Properties of nonfused liposomes immobilized on an L1 Biacore chip and their permeabilization by a eukaryotic pore-forming toxin

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Abstract

The L1 chip is used intensively for protein–membrane interaction studies in Biacore surface plasmon resonance systems. The exact form of captured lipid membranes on the chip is, however, not precisely known. Evidence exists that the vesicles both remain intact after the binding to the chip and fuse to form a large single-bilayer membrane. In this study, we were able to bind up to approximately 11,500 resonance units of zwitterionic liposomes (100 nm in diameter) at a low flow rate. We show by fluorescence microscopy that the entire surface of the flow cell is covered homogeneously by liposomes. Negatively charged vesicles (i.e., those composed of phosphatidylcholine/phosphatidylglycerol [1:1]) always deposited less densely, but we were able to increase the density slightly with the use of calcium chloride that promotes fusion of the vesicles. Finally, we used zwitterionic liposomes loaded with fluorescent probe calcein to show that they remain intact after the capture on the L1 chip. The fluorescence was lost only after we used equinatoxin, a well-studied pore-forming toxin, to perform on-chip permeabilization of vesicles. The characteristics of permeabilization process for chip-immobilized liposomes are similar to those of liposomes free in solution. All results collectively suggest that liposomes do not fuse to form a single bilayer on the surface of the chip.
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Keywords: Biacore; L1 chip; Liposomes; Pore-forming toxin; Equinatoxin

Biosensor technology based on surface plasmon resonance (SPR)[1] is accepted as a standard technique in biochemistry and other related sciences because it can give reliable kinetic data on the interaction of various molecular partners. One of the less explored interactions is that of proteins and membranes. These are hugely important for many biological processes, but only a fraction of published biosensor literature is devoted to them [1]. Advantages of SPR over other biochemical and biophysical techniques include speed, direct monitoring of binding without the need for labeling ligands, direct determination of association and dissociation rates, and small amounts of sample needed (usually nanomolar concentrations) [2]. In a typical experiment, a membrane is immobilized on the surface of the chip and the protein of interest is passed over the surface. Different approaches to membrane immobilization are described in the literature. It is possible to immobilize intact liposomes on the chip by binding to specific proteins that recognize a particular membrane component, for example, biotinylated lipid [3,4] or lipopolysaccha-

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ride [5]. Biacore provides the L1 chip that has covalently linked lipophilic anchors on a dextran matrix. Liposomes are stably retained on this surface at low flow rates [6,7]. A variant of this approach tethers bilayer membranes with the use of thiolipids attached on the surface of the gold chip [8,9]. It is easy to prepare stable lipid surface reproducibly under various experimental conditions, such as temperature, pH, and dimethyl sulfoxide concentration [10], by these approaches. Such surfaces provide a chemically and physically stable environment that mimics cell membranes. During recent years, therefore, SPR has been used for monitoring of membrane binding of peptides [11], pore-forming toxins [12], and peripheral proteins participating in membrane-mediated cell signaling [2,13]. It has also been used for the screening of drugs binding to lipid membranes, that is, as an important step in preclinical drug discovery [10,14–16].

The L1 chip is probably the most popular SPR approach in protein–membrane interaction studies. However, the exact nature of the lipid membrane preparation on the surface of the L1 chip is controversial. Cooper et al. [6] performed the first characterization of surface generated on the L1 chip after liposome injection. By confocal microscopy, they were able to show that sulforhodamine-loaded liposomes stay intact on the surface of the chip. In the same study, they showed that such prepared surfaces enable kinetic analysis of binding to ligands captured in the vesicles [6]. However, during that same year, Erb et al. [7] showed that liposomes do not stay intact but rather form a continuous bilayer on the surface of the chip. In the current study, we extended these previous studies. Here we show, by the use of calcein-loaded vesicles, that liposomes do not fuse onto the surface of the L1 chip. Liposomes with excess negative charge were always deposited less on the chip in comparison with zwitterionic liposomes due to electrostatic repulsion. Furthermore, we were able to permeabilize chip-immobilized vesicles with the well-characterized pore-forming toxin equinatoxin under conditions closely resembling free liposomes in solution, that is, performing binding and permeabilization experiments simultaneously. This approach might be particularly useful for pore-forming proteins that are difficult to obtain in large quantities.

Materials and methods

Materials

All materials were obtained from Sigma (USA) unless stated otherwise. Lipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DOPG), and bovine brain

sphingomyelin (SM) were obtained from Avanti Polar Lipids (USA). 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (β-pyr-C10-HPC) was from Molecular Probes (USA). Native isoform of equinatoxin, equinatoxin II (EqtII), was isolated as described previously [17]. Mutant EqtIIG27C was isolated and labeled with N-[(2-(iodoacetoxy)ethyl]-N-methyl)amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD, Molecular Probes), to obtain EqtIIG27C-NBD, exactly as described previously [18].

