Giant liposomes in physiological buffer using electroformation in a flow chamber

Daniel J. Estes, Michael Mayer*

Department of Biomedical Engineering and Department of Chemical Engineering, University of Michigan, Gerstacker Building, Room 1107, 2200 Bonisteel Boulevard, Ann Arbor, MI 48109-2099, USA

Abstract

We describe a method to obtain giant liposomes (diameter 10–100 μm) in solutions of high ionic strength to perform a membrane-binding assay under physiological conditions. Using electroformation on ITO electrodes, we formed surface-attached giant liposomes in solutions of glycerol in a flow chamber and then introduced solutions of high ionic strength (up to 2 M KCl) into this chamber. The ionic solution exchanged with the isoosmolar glycerol solution inside and outside the liposomes. An initial mismatch in index of refraction between the inside and outside of liposomes allowed for the observation of solution replacement. Ions and small polar molecules exchanged into and out of surface-attached liposomes within minutes. In contrast, liposomes formed in solutions of macromolecules retained molecules larger than 4 kDa, allowing for encapsulation of these molecules for hours or days even if the solution outside the liposomes was exchanged. We propose that solutes entered liposomes through lipid tubules that attach liposomes to the film of lipids on the surface of the ITO electrode. The method presented here makes it straightforward to perform flow-through binding assays on giant liposomes under conditions of physiological ionic strength. We performed a membrane-binding assay for annexin V, a calcium-dependent protein that binds to phosphatidylserine (PS). The binding of annexin V depended on the concentration of PS and decreased as ionic strength increased to physiological levels.
© 2005 Elsevier B.V. All rights reserved.

Keywords: Giant liposome; Electroformation; Physiological buffer; Membrane-binding assay; Annexin V

1. Introduction

Giant liposomes with dimensions of living cells are useful models to: (1) study membrane curvature and elasticity under varying conditions [1–4]; (2) examine interactions between lipid membranes and surfaces [5]; (3) record the activity from reconstituted ion channels [6–9]; (4) form planar lipid bilayers over microfabricated pores [10–12]; (5) create nano-fluidic networks [13,14]; and (6) make microscale bioreactors [15,16]. While there are several techniques for preparing giant liposomes [17], electro-formation is particularly useful for the formation of unilamellar giant liposomes with diameters between 10

and 100 μm [17–19]. In this method, AC electric fields induce the formation of surface-attached [20,21], giant unilamellar liposomes from a film of lipids on one or both electrodes that are separated by an aqueous solution [22].

Electroformation has been reported to succeed only in ionic strengths below 50 mM [17–20,23–27], except for one report in which the incorporation of negatively-charged lipids allowed the formation of a few giant liposomes in 100 mM KCl, 10 mM CaCl$_2$ [28]. The initial step in electro-formation involves the separation of bilayers in the multi-layered film of lipids on the electrode surface [17,29]. Ions impede electroformation by entering the space between bilayers in the film of lipids and hindering bilayer separation [27]. Biophysical assays on giant, unilamellar liposomes are therefore limited to ionic conditions that are not always representative for cellular membranes. To study accurately physiological processes that depend on ionic strength, such

---

* Corresponding author. Tel.: +1 734 763 4609; fax: +1 734 763 4371.
E-mail address: MiMayer@UMich.edu (M. Mayer).

as membrane-binding, vesicle fusion, and ion channel activity, giant liposomes in solutions of physiological ionic strength ($\geq$140 mM) would be very useful.

Methods other than electroformation to obtain giant liposomes in solutions of high ionic strength have used chemically modified or negatively charged phospholipids to promote separation of the membranes of liposomes. Yamashita et al. [30] produced giant liposomes in ionic solutions up to 2 M KCl by incorporating 0.2%–5% phosphatidylethanolamine (PE) with covalently linked polyethylene-glycol (PEG) groups into liposome membranes. The addition of PEGylated lipids favored bilayer separation and liposome formation, even at high ionic strengths, because of steric repulsion induced by the large PEG moiety [30]. Akashi et al. showed that the dehydration–rehydration technique succeeded in forming unilamellar and multilamellar liposomes in solutions up to 100 mM KCl by incorporating $\geq$10% negatively charged lipids in the liposomes [31]. The negatively charged head groups of lipids increased electrostatic repulsion between bilayers in the film of lipids to facilitate the separation of bilayers [31]. Ideally, giant, unilamellar liposomes could be formed in solutions of high ionic strength from a variety of lipids (charged and uncharged) without the addition of chemically-modified lipids.

Here, we obtained giant liposomes in solutions of high ionic strength by employing electroformation in a flow chamber. We bypassed the problem of reduced bilayer separation by adding solutions of high ionic strength after giant liposomes had already formed in a solution of low ionic strength. We succeeded in obtaining giant liposomes in a physiological buffer from a variety of charged or uncharged lipids without the addition of PEGylated or otherwise modified lipids. We employed these liposomes to perform a membrane-binding assay for fluorescently-labeled annexin V under physiological conditions. Annexin V binds strongly to phosphatidylserine (PS) in the presence of calcium [32] and may play a role in disrupting the coagulation cascade after cell injury by binding and reducing access to exposed anionic surfaces [33]. The strength of this binding interaction, however, decreases with increasing ionic strength [34]. Here, we use the interaction between annexin V and PS as a model system to demonstrate the importance of ionic strength for assays of binding interactions on giant liposomes.

2. Experimental

2.1. Formation of films of lipids on indium tinoxide (ITO) electrodes

We prepared films of lipids by spin-coating lipid solutions using a technique developed recently in our lab [22]. Solutions of lipids consisted of L-α-Phosphatidylcholine (egg, chicken) (eggPC), 1,2-Dioleoyl-sn-Glycero-3-[Phospho-L-Serine] (DOPS), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine (POPE) (all from Avanti Polar Lipids, Alabaster, AL), or asolectin lipids (Fluka, Switzerland) dissolved in 95% chloroform (Acros, NJ)/5% acetonitrile (HLPC grade, Fisher Scientific). All lipid solutions had a concentration of 3.75 mg mL^{-1}. Films were formed by spin-coating these solutions (at 600 rpm) onto 50 mm $\times$ 50 mm $\times$ 1.1 mm aluminosilicate glass slides coated with ITO (R_s=5–15 Ω, Delta Technologies, Stillwater, MN).

