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Assessing the efficacy of vesicle fusion with planar membrane arrays using a mitochondrial porin as reporter

Kamila Pszon-Bartosz [a,b], Jesper S. Hansen [a,b,1], Karin B. Stibius [a,c,1], Jesper S. Groth [a], Jenny Emnéus [b], Oliver Geschke [b], Claus Hélix-Nielsen [a,c,*]

[a] Aquaporin A/S, Ole Maaløes Vej 3, DK-2200 Copenhagen N, Denmark
[b] Technical University of Denmark, Department of Micro- and Nanotechnology, DK-2800 Kongens Lyngby, Denmark
[c] Technical University of Denmark, Department of Physics, DTU Physics, DK-2800 Kongens Lyngby, Denmark

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ABSTRACT

Reconstitution of functionally active membrane protein into artificially made lipid bilayers is a challenge that must be overcome to create a membrane-based biomimetic sensor and separation device. In this study we address the efficacy of proteoliposome fusion with planar membrane arrays. We establish a protein incorporation efficacy assay using the major non-specific porin of *Fusobacterium nucleatum* (FomA) as reporter. We use electrical conductance measurements and fluorescence microscopy to characterize proteoliposome fusion with an array of planar membranes. We show that protein reconstitution in biomimetic membrane arrays may be quantified using the developed FomA assay. Specifically, we show that FomA vesicles are inherently fusigenic. Optimal FomA incorporation is obtained with a proteoliposome lipid-to-protein molar ratio (LPR) = 50 more than 10^5 FomA proteins could be incorporated in a bilayer array with a total membrane area of 2 mm^2 within 20 min. This novel assay for quantifying protein delivery into lipid bilayers may be a useful tool in developing biomimetic membrane applications.

1. Introduction

There is a growing interest in mimicking biological membranes and create membrane-based sensor and separation devices. However, there are many challenges that must be overcome in order to build biomimetic membrane devices for industrial applications. One of the challenges is to ensure sufficient delivery of functional protein to the lipid matrix [1]. Fusion of lipid vesicles containing proteins (proteoliposomes) with a planar membrane has been used for many years and it is a powerful technique to incorporate membrane proteins into planar lipid bilayers [2,3]. In particular this method has been valuable for studies were incorporation of only few proteins (or even single proteins) is sufficient. However, large scale systems based on lipid bilayers will often require substantial amounts of incorporated proteins. It therefore is of interest to evaluate the up-scaling potential of vesicle based protein delivery.

Optimization of proteoliposomes fusion with biomimetic membranes depends on increasing proteoliposome fusogenicity. Theoretical studies indicate that the early stages of fusion involve formation of a fusion pore—or intermediate, a neck-like connection between two bilayers, with an initial size of about 10 nm [4,5]. The fusion time scale has not been measured experimentally, but patch-clamp electrophysiology [6] and ultrafast optical microscopy of giant vesicles [7] indicate that the fusion pore can be formed in less than 100 μs.

Fusogenicity may be increased by lowering the energetic barriers for formation of fusion intermediates. Vesicle fusion may be induced by lipids having opposite charges in vesicle and receiving planar membrane, respectively [8,9]. Another strategy involves the use of fusogenic peptides in the two fusing membranes as demonstrated by the use of soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) and associated (SNARE) receptor

Abbreviations: BFS, bilayer forming solution; BLM, black lipid membrane; DOPC, 1,2-dioloeyl-*sn*-glycero-3-phosphocholine; DPhPC, 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine; ETFE, ethylene tetrafluoroethylene; FomA, major outer membrane protein of *Fusobacterium nucleatum*; LDAO, *N*-lauryl-*N*,*N*-dimethylammonium-N-oxide; LPR, lipid to protein molar ratio; NBD-PC, 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine; NTA, nanoparticle tracking analysis; OG, octyl-β-D-glucopyranoside; PBS, phosphate buffered saline; PEG, polyethylene glycol; SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP); SNARE, SNAP receptor protein; Texas Red®, sulforhodamine 101 acid chloride; VDAC, mitochondrial voltage dependent anion channel.

* Corresponding author. Address: Technical University of Denmark, Department of Physics, DTU Physics, Fysikvej 309, Building 309, Office 138, DK-2800 Kongens Lyngby, Denmark. Fax: +45 45 93 16 69.
E-mail address: claus.helix.nielsen@fysik.dtu.dk (C. Hélix-Nielsen).