Preparation of large unilamellar vesicles

Lipids, at a desired mixture and concentration, were dissolved in chloroform. They were dried on the bottom of a round flask on a rotavapor for at least 3 h to remove all traces of chloroform. Multilamellar vesicles were generated by the addition of vesicle buffer (140 mM NaCl, 20 mM Tris–HCl, 1 mM ethylenediamine tetraacetic acid [EDTA], pH 8.5) to the dried lipid film. The buffer with lipids was vortexed vigorously in the presence of glass beads and was freeze-thawed six times. Multilamellar vesicles generated as such were extruded through polycarbonate filters with 100-nm pores as described previously [19]. Such preparation yields unilamellar liposomes with a well-defined and narrow distribution of size with an average of approximately 100 nm [20,21]. Vesicle buffer contained 60 mM calcein when large unilamellar vesicles (LUVs) loaded with calcein were prepared. The excess of calcein was removed after extrusion by passing the LUV solution through a small Sephadex G50 column. The concentration of lipids with a choline head group (DOPC and SM) was determined with a Phospholipids B Kit (Wako Chemicals, Germany).

Surface plasmon resonance measurements

A Biacore X apparatus (Biacore, Sweden) equipped with the L1 chip was used for surface plasmon resonance (SPR) measurements at 25 °C using vesicle buffer as the running buffer. The buffer solution was always filtered through a 0.22-μm filter and degassed thoroughly. The surface of the chip was conditioned with three consecutive injections for 1 min at 30 μl/min of isopropanol/50 mM NaOH (2:3, v/v, LUV wash solution). The same solution was also used for the regeneration of the chip surface after the experiment was finished. Liposomes were deposited on both flow cells for 10 min at a low flow rate of 1 μl/min. For the rest of the experiment, the flow rate was kept to 30 μl/min.

Fluorescence measurements

Fluorescence was measured with a Jasco FP-750 fluorimeter in vesicle buffer at 25 °C. The excitation and

emission wavelengths were 485 and 520 nm, respectively. Calcein release experiments were performed in 1.2 ml of the total volume. Liposomes at 25 μM lipid concentration were stirred in the cuvette, and then EqtII was added to obtain the desired concentration. The release was followed to reach a steady state, and then the maximal release was induced by the addition of 2 mM final Triton X-100. The percentage of permeabilization was calculated according to the following equation:

$$P(\%) = [(F_{90} - F_{init})/(F_{max} - F_{init})] * 100, \tag{1}$$

where F_{90}, F_{init}, and F_{max} stand for fluorescence values 1.5 min after the addition of EqtII, before the addition of EqtII, and after the addition of Triton X-100, respectively. Permeabilization of L1-chip immobilized LUVs was calculated from the fluorescence values according to the following equation:

$$P(\%) = [F_{eqt}/(F_{eqt} + F_{wash})] * 100, \tag{2}$$

where F_{eqt} and F_{wash} stands for fluorescence values of the buffer collected from the Biacore X after the addition of EqtII and desorption of lipids from the chip with LUV wash solution, respectively.

Fluorescence microscopy and image analysis

The L1 chip was imaged under a fluorescence microscope immediately after liposome binding. The chip was undocked, and vesicle buffer was applied over both flow cells and covered by a glass cover slide. The chip prepared as such was photographed using an Axioskop 2 MOT microscope with 10× objective and an AxioCam MRc color digital camera (Carl Zeiss, Germany). β-pyr-C10-HPC-containing LUVs were detected by UV light excitation (365 nm bandpass excitation and 397 nm longpass emission filter). LUVs with EqtIIG27C-NBD-bound or calcein-loaded immobilized liposomes were detected using blue light excitation (450–490 nm bandpass excitation and 515 nm longpass emission filter).

Fluorescence images were acquired with AxioVision 3.1 software (Carl Zeiss Vision, Germany) and further processed in ImageJ 1.34 [22]. Different experiments were analyzed by comparing the gray value profiles in the direction perpendicular to the flow cell on the L1 chip. The gray value profile represents the relative fluorescence intensity of the vertical pixel lines in the area where the profile is measured. Images used for semiquantitative comparisons of gray value profiles were acquired with the same microscope and camera settings. Color images were split into three gray scale images representing the red, green, and blue channels, and only the channel with the strongest signal was used in subsequent analyses. Uneven background in the image was corrected with the Shading Corrector plug-in in ImageJ using the image of an empty field on the chip showing background fluorescence. On each of these corrected images, the profile of gray values was measured. In some cases, the fluorescence signal was very strong and the background was still significantly uneven after shading correction. In these cases, the profile of gray values was measured on uncorrected images, and a polynomial function was fitted to the background segments of the profile and then subtracted from the profile using GraphPad Prism 4.02 (GraphPad Software, USA) to get a profile with a flat background signal.

Results

The amount of liposomes deposited on the L1 Biacore chip is dependent on the concentration of liposomes injected over the chip (Fig. 1A). By using zwitterionic lipids (the mixture of DOPC/SM [1:1, mol/mol] in Fig. 1A), a maximal response of approximately 11,000 resonance units (RU) was obtained routinely. This was achieved with 2 mM lipids injected over the chip at 1 μl/min for 10 min. Similar responses were obtained with other zwitterionic liposomes, that is, those composed solely of POPC or DOPC. At high concentrations of lipids (i.e., ∼1 mM), longer contact times did not increase the amount of bound LUVs. There is a limited number of liposome binding sites on the chip given that subsequent injections of the same concentration of lipids for the same duration did not significantly increase the amount of bound material (Fig. 1B). Therefore, response rarely exceeded 12,000 RU. In addition, no increase in signal was obtained when 0.1 mg/ml bovine serum albumin (BSA) was used to block an L1 chip previously saturated with liposomes (inset in Fig. 1B), further indicating that the surface is fully covered by lipids. In contrast, when BSA was injected over the L1 chip alone, there was a considerable increase in the resonance signal. We used washes of 100 mM NaOH and water to remove loosely bound liposomes. After the NaOH wash, the decrease was very small (Table 1); therefore, bound zwitterionic LUVs were stably retained on the chip.