2.2. Electroformation setup with a flow chamber

We formed giant liposomes from films of lipids by electroformation. A poly(dimethylsiloxane) (PDMS, Sylgard 184 Silicone, Dow Corning, Midland, MI) spacer with an interior chamber separated the opposing ITO electrodes (Fig. 1). Films of lipids covered both surfaces of ITO. To provide flow-through capability, we formed sealed inlet and
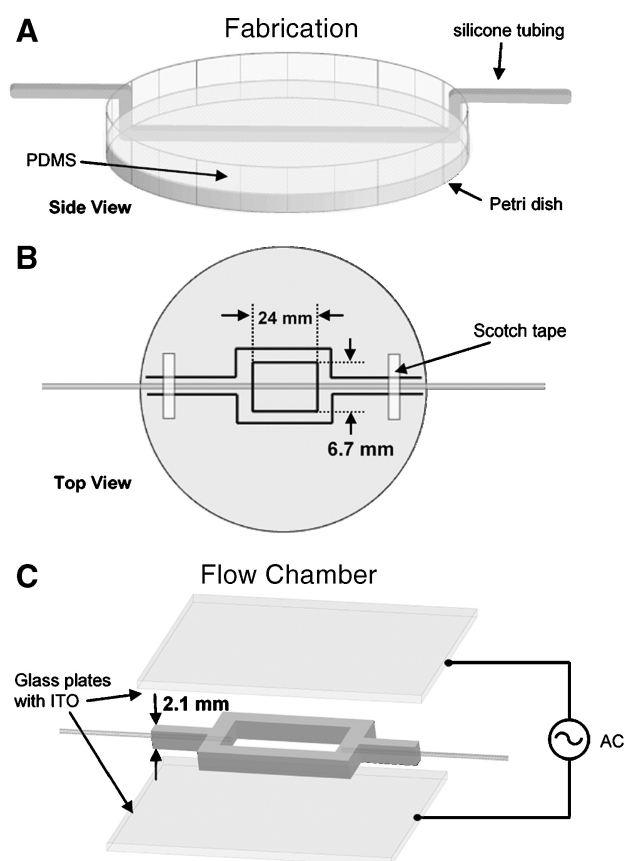

Fig. 1. Fabrication of an electroformation setup with flow chamber. (A) Silicone tubing (inside diameter=0.60 mm, Dow Corning, Midland, MI) was taped to the bottom of a Petri dish (150 $\times$ 15 mm, VWR International) before PDMS prepolymer was poured into the dish. (B) After curing of PDMS at 60 °C, the embedded tubing formed a tight bond with the PDMS, and a rectangular chamber (interior dimensions=23.6 $\times$ 6.7 $\times$ 2.1 mm) was cut out (along the solid lines) using a surgical blade. (C) The resulting PDMS spacer contained two opposing tubes and was sandwiched between two ITO plates to allow for the flow of solutions through the chamber.

outlet tubes in the interior chamber of the PDMS by the fabrication procedure shown in Fig. 1.

The PDMS spacer was held between the two plates of ITO using binder clips (Officemate International Corp., Edison, NJ). A needle (20G1½, Becton Dickinson Company, Franklin Lakes, NJ) was inserted into one end of the tubing, and a non-ionic solution (here defined as an aqueous solution that does not contain any salt molecules) was introduced into the chamber at a rate of $\sim$0.5 mL min^{-1} using a syringe while an electric field (sine-wave, 1.6 V peak-to-peak at 10 Hz) was applied between the two surfaces of ITO using a function generator (Circuitmate FG2, Beckman Coulter, Fullerton CA). After the chamber (volume=330 μL) was filled, the syringe was kept attached to the inlet tube to prevent backflow. The electric field was applied for at least 1.5 h to form surface-attached giant liposomes.

The same flow chamber was used throughout all experiments. We cleaned the chamber by flushing the silicone tubes with ethanol (95%, VWR International) (1× with 3 mL), followed by de-ionized water (18.2 MΩ cm) (2× with 3 mL), and we dried the chamber under vacuum ($\sim$−740 Torr) for 30 min.

2.3. Flow-through procedure

We used a programmable syringe pump (kd Scientific, Holliston, MA) to replace solutions in the electroformation chamber at a controlled rate. Whenever solutions were exchanged, at least four times the volume of the chamber was flowed through the chamber to ensure complete replacement. Introducing a new solution involved exchanging syringes at the inlet tube; during this procedure, we closed the inlet tubing with a binder-clip to prevent backflow.

We performed electroformation in aqueous solutions of glycerol and then replaced these solutions with ionic solutions. The osmolarity of the initial glycerol solution matched the osmolarity of the electrolyte solution that was subsequently introduced. After giant liposomes were formed, the salt solution was introduced into the chamber. We employed the following solutions to replace the initial glycerol solution both inside and outside the liposomes made from pure eggPC: up to 2 M KCl (Sigma, St. Louis MO) replacing 3.68 M glycerol; up to 300 mM NaCl (Sigma, St. Louis MO) replacing 558 mM glycerol; phosphate buffered saline (PBS) (Sigma, St. Louis MO) replacing 300 mM glycerol; and up to 200 mM CaCl$_2$ (EMD Chemicals, Gibbstown NJ) replacing 600 mM glycerol.

2.4. Examining exchange of solutions using an ion-sensitive fluorescent dye

We monitored the exchange of solutions inside and outside of liposomes by using sodium green tetra(tetrame-

thylammonium) salt, (cell impermeant, Molecular Probes, Eugene, OR), a fluorescent indicator for sodium, to quantify the concentration of Na$^+$ inside and outside the liposomes. Liposomes consisting of 99% eggPC, 1% 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-(Lissamine Rhodamine B Sulfonyl) (ammonium salt) (liss-rhodamine DPPE, Avanti Polar, Alabaster, AL) were formed in 251 mM glycerol, 0.2 mM TRIS (Hydroxymethyl) Aminomethane (TRIS, Shelton Scientific, Inc., Shelton CT) buffer pH 7.4, and 2.5 μM sodium green. This solution was then replaced by an ionic solution of 135 mM NaCl, 0.2 mM TRIS, 2.5 μM sodium green at a flow rate of 5.0 mL h^{-1}. Fluorescence intensities were measured inside and outside the liposomes after 1 h of flow by confocal microscopy.