[1] These authors have contributed equally to this work.

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leading to the formation of SNAP–SNARE complexes and vesicle fusion [10,11]. Fusion can also be driven solely by osmotic gradients across the planar receiving membrane provided that the vesicles are permeable to solute [12]. The latter has been demonstrated using vesicles with the antibiotic nystatin and ergosterol [2,3,13]. Nystatin–ergosterol channel complexes are weakly anion-selective and thus render the vesicle solute permeable. Upon fusion with an ergosterol-free planar membrane fusion events are evident as ion current transients reflecting the dissolution of the nystatin–ergosterol channel complexes.

The nystatin–ergosterol method is a powerful technique for protein incorporation but as the single-channel conductance for the nystatin–ergosterol complex is not a well-defined entity one cannot determine quantitatively the amount of protein delivered to the planar membrane.

However, by using ion channels that remain open after incorporation into the planar membrane one may asses the amount of inserted protein by detecting the increase in bilayer macroscopic conductance *G* after transfer of ion channels from the vesicle membrane to the bilayer. When the single-channel conductance *g* is known one may use the ratio *G/g* to estimate the total number of (ion channel) proteins that has been incorporated by fusion during this process (the fusion efficacy). This method has been demonstrated by using the voltage dependent anion channel (VDAC) [14,15]. With this method about ~100 fusion events corresponding to the insertion of about 500 VDACs in 10 min per mm^2 membrane area could be obtained. Still VDAC incorporation has to be driven by applying an osmotic gradient across the planar membrane and by addition of divalent cations.

Here we speculated if one could use the major outer membrane protein of *Fusobacterium nucleatum* (FomA) as a reporter for vesicle fusion with planar membranes—even in the absence of a transmembrane osmotic gradient. The rationale behind this comes from the suggestion that FomA participates in coaggregation between periodontal *F. nucleatum* and *Streptococcus sanguis* bacteria by direct binding [16]. FomA forms trimeric β-barrels where each barrel is predicted to consist of 14 β-strands [17] and the single-channel conductance is known [18]. Thus it should be possible to quantify proteoliposome fusion with planar membranes via the *G/g* ratio. Since porins can be tailored to specific functions [19] vesicles with fusigenic engineered FomA porins could also constitute building blocks for functional planar biomimetic membranes *per se*. In this study we assessed FomA vesicle fusogenicity as quantified by *G/g* and how FomA incorporation into planar membranes depends on proteoliposome lipid-to-protein ratio (LPR) and incubation time.

2. Materials and methods

2.1. Reagent and materials

Tefzel ETFE LZ200 Fluoropolymer for the fabrication of multi-aperture partitions, and Viton A Fluoroelastomer for the production of rubber chamber sealing rings were from DuPont Fluoropolymers (Detroit, USA). Uncoated 35- and 50-mm glass-bottom culture dishes were purchased from MatTek Corporation (Ashland, MA, US). The lipids 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC), 1,2-dioloeyl-*sn*-glycero-3-phosphocholine (DOPC), and 1-oleoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphocholine (NBD-PC) were from Avanti Polar Lipids Inc. (Alabaster, AL, USA). *n*-Decane (Fluka) was purchased from Sigma–Aldrich Denmark (Brøndby, Denmark). The *F. nucleatum* outer membrane protein A (FomA) was kindly provided by Dr. Jörg H. Kleinschmidt (Universität Konstanz, Konstanz, Germany). All other chemicals were of analytical grade and purchased from commercial sources.

2.2. Preparation of bilayers forming solution (BFS) and proteoliposome suspensions

The lipid bilayer forming solution (BFS) consisted of DPhPC in *n*-decane (25 mg/ml) doped with 1 mol% NBD-PC. The lipid solutions were prepared the day before the experiment, and stored at −20 °C until use.