It is possible to visualize the surface of the chip under the fluorescence microscope using LUVs with a small amount of fluorescent lipid (in our case, we used DOPC/SM/β-pyr-C10-HPC [50:50:0.5, mol/mol/mol] LUVs, Fig. 2A). A homogeneous fluorescence signal was observed across the entire flow cell when the surface was saturated with the liposomes by using the above conditions. The intensity of the signal was dependent on the concentration of LUVs injected over the surface of the chip. So, when lower concentrations of LUVs were injected over the chip, the surface was stained homogeneously but at a much lower intensity (trace b in Fig. 2D).
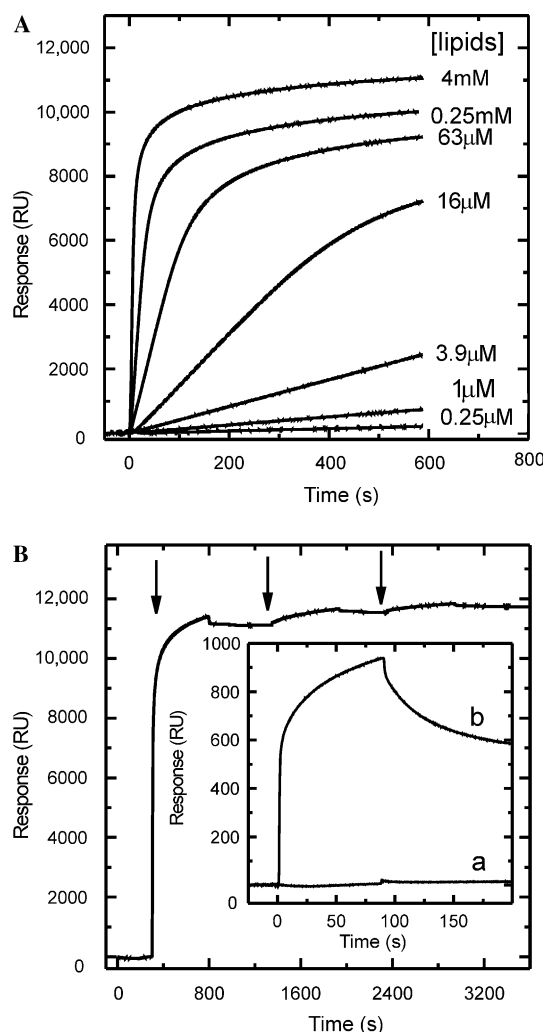
Fig. 1. Binding of LUVs to the L1 chip. (A) Binding of LUVs at different lipid concentrations to the L1 chip. LUVs were bound for 10 min at 1 μl/min. (B) Three subsequent injections of 2 mM DOPC/SM (1:1). The conditions were as in (A). The injections are marked by arrows. The inset shows response obtained after 1 min injection of 0.1 mg/ml of BSA over the L1 chip surface covered with approximately 11,000 RU lipids (a) or the L1 chip without lipids (b). The running buffer in all three cases was 140 mM NaCl, 1 mM EDTA, and 20 mM Tris–HCl (pH 8.5).

We routinely obtained lower responses when negatively charged vesicles (i.e., DOPC/DOPG [1:1, mol/mol] or DOPG) were used (Table 1). After 100 mM

NaOH wash, there was only approximately 7800 RU of LUVs deposited on the chip (Table 1 and Fig. 3). This result indicates that excess of negative charges prevents crowding of liposomes on the surface of the chip due to electrostatic repulsion between vesicles. We tested this by washing chip-bound liposomes by solutions without salt that screens the negative charge of liposomes in vesicle buffer. Water alone or solution of glucose (0.31 M glucose in water) was used. Such treatment significantly reduces the amount of bound negatively charged vesicles as opposed to zwitterionic ones (Table 1 and Fig. 3). The treatment with water was the most rigorous in that only one-fourth of the initially bound negatively charged vesicles remained bound. In contrast, 75% of zwitterionic lipids remained bound after water treatment. However, water treatment might destruct vesicles due to osmotic imbalance, and 0.31 M solution of glucose was also used after NaOH wash. This concentration was found to osmotically match the vesicle buffer used in experiments. Indeed, the amount of stably bound vesicles was higher in comparison with water wash. Approximately 50% of charged vesicles remained on the surface, whereas the change in the case of zwitterionic vesicles was nearly negligible. Water, then, indeed destroys more fragile negatively charged vesicles, but zwitterionic vesicles seemed to be more resistant to osmotic changes.

