at 4°C without mixing during incubation, resulting in the incorporation of the vesicle membranes into the GUV membranes. In order to determine the GUV number and diameter, pictures of vesicles were recorded and each picture was divided in uniform columns; the size of the vesicles was measured for each column, until 300 vesicles were measured. Thus, the size proportion can be compared before and after fusion. Two measurements were performed in each case.

In order to assess the unilamellarity membrane aspect of proteo-GUVs in such a study, we performed observations by phase-contrast microscopy and confocal imaging using the Nile red fluorescent lipid label. To stain the lipid membrane, 20 µl of Nile red in ethanol was added to 200 µl of proteo-GUV solution. Proteo-GUVs were observed with a confocal microscope (TCS-SP2; Leica, Deerfield, IL). Fluorescent and transmitted light images were acquired using the 488 nm wavelength of an argon laser, with a 40× (NA1, 25) objective. Nile red fluorescence was collected between 500 and 570 nm.

Patch-Clamp Experiments

The proteo-GUV solution was added to a Petri dish filled with a buffer solution (5 mM HEPES [pH 7.2], 100 mM NaCl, 5 mM $CaCl_2$). Most of the sucrose-containing GUVs were immobile on the bottom of the plastic dish. The micropipette was filled with the same buffer solution and lowered to isolated liposomes. Recordings of ion channels inserted in GUVs were done in cell-attached mode. Micropipettes were pulled from borosilicate glass capillaries (Kimax capillaries; Kimble Glass, Vineland, NJ) using a P-97 puller (Sutter Instrument, Novato, CA).

Their resistance in patch-clamp conditions was 2–10 MΩ. The micropipette was pressed against the lipid membrane, and a seal was obtained without disrupting it. The currents were recorded using a Biologic RK-300 amplifier, sampled at 1 kHz, filtered at 300 Hz with a low pass filter and analyzed with Clampfit10 (Molecular Devices, Palo Alto, CA).

Results

Sedimentation of GUVs

Concentration of GUVs was induced by a density difference between buffer solutions inside and outside the GUVs but with identical osmolarity to preserve GUV stability. To optimize GUV sedimentation, a theoretical model of the rate of sedimentation of GUVs was developed. The rate of GUV sedimentation, given by Stokes' law (Eq. 1), is a function of gravity acceleration (g), density differences between the solutions inside and outside the GUVs (ρ_{GUV} and ρ_{buffer}, respectively), GUV radii (r) and viscosity of the solution surounding the GUVs (μ). The viscosity of the glucose solution depends on the mole fraction of glucose (m) and on the temperature (T) as expressed in (Eq. 2) (Viet Bui and Nguyen 2004).

$$v = 2r^2 g(\rho_{GUV} - \rho_{buffer})/9\mu \quad (1)$$

$$\mu = 10^{-3} \times 2.0041 \times \exp(-0.4324m) \times \exp[(242.58m + 1889.3)/T] \quad (2)$$

From Eqs. 1 and 2, the rate of sedimentation was calculated as a function of vesicle sizes, with a diameter varying from 2 to 100 µm. Figure 2 shows that sedimentation rates increase significantly with the sugar concentration varying from 100 to 300 mM and with temperature rising from 4 to 25°C.

For both electroformation and sedimentation, a concentration of 200 mM sugar solution and room temperature were chosen: This represents the best compromise between a relatively fast sedimentation and a large amount of GUVs since we noticed that more GUVs were obtained with a reduced sucrose concentration. After 2 h sedimentation, the purified fluorescently labeled GUVs had diameters of 15–120 µm, with a main population at 50 µm and only few (one or two proteo-GUVs in each experiment) around 200 µm and a final theoretical concentration of 2 mg/ml, as shown in Fig. 3a. This size of GUVs was 15–120 µm and was in the range of GUV sizes usually obtained with other methods (diameter ~5–100 µm) (see references in Doeven et al. 2005).