To explore the mechanism of solution-exchange in surface-attached liposomes, we monitored the diffusion of 5(6)-carboxyfluorescein (Fluka, Switzerland) into giant liposomes of 99.5% eggPC, 0.5% liss-rhodamine DPPE. After growing liposomes in 0.1 mM TRIS buffer (pH 7.4), we introduced 0.2 μM carboxyfluorescein, 0.1 mM TRIS (pH 7.4) into the electroformation chamber at a flow rate of 5.0 mL h^{-1} for 2 h and monitored the fluorescence intensities inside and outside the liposomes over a period of 24 h, without flow.

2.5. Encapsulation inside surface-attached giant liposomes

We encapsulated fluorescein-conjugated avidin (MW=68 kDa, ImmunoPure, Pierce Biotechnology, Inc., Rockford, IL), fluorescein (FITC)-conjugated dextran-4000 (MW=4 kDa), fluorescein-conjugated dextran-70,000 (MW=70 kDa), and fluorescein-conjugated dextran-150,000 (MW=150 kDa) (the three dextrans were from Fluka, Biochemika, Switzerland) inside giant liposomes of eggPC. We first formed giant liposomes in solutions containing 0.5 μM avidin or dextran in 0.1 mM TRIS buffer (pH 7.4). We then flowed 0.1 mM TRIS buffer through the chamber at a flow rate of 5.0 mL h^{-1} for 1 h to remove the solution of dextran or avidin around the liposomes.

2.6. Binding of annexin V to giant liposomes

To perform an assay for annexin V binding, we formed liposomes in a solution of 300 mM glycerol and 1 mM TRIS buffer (pH 7.4) from solutions of lipids with the following mass-ratios of eggPC and DOPS—80:20, 90:10, and 100:0. Using a flow-rate of 3.8 mL h^{-1}, we replaced the initial solution in the chamber with a solution containing 1 mM TRIS, 1 mM CaCl$_2$, and 1 nM annexin V labeled covalently with fluorescein (annexin V-FITC, Sigma, St. Louis MO). To compare annexin binding in the absence or presence of salt, the annexin V-FITC solution contained either 300 mM glycerol or 135 mM NaCl/15 mM KCl (to simulate extracellular ionic conditions), respectively.

2.7. Observation and characterization of liposomes

We observed electroformed liposomes using a Nikon Eclipse TE 2000-U inverted microscope using $10\times$ and $20\times$ objectives with extra-long working distance in phase-contrast mode. We captured images of liposomes using a CCD camera (Photometrics CoolSnap HQ, Roper Scientific, Trenton, NJ). Image analysis software (Metamorph from Universal Imaging Corporation, Downington, PA) allowed for the determination of the size of liposomes as well as digital contrast enhancement of fluorescent images. Fluo-rescently-labeled annexin V was observed using filter settings for fluorescein (excitation filter=460–500 nm, dichroic mirror=505 nm, emission filter=510–560 nm). We used the same microscope and camera settings (10 s exposure time, $20\times$ objective, $1\times$ binning, maximum intensity of excitation of the fluorescence source, Exfo X-Cite 120, Photonic Solutions, Mississauga, Ontario) for all experiments involving annexin V. For experiments with sodium green or carboxyfluorescein, we measured the intensities of fluorescence using a confocal setup (Nikon D-Eclipse C1, 543 nm and 488 nm lasers) with the inverted microscope and confocal analysis software (EZ-C1).

3. Results and discussion

3.1. Giant liposomes in solutions of high ionic strength

To produce giant liposomes in solutions of high ionic strength, we initially formed surface-attached giant lip-osomes in solutions of glycerol (up to 3.68 M) using electroformation in a flow chamber (Fig. 1). We then flowed a solution of high-ionic strength isoosmolar to the initial solution of glycerol into the chamber. The ionic solution exchanged with the solution of glycerol both outside and inside the surface-attached liposomes. Phase-contrast micro-scopy allowed the visualization of this replacement of solutions, as solutions with lower refractive indices appeared brighter than did the solutions with higher indices [35]. Initially, the introduction of a new solution resulted in a difference in index of refraction between the inside and outside of the giant liposomes, since the freshly introduced ionic solution surrounded liposomes that were still predom-inantly filled with a glycerol solution (Fig. 2B). After flowing a volume of 2 mL ($\sim$25 min), these differences in refractive indices disappeared (Fig. 2C), and a uniform index of refraction indicated replacement of solutions inside and outside the giant liposomes.

We confirmed quantitatively the replacement of glycerol by sodium chloride by measuring intensities of sodium green, a fluorescent indicator dye for sodium. After replacing 251 mM glycerol with 135 mM NaCl (each solution contained the same concentration of sodium green), the fluorescence intensity outside and inside the liposomes increased by a factor of 2.4 (Figs. 3 and 4). This increase
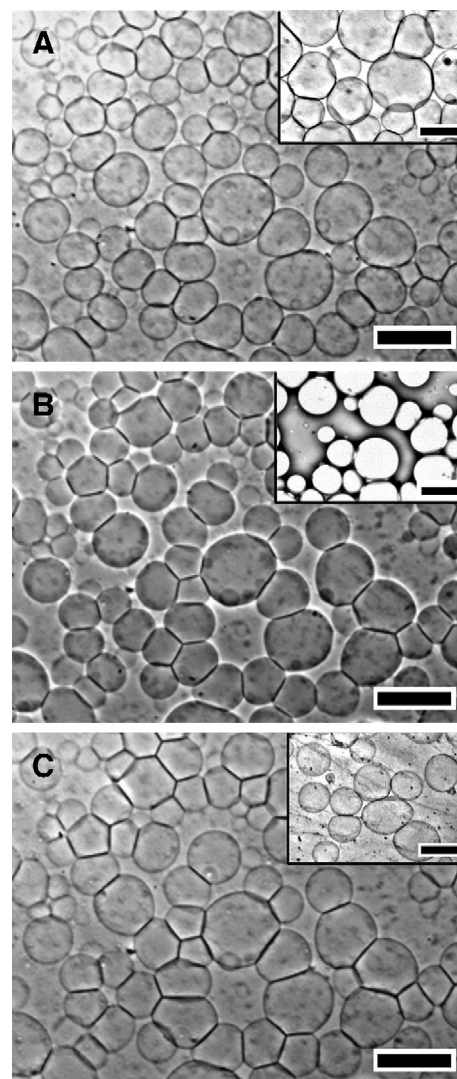