It would be difficult to desorb the negatively charged liposomes so efficiently if fusion of vesicles on the surface of the L1 chip had occurred, and presumably a larger bilayer surface would be anchored more tightly to the chip. We incubated the liposomes after deposition at a low flow rate (1 μl/min) for more than 1 h to check whether longer times are needed for the fusion of vesicles. Such treatment did not increase the amount of bound liposomes in zwitterionic and negatively charged liposomes (data not shown), indicating that there is no extensive fusion occurring on the chip. Slightly larger amounts of bound liposomes were obtained when they were deposited on the chip in high salt, in the presence of 5 mM CaCl₂, or when liposomes were fused on the chip by 20 min injection of 5 mM CaCl₂ after they were already bound on the chip (data not shown).

The above-mentioned results indicate indirectly that single liposomes are bound to the chip under used conditions. As a more direct approach, we used calcein-

Table 1
Deposition of LUVs (in RU) on the L1 chip

Lipid	Before NaOH wash	After NaOH wash	After 0.31 M glucose	After water wash
DOPC	$11,730 \pm 740$	$11,650 \pm 810$	9560 ± 480	8710 ± 560
DOPC:DOPG 1:1	7880 ± 740	7860 ± 730	4290 ± 1120	2320 ± 1010
DOPG	8050 ± 570	7890 ± 600	4370 ± 1470	1720 ± 980

Note. The values presented are averages and standard deviations of responses after injection of 1 mM lipids for 10 min at 1 μl/min and three 1-min consecutive washes with 100 mM NaOH, 0.31 M glucose in water, or water. Four independent experiments were done for DOPC vesicles, seven for DOPC:DOPG 1:1 vesicles, and three for DOPG vesicles. This set of data was performed on four different L1 chips. See Fig. 3 for sensorgrams.
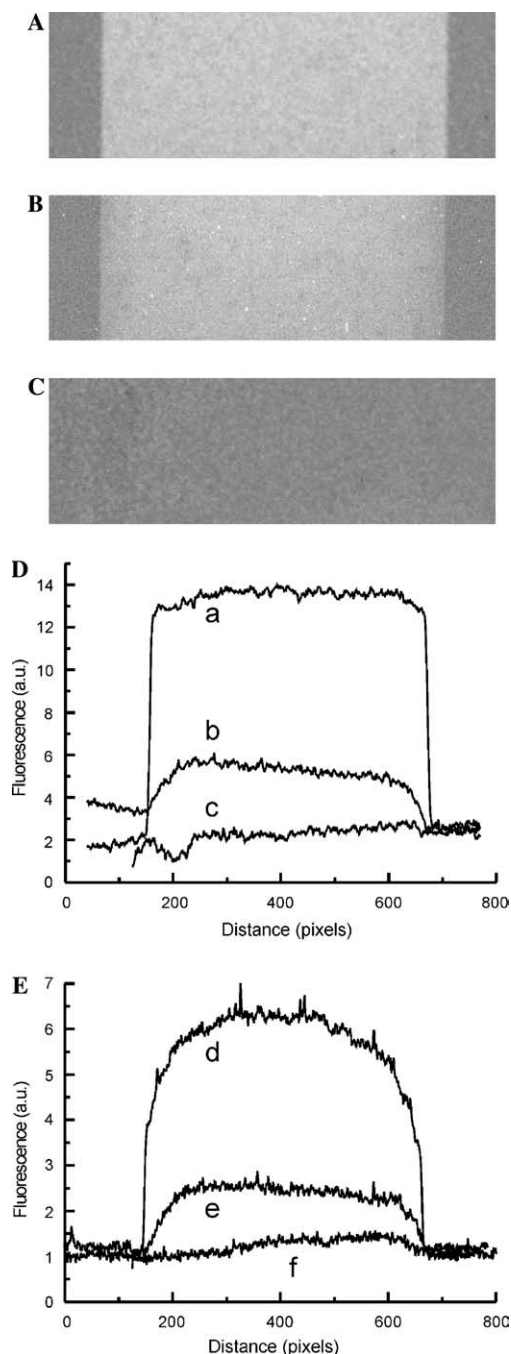
Fig. 2. Fluorescence of immobilized liposomes on the L1 chip. Images show part of a chip with the flow cell. Liposomes were immobilized by an injection of 4 mM DOPC/SM/β-pyr-C10-HPC (50:50:0.5, mol/mol/mol) LUVs for 10 min at 1 μl/min (A). In (B), 1 μM EqtIIG27C-NBD was bound to membranes after liposome immobilization. The chip was undocked after immobilization and was imaged immediately with the fluorescence microscope. Pyrene-containing LUVs (A) were visualized using UV light excitation, whereas LUVs with EqtIIG27C-NBD bound (B) were visualized using blue light excitation. Fluorescence intensity profiles across images in (A–C) are shown for pyrene-containing LUVs (D) and EqtIIG27C-NBD (E). Different amounts of liposomes were analyzed: approximately 10,000 RU (traces a and d) and 1400 RU (traces b and e). A blank cell (C) is also shown as a control in both cases (traces c and f). The surface of a flow cell was determined to be 1.23 mm^2 with a width of 0.54 mm. a.u., arbitrary units.

loaded vesicles to visualize them under a fluorescence microscope and check whether they remain intact. Calcein is a fluorescent dye that is negatively charged, has low membrane permeability, and is routinely used in studies of membrane-disrupting agents [23]. If liposomes rearrange to form a lipid bilayer, calcein should be released and little fluorescence should be observed under the fluorescence microscope. On the contrary, the L1 chip covered with calcein-loaded liposomes was brightly fluorescent (Fig. 4A). The fluorescence signal was dependent on the concentration of liposomes deposited on the chip, with low concentrations of calcein-loaded LUVs giving lower intensity (see trace c in Fig. 4D). But in both cases, the signal was homogeneously distributed over the surface of the chip.