Liposomes were prepared from thin film of DOPC lipids dissolved in PBS containing 1 wt% OG (octyl-β-D-glucopyranoside) (Anatrace, Affymetrix Europe, High Wycombe, UK) to a final lipid concentration of 10 mg/ml. The lipid solution was sonicated for 1 min and subjected to five then freeze–thaw cycles. After 1 min of sonication, the lipid solution was extruded 12 times through a barrel extruder (Lipex™ Extruder, Northern Lipids Inc., Burnaby, Canada) with two polycarbonate filters (Whatman, Maidstone, UK) with 200 nm pore sizes at 20 bar pressure. FomA dissolved in 10 mM borate buffer (9.6 mg/ml, pH 10 with 2 mM EDTA) was refolded in *N*-lauryl-*N*,*N*-dimethylammonium-N-oxide (LDAO) micelles (LDAO/FomA mol:mol ratio = 1000). Before mixing with liposomes the protein was labeled with Texas Red® (Sigma–Aldrich Denmark, Brøndby, Denmark) with a substitution degree of 0.4 mol dye/mol protein. Liposomes were mixed with the protein solution and dialyzed in Spectra/Por® MicroDialyzers (Spectrum Laboratories Inc., Breda, The Netherlands) for 24 h against PBS to remove the detergent. Samples with LPR = 25, 50, 100 and 200 were prepared and diluted with PBS to a final lipid concentration of 0.16 mg/ml.

2.3. Liposomes and proteoliposomes characterization

Size distribution analyses of liposomes and proteoliposomes were performed with NanoSight LM10 and NTA 2.0 Analytical software (NanoSight Ltd., Amesbury, UK). Around 1 ml of 0.001 mg/ml sample was needed to perform each measurement three times. The samples were measured for 90 s with manual shutter and gain adjustments. Mean and standard deviation values were obtained from mono-modal model fittings using the nanoparticle tracking analysis (NTA) 2.0 software.

2.4. BLM array formation and characterization

Planar horizontal lipid bilayers were established across microstructured ETFE partition arrays as previously described [20,21]. Briefly, ~0.5 μl of the BFS was deposited onto the partition array. To thin the membranes into bilayers, a sterile plastic inoculation loop with a 1 μl loop capacity (Sarstedt Nümbrecth, Germany) was used. The thinning process was carried out by gently sweeping the inoculation loop across the entire BLM array prior to addition of proteoliposomes.

Automated voltage-clamp measurements and data processing were carried out as previously described [22]. Fluorescent imaging was performed on a Zeiss Axiovert 200 M epifluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a monochrome Deltapix DP450 CCD camera (Deltapix, Maalov, Denmark). Images were acquired using Deltapix DpxView Pro Acquisition software (Deltapix, Maalov, Denmark). Objectives used were air-corrected Plan-Neofluar 2.5×/0.075 Numerical Aperture (NA) and 10×/0.25 NA, respectively.

Incorporation of proteoliposomes with different LPR values into an array of planar membranes was established by adding 2 μl of proteoliposomes with 0.16 mg/ml lipid concentration in close proximity to the lipid bilayer. The aqueous chamber solution

consisted of PBS buffer. The applied potential across the partition arrays was 60 mV.

3. Results and discussion

3.1. FomA proteoliposomes insert spontaneously into planar membranes

We first characterized FomA DOPC proteoliposome fusion with an array of planar membranes and assessed protein functional activity by voltage-clamp recordings of bilayers formed from (DPhPC)/decane solutions separating two aqueous compartments containing PBS. Stepwise increases of the current across the bilayer were observed when FomA proteoliposomes were applied in close proximity of the membrane (Fig. 1a, upper current trace). No transmembrane currents were observed for the DPhPC bilayer alone (Fig. 1a, middle current trace) or when protein-free DOPC liposomes were applied in close proximity of the DPhPC membrane (Fig 1a, lower current trace). The conductance amplitude histogram reveals a broad distribution with mean value of 0.07 nS for a total of 139 fusion events. This value can be compared with the 1.17 nS measured for FomA trimeric channel conductance in 1 M KCl [18]. Scaling the 1 M KCl FomA conductance value according to the conductivity ratio between PBS and 1 M KCl conductivity the scaled conductance value is 0.08 nS in good agreement with our data. These results demonstrate that functionally active FomA was successfully reconstituted into an array of planar membranes at physiologically relevant electrolyte concentrations. Fusion occurred spontaneously when FomA proteoliposomes were placed close to the membrane array in the absence of osmotic gradients across the membrane needed in other fusion methods, e.g. [3].