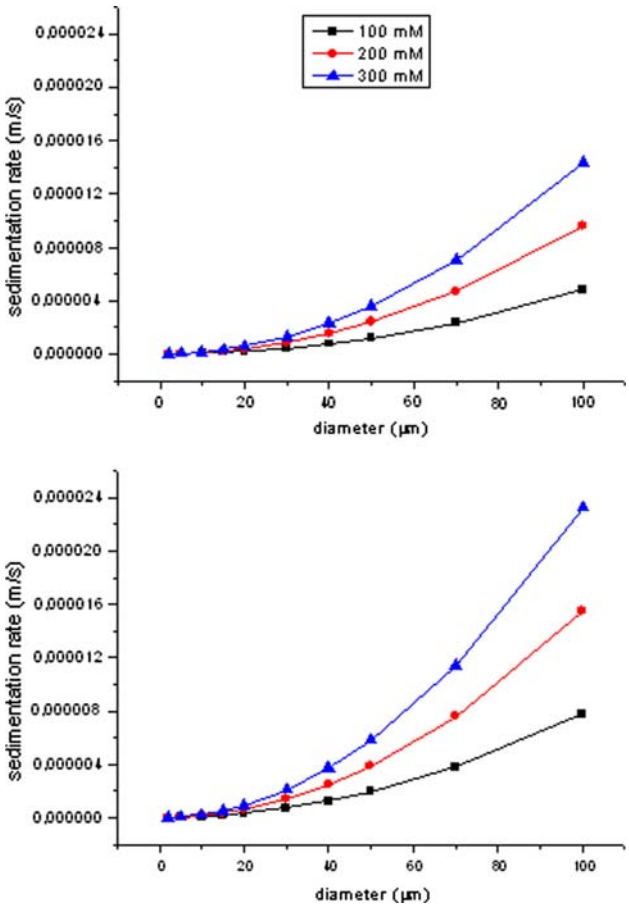

Fig. 2 Sedimentation rate of vesicles calculated from a theoretical model (see text) and represented as a function of vesicle diameter at different experimental conditions such as glucose concentration (100, 200 and 300 mM) and temperature (4°C [*upper panel*] and 25°C [*lower panel*])

Transfer of Material from Submicron-Size Proteoliposomes to GUVs

When purified and concentrated labeled GUVs were added to 1 µl small proteoliposomes, the formed proteo-GUVs had a diameter of 15–120 µm, with a mean size of 20 µm, as shown in Fig. 3b. Figure 3c shows the effect of the fusion of submicron proteoliposomes with labeled GUVs in terms of vesicle size distribution. The number of vesicles with diameter of 10–30 µm increased, while there were fewer vesicles >40 µm. This was done for two experiments. No statistical study was performed, but Fig. 3c is highly representative of what we usually observed during fusion. Moreover, observations in Fig. 4 using phase-contrast microscopy and confocal imaging with Nile red labeling allowed us to assess the unilamellar aspect of membranes. Direct observation revealed a weak but sharp contrast at the very edges of liposomes, probably due to the classical optical illusion about apparent differences in the color inside and outside of circles (even though the color is really the same).