Vesicles used in the above experiment were loaded with calcein at a concentration of approximately 60 mM. At this concentration, calcein self-quenching occurs and fluorescence is low. When vesicles are destroyed by the addition of detergent or some other substance that destroys membrane impermeability, calcein is released in the surrounding buffer and fluorescence increases. To see whether the material desorbed by the treatment with solutions without salt contained intact vesicles, we collected the buffer coming from the chip with attached calcein-loaded vesicles and measured the fluorescence in the presence and absence of Triton X-100. This ratio should be high (typically between 5 and 10) if intact liposomes are present in solution. As a control, we measured the ratio between fluorescence in the presence and absence of Triton X-100 (F_{Tx}/F) for LUVs in vesicle buffer. We found the following results: 6.0 ± 2.5, 7.0 ± 1.2, and 6.3 ± 1.4 for DOPC, DOPC/DOPG (1:1), and DOPG vesicles, respectively (results are averages of two independent vesicle preparations and standard deviations). The 100 mM NaOH washes always release some of the calcein, as was observed previously by Cooper and co-workers for sulforhodamine-loaded vesicles [6] (see also Fig. 5 inset on upper panel). This leakage of calcein was observed regardless of the membrane used, zwitterionic or negatively charged, and is probably due to increased membrane permeability at high pH [6]. In agreement with this proposition, the desorbed material contains very little intact vesicles given that the F_{Tx}/F ratio was 1.7 ± 1.0 ($n = 6$). In the case of DOPC/DOPG (1:1) vesicle, water and glucose washes removed quite a lot of material from the chip (Fig. 3). In this case, at least some vesicles are intact given that the F_{Tx}/F ratios were 2.9 ± 0.1 and 3.5 ± 1.4 ($n = 2$) for water and glucose washes, respectively. These values are roughly half of the values obtained for vesicles in solution, indicating that at least some liposomes are flushed intact from the chip. The F_{Tx}/F ratio for water wash was always found to be lower than that for the glucose wash, further supporting the above proposition that water treatment disrupts some
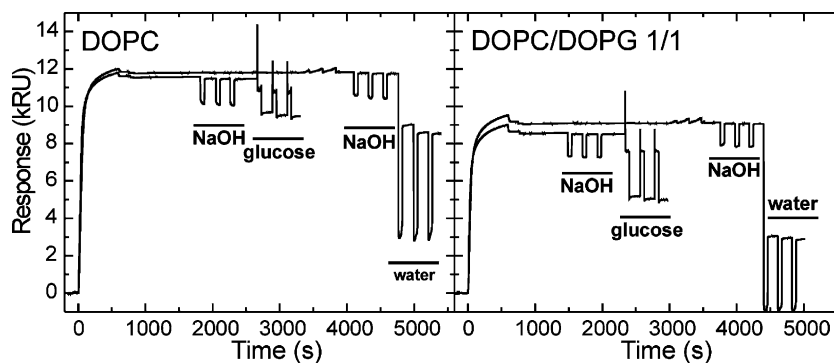
Fig. 3. Deposition of liposomes on the L1 chip. LUVs were deposited on the L1 chip for 10 min at 1 μl/min in the running buffer (140 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 8.5). The concentration of lipids in all cases was 1 mM. The flow rate was changed to 30 μl/min after the deposition, and liposomes were washed with three 1-min consecutive injections of NaOH and, finally, with three 1-min consecutive injections of 0.31 M glucose in water (this solution osmotically matches the running buffer) or of water (denoted by lines). All vesicles were in running buffer (140 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 8.5) during the injection.

of the vesicles. The F_{Tx}/F ratio for the treatment with LUV wash solution (isopropanol/50 mM NaOH [2:3, v/v]) that was used at the end of the experiment to remove everything from the chip was, as expected, approximately 1 (1.11 ± 0.25, $n = 6$).

We further tested the liposome-coated chip for the binding of EqtII. EqtII is a potent pore-forming protein from sea anemone that binds to lipid membranes and then oligomerizes in the plane of the membrane to form transmembrane pores [24]. Pores are permeable to small molecules that are rapidly released from liposomes [25,26]. An NBD-labeled mutant, EqtIIG27C-NBD, was used to show that under the conditions used (injection of 1.5 min at 30 μl/min), the toxin binds across the whole surface homogeneously (Fig. 2B). However, the calcein was released from the chip-immobilized liposomes only when the concentration of liposome-bound EqtII was high enough (Fig. 4B). For example, 25 nM EqtII bound fully to the liposomes. However, the calcein-loaded chip showed fluorescence intensity similar to one without toxin; therefore, the amount of bound toxin was not enough to permeabilize the liposomes (trace b in Fig. 4D). In contrast, when a 1 μM concentration of EqtII was used, the calcein was depleted from the vesicles and the surface was much less fluorescent (Fig. 4B and trace d in Fig. 4D).