Fig. 2. Giant liposomes from eggPC at different stages during the replacement of a non-ionic solution (300 mM glycerol) by an ionic solution (PBS). Image (A) shows giant liposomes formed after electro-formation in glycerol solution for 2 h. PBS was introduced slowly into the chamber (flow rate=5.0 mL h^{-1}) to replace the solution of glycerol. The darker regions inside of liposomes in image (B) illustrate the initial difference in refractive indices between liposomes filled with glycerol solution (n=1.3365) [43] and the surrounding solution of PBS (n=1.3339) [43]. Image (C) shows the complete replacement of glycerol solution by PBS inside the liposomes after 30 min; no difference in the refractive indices inside and outside the liposomes could be discerned. The figure insets show the same steps of solution exchange by flowing 300 mM sucrose into the chamber instead of PBS. Note the dramatic difference in refractive indices (B inset) between liposomes filled with glycerol (bright interior of liposomes) surrounded by the solution of sucrose (n=1.3478 for 0.3 M) [43], and the uniform indices of refraction before (A inset) and after (C inset) exchange of solutions. All scale bars=100 μm.

indicated that the solution of NaCl did replace the solution of glycerol in the chamber. In addition, since the fluo-rescence intensities were the same inside and outside the liposomes (Fig. 3), we concluded that after $\sim$200 s, most liposomes contained the same sodium concentration as did the surrounding medium (Fig. 4).
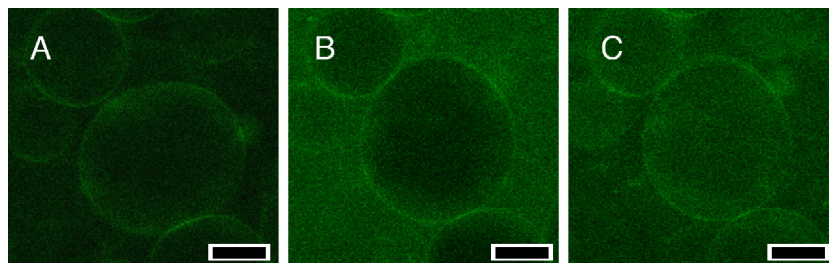
Fig. 3. Replacement of glycerol by NaCl using sodium green as a fluorescent indicator dye for Na^+. (A) Liposomes were formed in 251 mM glycerol, 2.5 μM sodium green. Initially, the average fluorescence intensity inside liposomes was 207 ± 10, $N=5$, and the average intensity outside was 204 ± 10, $N=5$. (B) Immediately after introduction of 135 mM NaCl, 2.5 μM sodium green into the chamber, the fluorescence intensity outside the liposomes increased to 461 ± 17, $N=5$, whereas the intensity inside was 294 ± 18, $N=6$. (C) Approximately 3 min later, NaCl exchanged with glycerol inside and outside the liposomes (time course in Fig. 4), as indicated by the uniform intensity of fluorescence inside and outside the liposomes (intensity inside$=513 \pm 19$, $N=6$; intensity outside$=496 \pm 48$, $N=5$). Scale bars$=40$ μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

At slow flow rates (below threshold values in Table 1), the size and position of the giant liposomes attached to the surface of ITO did not change (Fig. 2); we observed, however, a marked increase in the membrane–membrane interactions between adjacent liposomes in the ionic solutions. Adjacent giant liposomes assumed connected configurations consisting of straight lines and sharp angles differing from the originally spherical shapes of liposomes (Fig. 2B, C). Presumably, electrostatic interactions of ions with the zwitterionic or negatively charged head groups of lipids in the liposomes induced the lipid membranes to adopt conformations of increased inter-membrane interaction [36]. We did not attribute the changes in shape of the liposomes to osmotic pressure since we observed shape
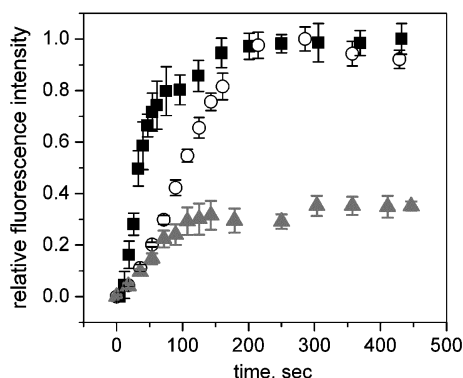

Fig. 4. Time course of solution exchange by measuring relative intensities of fluorescence inside surface-attached liposomes. (■) Replacement of 251 mM glycerol, 2.5 μM sodium green inside and outside of liposomes with 135 mM NaCl, 2.5 μM sodium green. During the exchange, fluorescence intensity increased by a factor of 2.4. (○) Replacement of 0.1 mM TRIS buffer with 0.1 mM TRIS buffer, 0.1 μM carboxyfluorescein in liposomes that were located closely (distance <5 μm) to the ITO electrode. During the exchange, the absolute fluorescence intensity increased by a factor of 4 compared to background. (▲) Replacement of 0.1 mM TRIS buffer with 0.1 mM TRIS buffer, 0.1 μM carboxyfluorescein in liposomes that were located ~20 μm away (in *z*-direction, see Fig. 6B) from the ITO electrode. The zero time value represents the onset of the increase in fluorescence in the solution surrounding the liposomes. Data were normalized to the maximum fluorescence observed outside the liposomes for each solution. All data represent mean intensity values of four measurements; the error bars reflect standard deviation.

changes of membranes even when flowing an ionic solution (PBS) hypertonic to the original solution of glycerol (210 mM) into the chamber (manuscript in preparation).