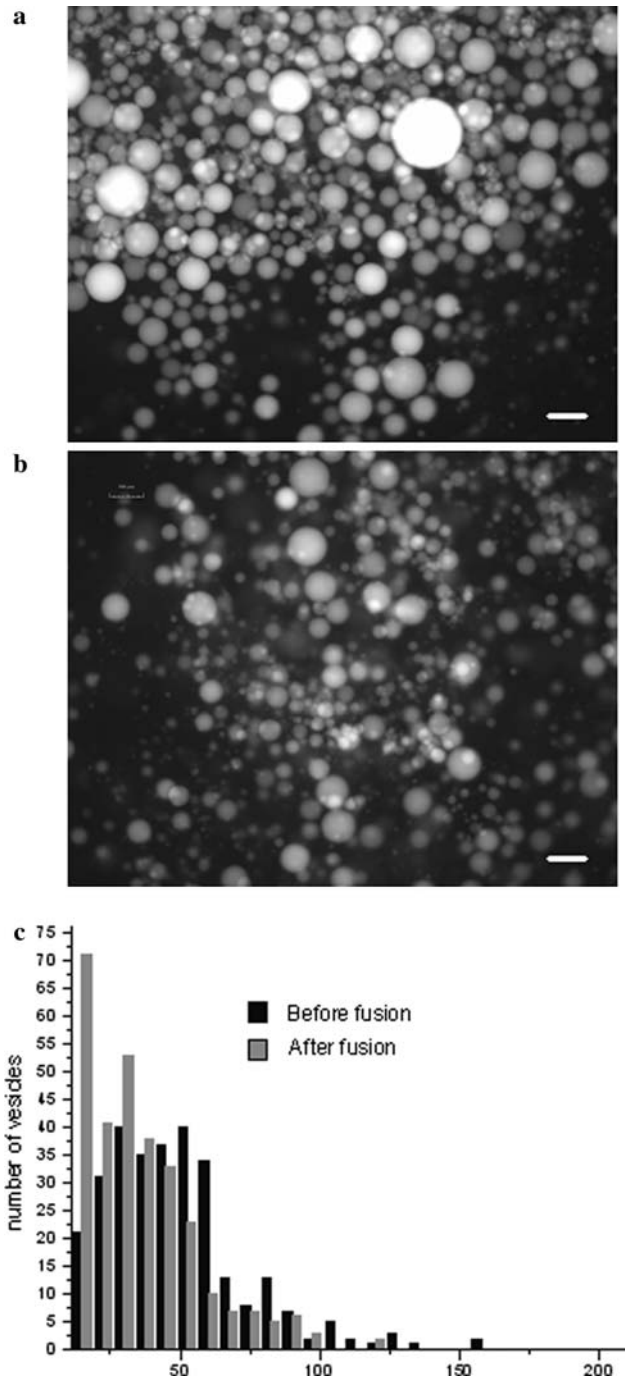

Fig. 3 Purified and concentrated TRITC-labeled GUVs (scale bar = 100 μm) before (**a**) and after (**b**) fusion with submicron proteoliposomes. Histogram (**c**) of vesicle size distribution before (*black*) and after (*gray*) fusion with submicron proteoliposomes

Patch-Clamp Recordings

Electrical Seal

High-resistance seals (>1 GΩ) were obtained either by applying negative pressure or by simply getting the pipette tip close to the vesicles. This last point can contribute to the simplification of electrophysiological measurement robots, circumventing the use of a pressure-control device.

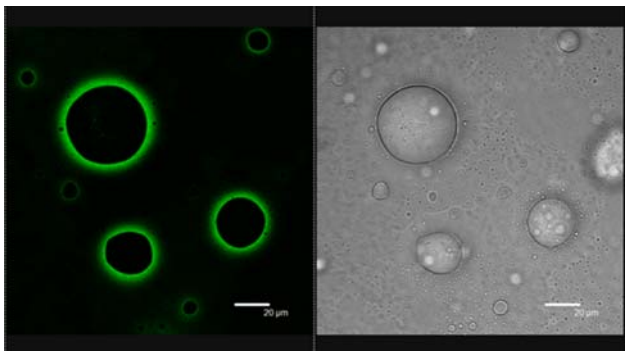

Fig. 4 Nile red–labeled proteo-GUVs observed by confocal imaging. Comparison between transmitted light images (*right*) and fluorescent images (*left*). GUVs were formed by the electroformation method and fused with submicron vesicles including VDAC. Bar = 20 μm

Patch-Clamp Recordings in Cell-Attached Mode

With 50 μl GUVs incubated with 4 μl VDAC proteoliposomes, conductance measurements at constant applied voltage showed the presence of active proteins for around 50% (8 out of 15) of the high-resistance seals obtained (Fig. 5), which gives functional evidence of fusion events. However, the number of fusion events could not be quantitatively evaluated here. High VDAC content in the membrane was previously reported to induce no differences in measurements in the attached or excised patch-clamp mode (Wunder and Colombini 1991). By decreasing the potential from 0 to −70 mV, the current amplitudes increased proportionally with clear opening and closing characteristics of porines such as VDAC. The observed potential dependence confirms the integrity of the membrane protein activity.