We collected the buffer coming from the flow cell and measured the fluorescence of released calcein in a fluorimeter to determine the percentage of liposome permeabilization for different concentrations of injected EqtII. The typical experiment, shown in Fig. 5, presents the results for low (25 nM) and high (1 μM) EqtII concentrations. In agreement with fluorescence microscope data (Fig. 4), at a low EqtII concentration, nearly all of the fluorescence is obtained after the wash with LUV wash solution that removes all remaining liposomes from the chip at the end of the experiment; therefore, permeabilization was negligible (1.6%). At a high toxin con-

centration, the majority of the fluorescence is released during the toxin injection and very little at the washing step; therefore, the permeabilization is high (83.6%). The data also show that the majority of the vesicles are removed from the chip during the first injection of LUV wash solution. The data for all EqtII concentrations tested are presented on Fig. 6 together with amounts of EqtII bound measured by SPR after 3 min of dissociation (inset). There was a saturation of EqtII binding at concentrations greater than 1 μM. To compare permeabilization of chip-immobilized liposomes with those free in solution, we measured fluorimetrically the release of calcein from liposomes in a stirred cuvette setup (Fig. 7). To compare permeabilization values at similar EqtII concentrations on the Biacore, we determined the permeabilization of vesicles 1.5 min after the addition of the toxin (Fig. 6, open squares). The results are in good agreement despite the fact that it is hard to quantitatively collect the solution from the port of the Biacore apparatus.

Discussion

Our results, as well as those of other authors, showed that there is a limit in binding capacity of the L1 Biacore chip. We could not bind more than approximately 11,500 RU of zwitterionic LUVs to the chip surface (Table 1). The refractive index of lipid membranes is estimated to be 1.4–1.5 [27–30] and is similar to the refractive index for proteins (i.e., 1.35–1.6 [31–33]). Therefore, it is possible to estimate the number of liposomes on the chip surface and the amount of bound EqtII on a single vesicle. We estimated that 125×10^6 liposomes are bound on a chip, assuming that, in a Biacore setup, 1000 RU corresponds roughly to 1 ng of matter (lipid or protein) per 1 mm^2, one liposome of 100 nm in a diameter contains 8.97×10^4 molecules
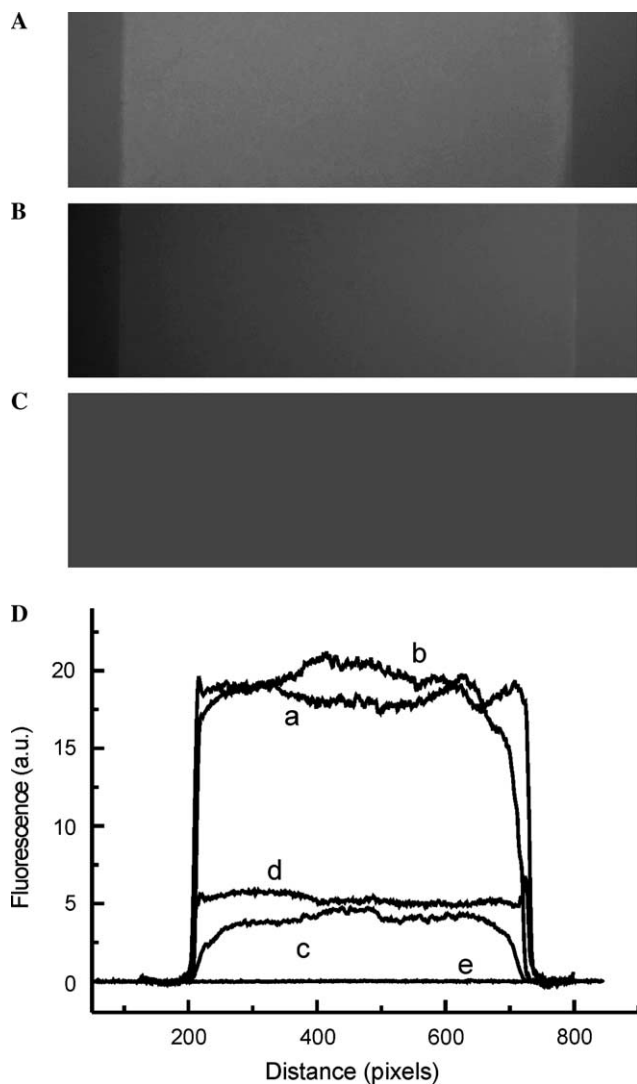
Fig. 4. Fluorescence of calcein-loaded immobilized liposomes on the L1 chip. Images show part of a flow cell. Liposomes were immobilized by an injection of 2 mM DOPC/SM (50:50, mol/mol) LUVs loaded with 60 mM calcein for 10 min at 1 μl/min. Approximately 11,000 RU were immobilized (A). After immobilization, the chip was washed with NaOH and BSA as shown in Fig. 5, and then 25 nM (trace b) or 1 μM EqtII (B and trace d) was injected over the surface for 90 s. Afterward, the chip was undocked and immediately imaged with the fluorescence microscope using blue light excitation. An empty flow cell is shown in (C) as a control. Fluorescence intensity profiles across images are shown in (D). The labels on the curves denote the following: a, 11,000 RU calcein-loaded LUVs (derived from A); b, 11,000 RU calcein-loaded LUVs and 25 nM EqtII bound afterward; c, 1100 RU calcein-loaded LUVs; d, 11,000 RU calcein-loaded LUVs and 1 μM EqtII bound afterward (derived from B); e, blank cell as a control (derived from C). a.u., arbitrary units.