Exchanging solutions after electroformation made it possible to obtain giant liposomes in phosphate buffered saline (PBS) from a variety of lipids, both charged (e.g. asolectin, which contains ~25% negatively charged lipids; 80% eggPC/20% DOPS; or 90% eggPC/10% DOPS) and zwitterionic (pure eggPC; pure POPC; or 50% eggPC/50% POPE). Liposomes in physiological ionic solutions were stable for several days, and lipids grafted with polyethylene glycol groups were not required to stabilize giant liposomes in the salt solutions. We obtained giant liposomes in a range of electrolyte solutions including 2 M KCl, 300 mM NaCl, and 200 mM $CaCl_2$. We believe that electroformation in a flow chamber may offer a general strategy to produce giant, unilamellar, liposomes from a wide range of lipids in solutions of high ionic strength.

3.2. Encapsulation of large molecules inside surface-attached liposomes

While solutions of ions and small polar molecules (e.g. sucrose or carboxyfluorescein) replaced solutions of glycerol in surface-attached liposomes, we found that molecules ≥ 4 kDa did not exchange during flow (Fig. 5). After forming giant liposomes of eggPC in solutions of avidin (MW$=68$ kDa), dextran-4000, dextran-70000, or dextran-150000 (all four conjugated with fluorescein), we encapsulated these molecules by flowing 0.1 mM TRIS buffer through the chamber. While solutions replaced outside the liposomes, fluorescently-labeled avidin and all three types of fluorescently-labeled dextran remained inside the liposomes. Using confocal microscopy, we showed that the fluorescence of FITC-conjugated avidin and dextran-4000 inside the liposomes did not decrease after 24 h of encapsulation (See Supporting Information). Electroformation in a flow-chamber, therefore, offers a means to encapsulate proteins and other large molecules inside of surface-attached liposomes. This capability may be useful for employing giant liposomes as bioreactors.

Table 1
Maximum linear flow rates (u) for vesicles attached to the ITO surface before liposomes detached or ruptured

Lipid	Flow solvent	u_1 (mm min^{-1})[a]	u_2 (mm min^{-1})[b]	Effect of flow rate above threshold
EggPC	Sucrose	17.2	54.4	Detachment with tubules; elongation of membrane until vesicles ruptured
EggPC	PBS	21.2	64.9	Elongation of liposomes until vesicles ruptured
50% POPE−50% eggPC	Sucrose	8.3	17.7	Detachment of liposomes with elongation of liposomes and extraction of tubules
50% POPE−50% eggPC	PBS	20.6	–	Strong interaction between liposomes and surface—liposomes did not detach
Asolectin	Sucrose	1.5	4.7	Detachment of liposomes, extraction of tubules
Asolectin	PBS	2.9	13.6	Extraction of tubules, some liposomes ruptured

The conversion between volumetric flow rate (mL h^{-1}) and linear flow rate (mm min^{-1}) for our flow chamber (width=6.7 mm, height=2.1 mm) is: 1 mL h^{-1}=1.18 mm min^{-1}.

[a] u_1 represents the flow rate at which liposomes started to show effects due to flow (detachment or some elongation).

[b] u_2 represents the flow rate at which >50% liposomes showed effects due to flow of solvent.

3.3. Proposed mechanism of exchange of solutions

The replacement of solutions inside surface-attached liposomes conflicts with traditional models of transport through the membranes of giant vesicles. While lipid bilayers usually prohibit or dramatically attenuate the transfer of ions and polar molecules [36,37], we found that these molecules could pass into and out of surface-attached liposomes. According to a model of electro-formation proposed by Angelova, surface-attached giant liposomes consist of liposomes, which taper into open lipid tubules that attach to the film of lipids on the surface of the electrode [20] (Fig. 6). Orwar et al. demonstrated that lipid tubules provide networks through which molecules can enter liposomes [13,14]. We propose, therefore, that the exchange of solutions in surface-attached giant liposomes occurs through lipid tubules that connect liposomes to the film of lipids on the surface of the electrodes (Fig. 6).

To support this hypothesis, we first demonstrated the existence of the lipid tubules between liposomes and the film of lipids. While these lipid tubules were not visible for liposomes attached closely to the film of lipids, we observed tubules by extending their length through flow. When surface-attached liposomes were subjected to high

Fig. 5. Encapsulation of fluorescein-conjugated dextran-70000 inside giant liposomes 2 h after flow. Scale bar=100 μm. Inset: encapsulation of fluorescein-conjugated dextran-4000 after 24 h. Inset scale bar=50 μm.

rates of linear flow (see Table 1), they detached and extracted tubules between themselves and the film of lipids (Fig. 7). These tubules extruded behind the liposomes, parallel to the direction of flow, and we typically observed only one tubule attached to each liposome. Previous studies have reported that liposomes could extract tubules under shear-flow [38–41].

Another result that reinforces the mechanism proposed here is the observation that solution exchange occurred faster in liposomes that were located close to the surface of the electrode compared to liposomes located further away from the film of lipids. We monitored the influx of carboxyfluorescein into pre-formed giant liposomes, which grew in several different "layers" (Fig. 6B); some liposomes appeared above others and were attached either to the ITO surface with long tubules or to other liposomes [19]. After 2 h of flow of carboxyfluorescein solution (10 mL), we observed different intensities of fluorescence in different layers of liposomes using confocal scans in the z-direction. Liposomes closest to the surface of ITO exhibited complete replacement of solution in >80% of vesicles (Fig. 6A); in contrast, liposomes farther away (∼20–50 μm) from the surface of ITO showed carboxyfluorescein in <50% of vesicles (Fig. 6C). After 48 h, we found that most liposomes (>75%) throughout the chamber were filled with carboxyfluorescein (see Supporting Information).