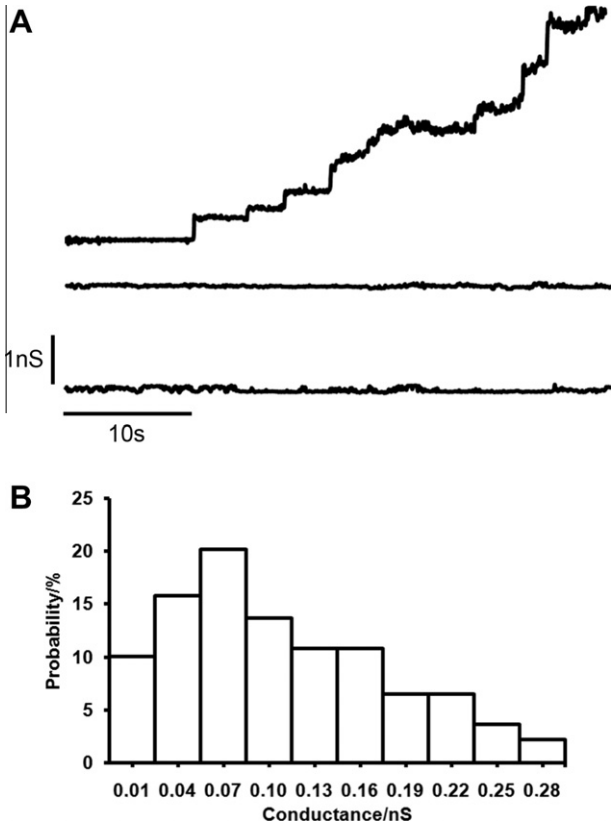

Fig. 1. FomA channels recordings demonstrate proteoliposomes fusion into an array of planar membranes. (A) Conductance across: an array of DPhPC membranes in PBS buffer after addition of DOPC–FomA proteoliposomes (upper trace), an array of DPhPC membranes alone in PBS buffer (middle trace), and after addition of FomA free liposomes (lower trace). (B) Histogram of the probability of the occurrence of a given conductivity unit (139 fusion events were recorded) during DOPC–FomA proteoliposomes, with LPR 200, fusion into 8 × 8 array of DPhPC planar membranes. The applied potential across the partition arrays was +60 mV. The conductance of single FomA channel in PBS was in the range 0.01–0.28 nS with a maximum probability for a conductance of 0.07 nS.

3.2. FomA proteoliposomes have an upper limit LPR

In order to evaluate the influence of the protein content of the proteoliposomes we performed fusion experiments with proteoliposomes prepared with different LPRs and this is shown in Fig. 2a. The highest fusion efficacy (measured as G/g) was achieved with LPR 50 (see Table 1). Fusion efficacy increases when the LPR value decreases from 200 to 50. However, with LPR = 25 functional protein incorporation is close to that observed for LPR = 200. A reason for the low efficacy at low LPR may due to the fact that an LPR = 25 corresponds to a protein area coverage of 64%. This may be the upper limit for FomA protein reconstitution in proteoliposomes before protein precipitation occurs. It may thus be that only small amounts of protein were reconstituted into proteoliposomes at the nominal LPR = 25 leading to concomitant low protein incorporation with the planar membranes. There were no significant size differences between protein-free liposomes and proteoliposomes with nominal LPR = 200, 100 and 50 as evidenced by nanoparticle tracking analysis (see Fig. 2b). Proteoliposomes with nominal LPR = 25 has a mean diameter of 200 nm which is significantly different from the observed liposome diameter of 150 nm. Although we do not know the reason for the apparent size difference it may be related to the very high protein concentration (and potential precipitation) during the mixing and dialysis steps in proteoliposome production.

3.3. Proteoliposome fusion kinetics and membrane stability

In order to assess how proteoliposome LPR is related to fusion kinetics and membrane stability we analyzed membrane currents I ($I \sim G/g$) over time with the two LPR values giving the highest efficacy (LPR = 50 and 100) and this is presented in Fig. 3a. After