Discussion

Membrane fusion events are known to be induced by specific fusogenic proteins (Tareste et al. 2008; Kahya et al. 2001). Membrane fusion can be also obtained by physical or chemical induction (Cevc and Richardsen 1999; Haluska et al. 2006). Spontaneous membrane protein insertion into GUVs up to 10 μm in size has been previously reported under specific conditions (Scotto and Gompper 1990), where crystalline arrays of bacteriorhodopsin formed in purple membranes spontaneously incorporated into preformed vesicles.

We have presented here a fusion process between submicron proteoliposomes and GUVs that can occur in more

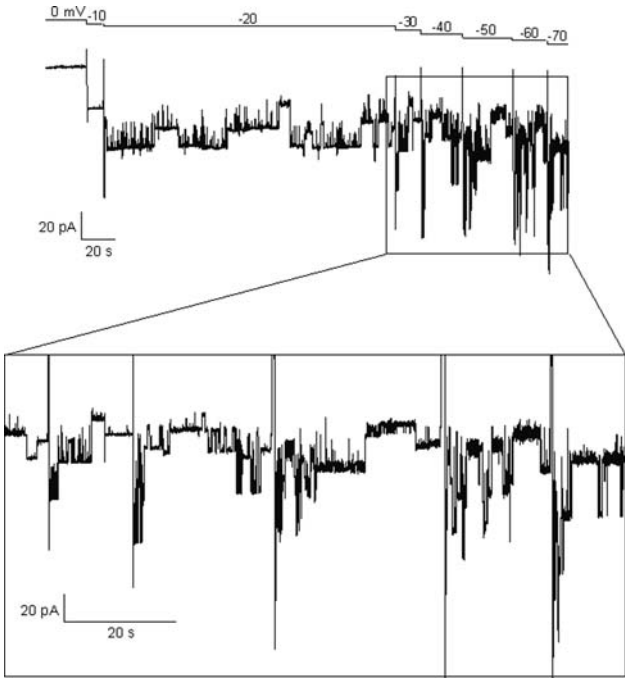

Fig. 5 Patch-clamp recording of porins inserted in GUVs: current time course at voltages from 0 to −70 mV by steps of −10 mV. *Lower panel* is current recording between −30 and −70 mV on an expanded time scale

general conditions, even if the intrinsic mechanism of the process remains unclear. In fact, the observed spontaneous fusion process between submicron proteoliposomes and GUVs can be influenced by many parameters in which membrane proximity and membrane defect density are the final determinant factors (Cevc and Richardsen 1999). Here, membrane proximity was favored by concentrating GUVs. Membrane proximity can be also obtained with the application of an electric field or by the use of intermediate ligands (Haluska et al. 2006). High-density bilayer defects could have many origins. By decreasing the temperature to 4°C, which is closer to the lipid phase transition temperature, bilayer defects could be induced (Cevc and Richardsen 1999). Lipid composition of the vesicles might be important as well: Cholesterol could induce membrane fusion events (Tenchov et al. 2006). In this case, the lipid composition of the vesicles was optimized to be highly compatible with the cell-free production of VDAC. Indeed, lipid composition has been found to influence the mechanics of biological membrane fusion, e.g., the propensity of lipid bilayers to develop fusion pores and to hemifuse (Chernomordik and Kozlov 2008). In addition, osmotic stress of the membrane could be caused by the slight osmolarity difference between the solutions inside and outside the vesicles and could contribute to membrane defects or induce swelling and stressing of the liposomes, thus favoring the fusion of membranes as mentioned by Zagnoni et al. (2007). In the case of electroformation of GUVs from adjacent vesicles, the application of an AC electric field might contribute to the formation of bilayer defects since evidence of fusion of adjacent vesicles during electroformation was reported (Montes et al. 2007).