Presumably, liposomes closely attached to the ITO surface had short connecting tubules (Fig. 6B) that allowed for the rapid diffusion of carboxyfluorescein into the liposomes (Fig. 4). Vesicles farther away from the surface had long connecting tubules (Fig. 6B) that hindered the movement of carboxyfluorescein into the liposomes by narrowing or becoming blocked. Ions and small polar molecules could diffuse quickly into surface-attached liposomes through lipid tubules (Fig. 4). Large molecules such as dextran-4000 or avidin were too large to pass or moved very slowly through lipid tubules, making the encapsulation of these molecules possible. The proposed model of solution exchange through lipid tubules is in agreement with previously reported results [20] and offers an explanation
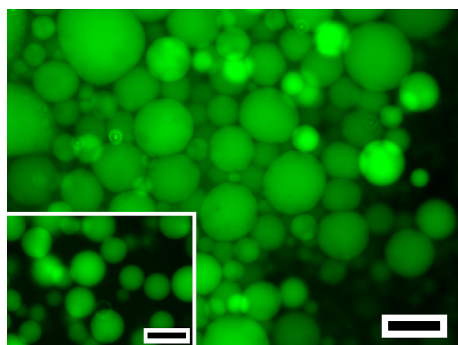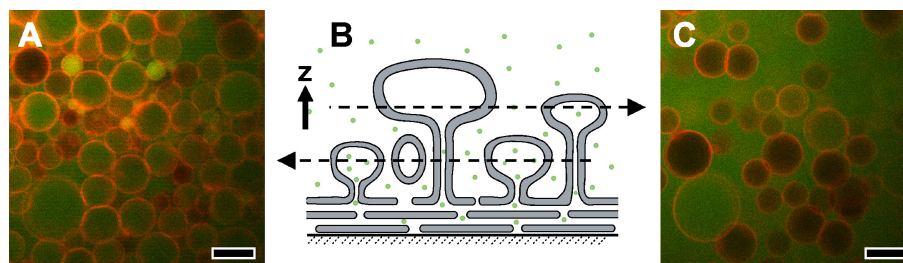
Fig. 6. Confocal micrographs of carboxyfluorescein fluorescence (green) inside and outside the liposomes with membranes containing fluorescently labeled lipids (liss-rhodamine-DPPE red) 2 h after introducing carboxyfluorescein into the chamber. The liposomes were initially formed in a solution without carboxyfluorescein. The cartoon in panel (B) depicts a model of surface-attached liposomes (adapted from Angelova [20]), with lipid tubules connecting liposomes to the plate of ITO and forming conduits through which molecules can diffuse into liposomes. The dashed lines represent horizontal planes from scans in z-direction of different lipid layers obtained by confocal microscopy. Image (A) shows the layer of liposomes closest to the ITO. Note that most liposomes are filled with the solution of carboxyfluorescein. Image (C) depicts liposomes ~30 μm above those shown in image (A). Most liposomes have a dark interior and are not yet filled with the solution of carboxyfluorescein. Scale bars=50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for the rapid exchange of ions as well as the encapsulation of macro-molecules in surface-attached liposomes.

3.4. Stability of surface-attached giant liposomes under flow

In order to assess how quickly new solutions could be introduced into the flow chamber, we determined the maximum tolerable flow rates for surface-attached liposomes of different mixtures of lipids under flow of ionic (PBS) and non-ionic (solutions of sucrose) solutions. The replacement of solutions was successful for flow rates below the threshold values (Table 1). Using different rates of flow below 5 mL h^{-1}, we did not observe differences in the rate of exchange of solutions inside the liposomes. Similarly, the efficiency of encapsulation of dextran-70000 inside eggPC liposomes did not depend on the rate of flow (for flow rates below 5.0 mL h^{-1}, see Supporting Information S3). Liposomes from zwitterionic lipids (eggPC; 50% eggPC/ 50% POPE) could withstand higher maximum flow rates
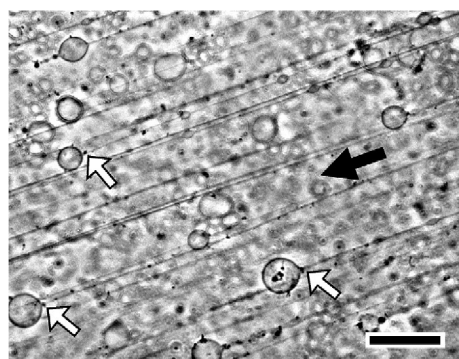

Fig. 7. Extraction of tubes with parallel orientation from giant liposomes under shear flow in an ionic solution. Tubes were extracted from giant liposomes formed from asolectin lipids at flow speeds of 11 mL h^{-1} of PBS. Some giant liposomes detached from the surface of ITO, and tubes were extracted from these vesicles (see white arrows). Extraction of tubes was observed in non-ionic solutions of sucrose and in ionic solutions of PBS. The black arrow indicates the direction of flow. Scale bar=70 μm.
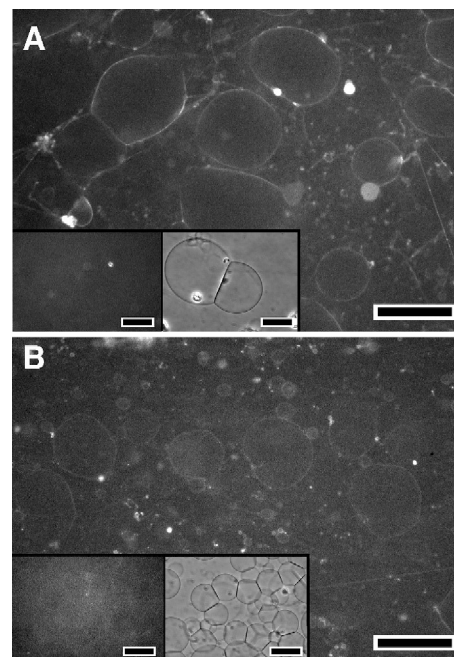

Fig. 8. Fluorescent micrographs showing binding of fluorescently-labeled annexin V to liposomes containing PS lipids. (A) Fluorescently-labeled annexin bound to giant liposomes containing 10% DOPS, 90% eggPC in the presence of 300 mM glycerol (difference of fluorescence intensity between membrane and interior of liposomes=9.1%±3.5%, N=4). The inset in panel (A, left) shows minimal annexin binding to liposomes containing 10% DOPS in the presence of physiological buffer; the inset in panel (A, middle) shows the corresponding phase-contrast micrograph of the same region (scale bars of inset=50 μm). (B) Annexin bound to giant liposomes containing 20% DOPS, 80% eggPC in the presence of 135 mM NaCl, 15 mM KCl (difference in fluorescence intensity between membrane and interior of liposomes=4.5%±0.2%, N=4). Note the decrease of annexin binding in conditions of physiological salt (B) despite the presence of twice the percentage of PS. The inset in panel (B) shows a control experiment of annexin binding to liposomes without PS (100% eggPC) in a solution of 0.3 M glycerol. The inset in panel (B, left) shows minimal binding of annexin to liposomes containing no PS; the inset in panel (B, middle) shows the corresponding phase-contrast view of the same liposomes (scale bars of inset=50 μm). Scale bars=50 μm.

than do liposomes containing lipid mixtures with approximately 25% negatively charged head groups (asolectin) (Table 1). Liposomes under flow of ionic solutions withstood higher flow rates than did liposomes under flow of non-ionic solutions; this result suggests enhanced stability from increased membrane interactions between neighboring liposomes and increased interaction of liposomes with the film of lipids on the ITO plate to which the liposomes are attached.