[34], and the surface of a flow cell is 1.23 mm^2, as determined from fluorescence microscopy images. This is slightly less than 142.7×10^6 liposomes, a theoretical maximal hexagonal packing of liposomes that should yield a response of 13,100 RU. Such tight packing is unrealistic; in fact, we have not observed such high values in any experimental conditions, nor have such high

values been, to our knowledge, reported in the literature. Thus, at 11,500 RU, one vesicle occupies 9.8×10^3 nm^2 of the chip surface, 25% more surface than does a vesicle of 100 nm in a diameter. Liposomes probably are slightly spaced given that electrostatic interactions prevented tighter packing. This is supported by the fact that there was a release of vesicles if high-salt buffer was replaced with water or glucose solution. This was even more evident if negatively charged lipids were incorporated into the liposomes. In that case, we always observed a lower amount of stably bound LUVs due to electrostatic repulsion between vesicles that prevented closer packing on the surface of the chip. Similarly, Frostell-Karlsson et al. [14] recently reported lower immobilization levels, approximately 8000 RU at saturation, when they used mixture of charged lipids (PE/PS/DOPC [5:3:2, w/w/w], consisting of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine [PE], 1,2-dioleoyl-*sn*-glycero-3-(phospho-L-serine) [PS], and DOPC), in comparison with liposomes composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine that yields approximately 11000 RU, similar to our results. Other researchers also have reported lower deposition of negatively charged liposomes in comparison with zwitterionic ones [7,35–37].

It was proposed that vesicles fuse on the surface of the L1 chip, thereby generating a continuous bilayer surface [7]. If the entire flow cell is covered by a single bilayer of lipid composition similar to the liposomes used in our study, a maximal response of 3600 RU is expected. This value is approximately three times lower than that actually observed at surface saturation. Furthermore, the calcein-loaded liposomes were clearly unperturbed given that calcein fluorescence was observed under the fluorescence microscope (Fig. 4A) and was lost only when chip-immobilized vesicles were treated with high doses of EqtII that formed pores releasing calcein. Similarly, Stahelin and Cho [35], in one of their controls, failed to detect any fluorescence from chip-immobilized liposomes loaded with 5-carboxyfluorescein, suggesting that they remained intact. Although fusion of vesicles was not monitored directly, we could confirm the above proposition by observing the amount of stably bound lipids after water or glucose wash. Presumably, fused large patches of the membranes would not be removed as easily because they would bind to the chip stronger than would smaller vesicles. However, we could not increase the amount of stably bound liposomes after prolonged incubation on the chip under a low flow rate. We could increase the amount of bound lipids only slightly if we treated negatively charged liposomes with 5 mM CaCl$_2$ (an agent known to induce vesicle fusion [38]), with prior injection, or when they were bound on the chip. Our data, then, argue strongly that vesicles remain intact once bound on lipophilic anchors on the surface of the L1 chip, as was shown first by Cooper et al. [6]. This might also be true for other vesicular preparations
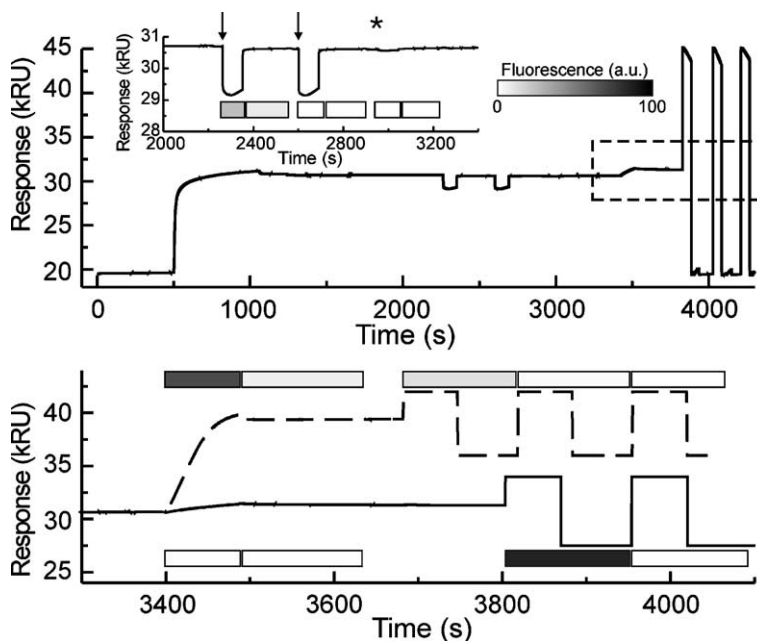
Fig. 5. EqtII binding and permeabilization experiment. The typical binding experiment is presented in the upper panel. It consists of injection of 2 mM LUVs loaded with 60 mM calcein (10 min at 1 μl/min, large arrow), two washings with 100 mM NaOH (small arrows), and one coating with 0.1 mg/ml BSA (asterisk), all for 1 min at 30 μl/min. The enlarged view of the washing is presented above the main sensorgram as an inset. EqtII was bound next (association for 90 s and dissociation for 180 s). The surface of the L1 chip was regenerated with three washings of LUV wash solution (isopropanol/50 mM NaOH [2:3, v/v]) at the end of the experiment (large changes in response at the end). The enlarged view (area denoted by dashed line rectangle in the main sensorgram) for the binding of EqtII is shown in the lower panel for 1 μM concentration (broken line) and 25 nM concentration (solid line). The responses from the washing step were trimmed for clarity. The rectangles show when the buffer was collected at the injection port. a.u., arbitrary units.
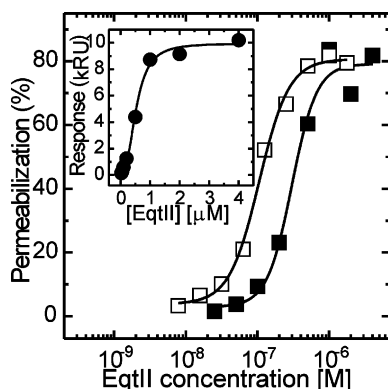