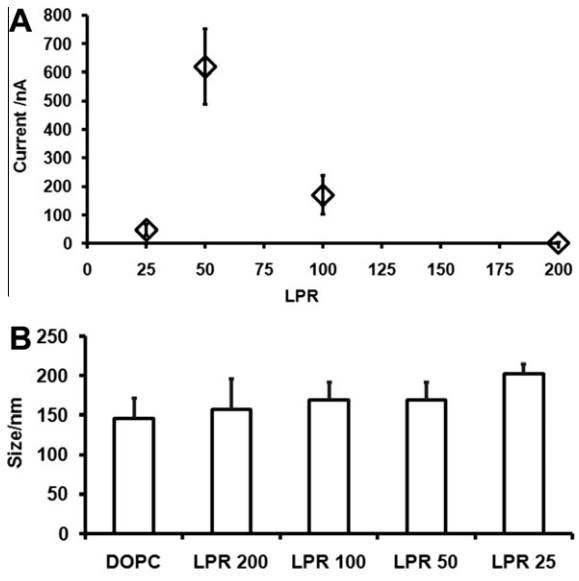

Fig. 2. (A) Maximal FomA proteoliposomes fusion into an array of planar membranes. The applied potential across the partition arrays was +60 mV. (B) Liposome/proteoliposome diameters. Mean values are based on mono-modal model fittings applied to the experimental data by NTA (nanoparticle tracking analysis) software (error bars represent SD, $n = 3$).

Table 1
Proteoliposome fusion efficacy.

LPR[a]	Proteoliposome FomA coverage[b] (%)	Number of FomA proteins per proteoliposome[c]	Maximal fusion efficacy G/g[d]
25	64	5146 ± 933	11,429 ± 4343
50	47	2682 ± 971	147,619 ± 31,527
100	31	1754 ± 635	40,417 ± 16,020
200	18	934 ± 618	524 ± 179

[a] Lipid to protein molar ratio at the step of proteoliposomes preparation.
[b] Assuming 0.72 nm^2 lipid polar head surface and 16 nm^2 FomA monomer area.
[c] Calculation based on the nominal LPR and the size of proteoliposomes determined by NTA measurements.
[d] Assuming single FomA conductance = 0.07 nS.

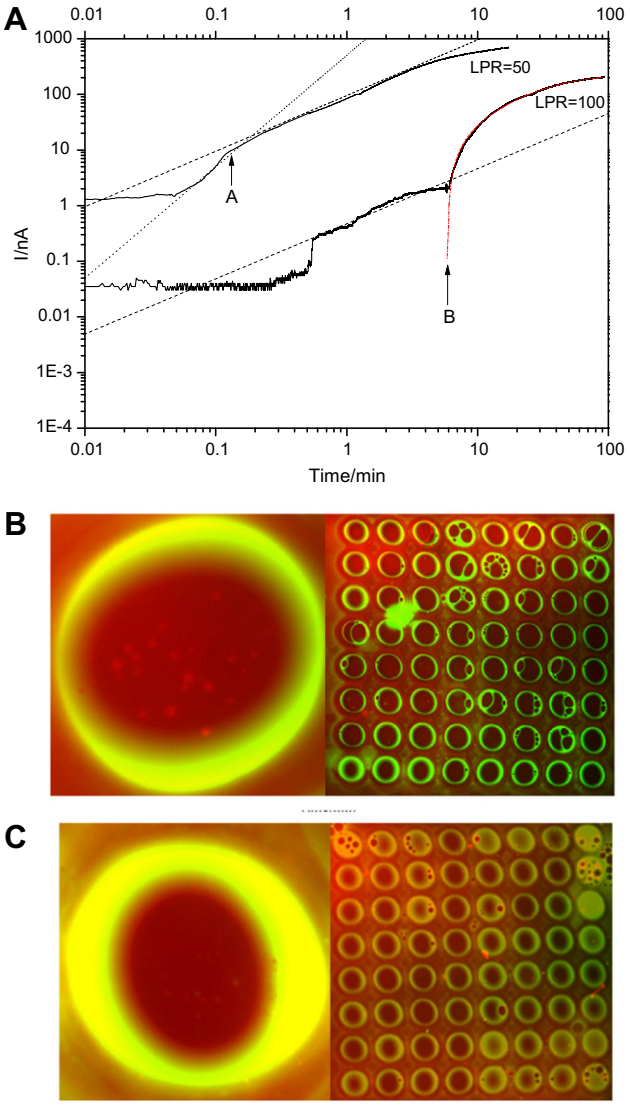

Fig. 3. Characterization of proteoliposome fusion into planar membranes. (A) Total membrane current I versus time t for proteoliposome LPR = 50 and 100. Straight (guiding) lines indicate first order ($I \sim t$) (dashed lines) and second order ($I \sim t^2$) (dotted lines) proportionality, respectively. Arrow A indicates transition from second to first-order kinetics and arrow B indicates onset of exponential kinetics: $I \sim A \cdot (1 - \exp(-t/\tau))$. Dashed-dot line (red) represents a fit to the exponential kinetics equation with τ = 30.1 min. (B) Combined fluorescent images of FomA (red) proteoliposomes with LPR = 50 fused into planar membranes (left: single aperture; right: the 8 × 8 bilayer array). (C) As in (B) with LPR = 100. Images were acquired with a 20× (B and C, left) and 2.5× (B and C, right) air-corrected objective. Average ± SD outer aperture diameter for the 8 × 8 array is 300 ± 5 μm. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