As previously discussed, liposomes, especially those from asolectin lipids, detached and extracted tubules when subjected to flow rates above 11 mL h^{-1}. Tubes extracted in both ionic and non-ionic solutions and were oriented parallel to the direction of flow (Fig. 7). The rate of extraction increased with increasing flow rate. Flowing solutions through a chamber of surface-attached liposomes of asolectin lipids thus offers a simple method for forming many parallel tubes in ionic or non-ionic solutions.

3.5. Membrane-binding assay for annexin V under conditions of physiological ionic strength

We employed electroformation in a flow chamber setup to perform a membrane-binding assay for fluorescently-labeled annexin V. Testing liposomes with 0%, 10%, and 20% PS, we found that in the absence of salt, annexin V bound strongly to liposomes containing PS (Fig. 8A), while in the presence of a buffer with physiological ionic strength, annexin V bound weakly to PS (Fig. 8B). In liposomes containing 0% PS, negligible binding occurred (Fig. 8B inset) in both the presence and absence of salt. These results show that: (1) membrane-binding assays can be performed on giant liposomes under conditions of physiological ionic strength; and (2) membrane-binding interactions can depend strongly on the ionic strength of the surrounding solution [34]. The flow-through technology presented here might be useful for studying a wide range of binding interactions on giant liposomes that depend on ionic strength.

In addition, the presented technique allows for changing the experimental conditions of assays with giant liposomes by sequentially introducing different solutions into the chamber. In a single experiment, we were able to exchange solutions several times in surface-attached liposomes. Multiple replacements of solutions may prove useful for examining membrane-binding events in a range of ionic solutions. Also, in contrast to traditional microinjection techniques involving giant liposomes in which a new solution is injected into a single liposome [27,42], the work presented here allows for the introduction of solutions into many surface-attached liposomes in parallel.

Although surface-attached liposomes do not permanently entrap ions and small polar molecules, our experiments with dextran-4000 and avidin indicated that large molecules (MW$\geq$4 kDa) can be entrapped inside liposomes for 24 h without significant loss. By growing liposomes in a solution containing large macromolecules (e.g. enzymes), it may be possible to perform bioreactions of entrapped macromolecules in parallel by flowing solutions containing small, reactive species (e.g. substrates) into the chamber.

Performing electroformation in a flow chamber allows for a range of new experimental possibilities, most notably a general method for forming giant liposomes in physiological conditions, which may bring giant liposomes one step closer to models of living cells.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2005.03.012.

References

[1] E. Farge, P.F. Devaux, Shape changes of giant liposomes induced by an asymmetric transmembrane distribution of phospholipids, Biophys. J. 61 (1992) 347–357.

[2] W. Rawicz, K.C. Olbrich, T. McIntosh, D. Needham, E. Evans, Effect of chain length and unsaturation on elasticity of lipid bilayers, Biophys. J. 79 (2000) 328–339.

[3] M.M. Claessens, B.F. van Oort, F.A. Leermakers, F.A. Hoekstra, M.A. Cohen Stuart, Charged lipid vesicles: effects of salts on bending rigidity, stability, and size, Biophys. J. 87 (2004) 3882–3893.

[4] D. Needham, R.S. Nunn, Elastic deformation and failure of lipid bilayer membranes containing cholesterol, Biophys. J. 58 (1990) 997–1009.

[5] P. Fromherz, V. Kiessling, K. Kottig, G. Zeck, Membrane transistor with giant lipid vesicle touching a silicon chip, Appl. Phys., A Mater. Sci. Process. 69 (1999) 571–576.

[6] G. Riquelme, E. Lopez, L.M. Garcia-Segura, J.A. Ferragut, J.M. Gonzalez-Ros, Giant liposomes: a model system in which to obtain patch-clamp recordings of ionic channels, Biochemistry 29 (1990) 11215–11222.

[7] B.U. Keller, R. Hedrich, W.L. Vaz, M. Criado, Single channel recordings of reconstituted ion channel proteins: an improved technique, Pflugers Arch. 411 (1988) 94–100.

[8] E. Lopez-Alonso, J. Canaves, M. Arribas, A. Casanova, J. Marsal, J.M. Gonzalez-Ros, C. Solsona, Botulinum toxin type A inhibits Ca2+-dependent transport of acetylcholine in reconstituted giant liposomes made from presynaptic membranes from cholinergic nerve terminals, Neurosci. Lett. 196 (1995) 37–40.

[9] P. Girard, J. Pecreaux, G. Lenoir, P. Falson, J.L. Rigaud, P. Bassereau, A new method for the reconstitution of membrane proteins into giant unilamellar vesicles, Biophys. J. 87 (2004) 419–429.

[10] M. Mayer, J.K. Kriebel, M.T. Tosteson, G.M. Whitesides, Micro-fabricated teflon membranes for low-noise recordings of ion channels in planar lipid bilayers, Biophys. J. 85 (2003) 2684–2695.

[11] C. Schmidt, M. Mayer, H. Vogel, A chip-based biosensor for the functional analysis of single ion channels, Angew. Chem., Int. Ed. 39 (2000) 3137–3140.

[12] M. Mayer, S. Terrettaz, L. Giovangrandi, H. Vogel, Functional analysis of ion channels: planar patch clamp and impedance spectroscopy of tethered lipid membranes, in: J.M. Cooper, A.E.G. Cass (Eds.), Biosensors: A Practical Approach, 2nd ed., Oxford Univ. Press, Oxford, 2003, pp. 153–184.

[13] M. Davidson, M. Karlsson, J. Sinclair, K. Sott, O. Orwar, Nanotube-vesicle networks with functionalized membranes and interiors, J. Am. Chem. Soc. 125 (2003) 374–378.