Fig. 6. Release of calcein from chip-immobilized LUVs. The percentages of permeabilization were calculated from the values of fluorescence intensity of the buffer collected during the experiment (solid squares) (see Fig. 5 for details). Percentages of permeabilization for the vesicles in solution are shown for comparison (open squares). The values were obtained from time traces (as in Fig. 7) of calcein release after the addition of EqtII. The inset shows the amount of bound EqtII after 3 min of dissociation.

Fig. 7. Release of calcein from LUVs in solution. The concentration of lipids was 25 μM in vesicle buffer (140 mM NaCl, 1 mM EDTA, 20 mM Tris–HCl, pH 8.5). LUVs were composed of DOPC/SM (1:1, mol/mol) ratio and were loaded with 60 mM calcein. They were stirred at 25 °C. Toxin at the desired L/T ratio, marked at the top of the figure, was added to the LUV solution at time 0. At the end of the assay, Triton X-100 at a concentration of 2 mM was added (designated by asterisks) to release completely encapsulated calcein.

prepared from whole-cell lysates; for example, synaptic vesicles also remain intact and do not fuse when bound on the chip, as demonstrated by electron microscopy [39].

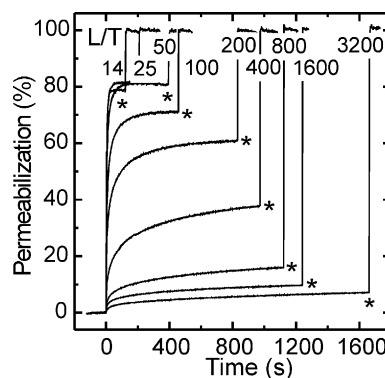To characterize the release of calcein from bound liposomes, we used a well-characterized pore-forming toxin, EqtII, from the sea anemone *Actinia equina*. EqtII forms transmembrane pores in lipid membranes by a sequential mechanism; it binds to the lipid membrane and then oligomerizes on the plane of the membrane and transfers the N-terminal region across the lipid bilayer to form final transmembrane pores. The first

binding step is noncooperative [9,20], but the pore formation on the plane of the membrane is cooperative and requires three or four monomers to build the final pore [20]. Therefore, EqtII forms pores only when the concentration of surface-bound toxin is high enough. Maček et al. [26] showed that when the lipid/toxin (L/T) ratio is greater than 20, the rate-limiting event is an aggregation of monomers on the surface of the vesicles. Therefore, at high L/T ratios (e.g., 1000), the toxin bound to the lipid membranes but formation of pores did not occur and vesicles remained intact [18]. This is exactly what happened on the chip-immobilized liposomes at a low EqtII concentration (25 nM), where approximately 50 molecules of toxin (calculated from the binding levels after 3 min of desorption) bound to a single vesicle at an L/T ratio of approximately 1800 but did not induce the release of calcein. On the contrary, at 1 μM EqtII, approximately 2700 molecules were bound to a single vesicle at an L/T ratio of 33; thus, permeabilization occurred to reach maximal values. The majority of released calcein was obtained during the injection phase that lasted 1.5 min, and almost none was obtained during the dissociation phase (Fig. 5). Therefore, pore formation of chip-immobilized liposomes is very fast and finished within the injection phase at high concentrations. This is exactly what is observed when stirred liposomes in a cuvette setup are used, where at large concentrations of toxin, maximal permeabilization is obtained within a few minutes (Fig. 7). EqtII bound tightly to liposomes with low dissociation (trace at 1 μM EqtII in Fig. 5) [9], indicating that, in addition to calcein release data, there is probably no redistribution of toxin molecules between the vesicles. This was also observed for EqtII previously [40]. In summary, with the use of a well-defined eukaryotic pore-forming toxin, we showed that chip-immobilized liposomes behave similarly to liposomes free in solution.

Taking into consideration all of the above observations, we propose the following general protocol for loading the L1 chip with lipid vesicles: deposit 2 mM lipids in the form of LUVs on the surface of the chip for 10 min at 1 μl/min, wash with three 1-min injections of 100 mM NaOH at 30 μl/min, and check for the quality of surface with a 1-min injection of 0.1 mg/ml BSA at 30 μl/min. This should yield a homogeneously covered surface with unperturbed liposomes.

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