addition of 2 μl proteoliposomes (0.16 mg/ml) with LPR = 50 fusion efficacy showed an initial lag phase followed by a second order rise phase ($I \sim t^2$) within the first 6 s. This is followed by a longer first order ($I \sim t$) rise phase and finally I is levelling off approaching a saturating level. For LPR = 100 the initial lag phase is shorter and followed by a longer first order rise phase until 6 min (the jumps just after 0.53 min reflect insertion of multiple FomA proteins, cf. Fig 1a). At 6 min an inoculation loop was swept across the entire membrane array. The induced stirring causes the incorporation to follows an exponential time course until a saturating level is reached after 90 min. In cases where some membranes in the array broke during the first order rise phase they could be recreated by careful local application of the inoculation loop and the efficacy level resulting in current levels returning to the level just prior to membrane breakage. Although LPR = 100 results in free-standing membranes reaching saturating incorporation levels, fusion with proteoliposomes having LPR = 50 reach several fold higher levels within 10 min.

Thus one may use either slow first-order kinetics corresponding to passive diffusion of the proteoliposomes up to the membrane, or use stirring for rapid delivery of vesicles to the receiving membrane. The two different kinetic schemes may be considered as part of a microfluidic design where flow conduits and rates may be adjusted for various types of flow, e.g. turbulent flows for creating efficient stirring of the membrane interfacial regions. Subsequently membranes may be stabilized, e.g. via hydrogel encapsulation [23].

Table 1 shows the maximal fusion efficacy (G/g) corresponding to the effective number of trimeric FomA channels incorporated. For LPR = 100 the total membrane conductance corresponds to about 40,000 FomA trimers whereas for LPR = 50 a total of 150,000 FomA trimers appears to have been inserted. With the theoretical numbers for single proteoliposome conductances based on their LPRs this corresponds to insertion of about 54 and 22 proteoliposomes, respectively. However, this is under the assumption that all proteoliposomes have functional protein content corresponding to their LPR and that all proteins retain their functionality after insertion. This may not be the case, and there may be non-functional protein material inserted the membrane together with the functional FomA channels. However, we note that the membrane conductance levels off with time for both LPR = 50 and 100 approaching a steady state. If the membrane stability was generally compromised due to the presence of non-functional (mis-folded) protein this would be manifest in large conductance fluctuations and membrane rupture. We do not see evidence of this and conclude that the receiving membrane may contain non-functional protein but that this does not compromise membrane overall integrity.

In order to further characterize proteoliposome fusion into planar membranes we simultaneously monitored the membrane array optically and electrically. Using the fluorescent lipid analog NBD-PC (1 mol%) in the membrane bilayer forming solution and Texas Red® labeled FomA we could observe bilayers formation and protein incorporation and this is shown in Fig. 3b. For both LPR = 50 and 100 we observed high-intensity dots in some membranes in the array moving within the membrane area delimited by the Plateau–Gibbs border. This heterogeneity may reflect that the incorporated protein aggregate in the membrane or that proteoliposomes (or proteins) aggregate on the membrane surface. The heterogeneity is most pronounced for LPR = 50 which may reflect the high protein concentration (and possible precipitation) for LPR = 50 proteoliposomes.

In summary, we established an outer membrane porin fusion efficacy assay and used this to quantify proteoliposomes fusion with an array of planar membranes. We showed the influence of LPR on FomA proteoliposomes reconstitution yield. Maximal fusion obtained was almost 150,000 porin insertions during 20 min with

proteoliposomes prepared with LPR = 50. Incorporation could be established as a process with either first order or exponential kinetics. This may be of interest to microfluidic designs involving protein delivery to biomimetic membranes developed for sensor and separation applications.

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