[14] A. Karlsson, M. Karlsson, R. Karlsson, K. Sott, A. Lundqvist, M. Tokarz, O. Orwar, Nanofluidic networks based on surfactant membrane technology, Anal. Chem. 75 (2003) 2529–2537.

[15] D.T. Chiu, C.F. Wilson, F. Ryttsen, A. Stromberg, C. Farre, A. Karlsson, S. Nordholm, A. Gaggar, B.P. Modi, A. Moscho, R.A. Garza-Lopez, O. Orwar, R.N. Zare, Chemical transformations in individual ultrasmall biomimetic containers, Science 283 (1999) 1892–1895.

[16] V. Noireaux, A. Libchaber, A vesicle bioreactor as a step toward an artificial cell assembly, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 17669–17674.

[17] L.A. Bagatolli, T. Parasassi, E. Gratton, Giant phospholipid vesicles: comparison among the whole lipid sample characteristics using different preparation methods—A two photon fluorescence microscopy study, Chem. Phys. Lipids 105 (2000) 135–147.

[18] M. Angelova, S. Soleau, P. Meleard, J.F. Faucon, P. Bothorel, Preparation of giant vesicles by external a.c. electric fields. Kinetics and applications, Prog. Colloid Polym. Sci. 89 (1992) 127–131.

[19] L. Mathivet, S. Cribier, P.F. Devaux, Shape change and physical properties of giant phospholipid vesicles prepared in the presence of an AC electric field, Biophys. J. 70 (1996) 1112–1121.

[20] M.I. Angelova, Liposome electroformation, in: P.L. Luisi, P. Walde (Eds.), Giant Vesicles, vol. 6, John Wiley and Sons, New York, 2000, pp. 27–36.

[21] B.M. Discher, Y.Y. Won, D.S. Ege, J.C.M. Lee, F.S. Bates, D.E. Discher, D.A. Hammer, Polymersomes: tough vesicles made from diblock copolymers, Science 284 (1999) 1143–1146.

[22] D.J. Estes, M. Mayer, Electroformation of giant liposomes from spin-coated films of lipids, Colloids Surf., B Biointerfaces 42 (2005) 27–35.

[23] S.D. Shoemaker, T.K. Vanderlick, Intramembrane electrostatic interactions destabilize lipid vesicles, Biophys. J. 83 (2002) 2007–2014.

[24] A. Moscho, O. Orwar, D.T. Chiu, B.P. Modi, R.N. Zare, Rapid preparation of giant unilamellar vesicles, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11443–11447.

[25] F.M. Menger, M.I. Angelova, Giant vesicles: imitating the cytological processes of cell membranes, Acc. Chem. Res. 31 (1998) 789–797.

[26] M.I. Angelova, D.S. Dimitrov, Liposome electroformation, Faraday Discuss. (1986) 303–311.

[27] P. Bucher, A. Fischer, P.L. Luisi, T. Oberholzer, P. Walde, Giant vesicles as biochemical compartments: the use of microinjection techniques, Langmuir 14 (1998) 2712–2721.

[28] T.G. D'Onofrio, A. Hatzor, A.E. Counterman, J.J. Heetderks, M.J. Sandel, P.S. Weiss, Controlling and measuring the interdependence of local properties in biomembranes, Langmuir 19 (2003) 1618–1623.

[29] F.M. Menger, J.S. Keiper, Chemistry and physics of giant vesicles as biomembrane models, Curr. Opin. Chem. Biol. 2 (1998) 726–732.

[30] Y. Yamashita, M. Oka, T. Tanaka, M. Yamazaki, A new method for the preparation of giant liposomes in high salt concentrations and growth of protein microcrystals in them, Biochim. Biophys. Acta 1561 (2002) 129–134.

[31] K. Akashi, H. Miyata, H. Itoh, K. Kinosita, Formation of giant liposomes promoted by divalent cations: critical role of electrostatic repulsion, Biophys. J. 74 (1998) 2973–2982.

[32] A.G. Buckland, D.C. Wilton, Anionic phospholipids, interfacial binding and the regulation of cell functions, Biochim. Biophys. Acta, Mol. Cell Biol. Lipids 1483 (2000) 199–216.

[33] P. Raynal, H.B. Pollard, S.W. Cushman, M. GuerreMillo, Unique subcellular distribution of five annexins in resting and insulin-stimulated rat adipose cells, Biochem. Biophys. Res. Commun. 225 (1996) 116–121.

[34] J.F. Tait, D.F. Gibson, C. Smith, Measurement of the affinity and cooperativity of annexin V-membrane binding under conditions of low membrane occupancy, Anal. Biochem. 329 (2004) 112–119.

[35] M. Born, E. Wolf, Principles of Optics, 3rd ed., Pergamon Press, Oxford, 1965, pp. 426–429.

[36] R.B. Gennis, Biomembranes: Molecular Structure and Function, Springer-Verlag, New York, 1989.

[37] H. Schonherr, J.M. Johnson, P. Lenz, C.W. Frank, S.G. Boxer, Vesicle adsorption and lipid bilayer formation on glass studied by atomic force microscopy, Langmuir 20 (2004) 11600–11606.

[38] N. Fa, C.M. Marques, E. Mendes, A.P. Schroder, Rheology of giant vesicles: a micropipette study, Phys. Rev. Lett. 92 (2004) 1–4.

[39] N. Borghi, O. Rossier, F. Brochard-Wyart, Hydrodynamic extrusion of tubes from giant vesicles, Europhys. Lett. 64 (2003) 837–843.

[40] J.P. Douliez, L. Lavenant, D. Renard, Formation of tubules and giant vesicles from large multilamellar vesicles, J. Colloid Interface Sci. 266 (2003) 477–480.

[41] K.H. de Haas, C. Blom, D. van den Ende, M.H.G. Duits, J. Mellema, Deformation of giant lipid bilayer vesicles in shear flow, Phys. Rev., E 56 (1997) 7132–7137.

[42] R. Wick, M.I. Angelova, P. Walde, P.L. Luisi, Microinjection into giant vesicles and light microscopy investigation of enzyme-mediated vesicle transformations, Chem. Biol. 3 (1996) 105–111.

[43] D.L. Lide (Ed.), CRC Handbook of Chemistry and Physics, 85th ed., CRC Press, Cleveland, 2004.