The properties of proteo-GUVs obtained by spontaneous fusion, in terms of size and seal formation, are consistent with previously reported data for proteo-GUVs formed with other methods. For the first point, the decrease of the size of the vesicles after fusion can be related to the relationship between the size of the liposomes and the concentration of inserted proteins. With the dehydration/rehydration method, it was reported that the size of proteoliposomes decreased when the content of inserted membrane proteins was high (Regueiro et al. 1996; Criado and Keller 1987) and that the protein/lipid ratio could modulate the amount of electroformed GUVs (Girard et al. 2004). The change in vesicle size partition induced by incorporation of membrane proteins can also be due to the fragility of the largest vesicles. The incorporation of membrane proteins could destroy the largest vesicles. For the second point, the rate of high-resistance seal formation observed in this study is similar to the one obtained with proteo-GUVs formed by dehydration/rehydration (Wunder and Colombini 1991).

To our knowledge, this is the first time fusion of submicron proteoliposomes with GUVs without the use of fusogenic peptide has been reported. This method is simple and does not use any conditions that could damage the conformation of the protein. The originality of our approach is based on the concentration of GUVs according to the difference of density inside and outside the GUVs, allowing simultaneously (1) membrane concentration of GUVs presenting homogeneous sizes (favorable for fusion) and (2) purification with elimination of vesicles <1 μm diameter. Both the presence and the functionality of the inserted ion channel protein in the GUVs were directly assessed using patch clamp. Patch-clamp experiments directly performed on fused vesicles provide the functional signature of the VDACs properly oriented into the GUV membrane. Moreover, even if the number of fusion events was not evaluated here, the patch clamp provides an electrical indication of vesicle fusion. However, it is not so obvious since a lack of current with the patch clamp would not necessarily indicate unsuccessful fusion but could also traduce a lack of VDAC inside the micropipette.

Our aim here was to validate a methodology using a functional test by patch clamp. However, in order to assess the unilamellarity membrane aspect of proteo-GUVs in such a study, we completed patch-clamp experiments with observations performed by phase-contrast microscopy and confocal imaging using the Nile red fluorescent lipid label. We judged the liposomes to be unilamellar by comparison

to phase-contrast images previously obtained from giant liposomes that were very similar to what we obtained (Akashi et al. 1996). Although the fluorescence images do not have the resolution to allow confirmation that we have unilamellar vesicles, previous work and personal communication support this argument about unilamellar vesicles (Di Maio et al. 2006). Thus, the results of the present work demonstrate the functionality of VDAC porins inserted in GUVs that were most likely unilamellar according to fluorescence and phase-contrast images.

The present method describing the incorporation of membrane proteins from submicron vesicles into GUV membranes represents the proof of concept toward a possible automation. One of the potential bottleneck steps in the process would be the production of functional channel molecules. However, the optimization of the cell-free expression system allowed us to produce in a "one-step" reaction functional proteoliposomes containing VDAC (Liguori et al. 2008, 2009). Here, proteo-GUVs were immobilized by sedimentation and, as often observed in experiments, easily patched without the need for applied suction. Thus, our reconstitution protocol could be further extended to planar substrates such as "patch-clamp on a chip" silicon devices (Sordel et al. 2006) or any other systems that replace conventional glass micropipettes by microsized apertures in a chip (Dunlop et al. 2008). By contrast to cells, such devices have only been recently explored for proteoliposomes (Kreir et al. 2008). Nevertheless, the residual presence of detergent remaining from the process of proteoliposome formation has been shown to hamper optimal seal formation. Since the development of patch-clamp robots is determinant for the screening of drug molecules on membrane receptors and ion channels, many efforts have been made in the last 10 years to automate and parallelize patch-clamp analysis (Dunlop et al. 2008). In this context our approach can open new insights in the rapid and simple integration of proteins from proteoliposomes into GUVs, without any detergent or other substances, and could be attractive to facilitate automated routine screening procedures.

Conclusion

This study reports an original and versatile approach for membrane protein reconstitution, based on spontaneous fusion between GUVs used as acceptors and VDAC-proteoliposomes used as donors. Compared with other proteo-GUV formation methods and due to its practicality, this work represents a useful approach for protein membrane reconstitution, suitable for automation and parallelization of patch-clamp analysis.

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