

# Preparation of Giant Liposomes in Physiological Conditions and Their Characterization under an Optical Microscope

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**ABSTRACT** Unilamellar liposomes with diameters of 25–100  $\mu\text{m}$  were prepared in various physiological salt solutions, e.g., 100 mM KCl plus 1 mM  $\text{CaCl}_2$ . Successful preparation of the giant liposomes at high ionic strengths required the inclusion of 10–20% of a charged lipid, such as phosphatidylglycerol, phosphatidylserine, phosphatidic acid, or cardiolipin, in phosphatidylcholine or phosphatidylethanolamine. Three criteria were employed to identify unilamellar liposomes, yielding consistent results. Under a phase-contrast microscope those liposomes that showed the thinnest contour and had a vigorously undulating membrane were judged unilamellar. When liposomes were stained with the lipophilic fluorescent dye octadecyl rhodamine B, fluorescence intensities of the membrane of individual liposomes were integer multiples (up to four) of the lowest ones, the least fluorescent liposomes being those also judged unilamellar in the phase-contrast image. Micropipette aspiration test showed that the liposomes judged unilamellar in phase and fluorescence images had an area elastic modulus of  $\sim 160$  dyn/cm, in agreement with literature values. The giant liposomes were stable and retained a concentration gradient of  $\text{K}^+$  across the membrane, as evidenced in fluorescence images of the  $\text{K}^+$ -indicator PBFI encapsulated in the liposomes. Ionophore-induced  $\text{K}^+$  transport and associated volume change were observed in individual liposomes.

## INTRODUCTION

Giant liposomes, greater than 10  $\mu\text{m}$  in diameter, have been utilized to study physical properties of lipid bilayers, such as mechanical properties (Servuss et al., 1976; Kwok and Evans, 1981; Hotani, 1984; Schneider et al., 1984; Engelhardt et al., 1985; Farge and Devaux, 1992) and electrical properties (Harbich and Helfrich, 1979; Büschl et al., 1982; Needham and Hochmuth, 1989; Teissié et al., 1989; Itoh et al., 1990; Kinoshita et al., 1992; Stoicheva and Hui, 1994). They have also served as a cell model involving proteins or intact cells (Ketis et al., 1980; Hotani and Miyamoto, 1990; Longo et al., 1992; Miyata and Hotani, 1992; Miyata and Kinoshita, 1994; Evans et al., 1995; Soltesz and Hammer, 1995; Noppl-Simson and Needham, 1996).

For giant liposomes to serve as a cell model, they should be prepared under an ionic condition close to the physiological one, and their unilamellarity must be proved. However, preparation of the liposomes based on the “gentle hydration method” (Reeves and Dowben, 1969; Mueller et al., 1983; Needham and Evans, 1988) was successful only at low ionic strengths, e.g., up to  $\sim 10$  mM NaCl, far lower than the physiological level. The lamellarity of the liposomes was not always investigated. The preparation protocol of giant unilamellar liposomes under physiological ionic conditions should be established.

Here we report on the preparation of giant liposomes at an ionic strength close to the physiological level, using an improved gentle hydration method. Unilamellarity of the liposomes was confirmed by three independent methods, phase-contrast microscopy (Servuss and Boroske, 1979), fluorometric assay (Schneider et al., 1984), and the measurement of the area elastic modulus (Kwok and Evans, 1981). Fluorescence imaging of ionophore-induced  $\text{K}^+$  transport in individual liposomes indicated that the giant liposomes were stable and did not allow the passage of  $\text{K}^+$  in the absence of ionophores.

## MATERIALS AND METHODS

### Materials

Phosphatidylcholine (egg), phosphatidylglycerol (egg), phosphatidic acid (egg), phosphatidylserine (bovine brain), and cardiolipin (bovine heart), all sealed in ampoules, were obtained from Avanti Polar Lipids Co. (Alabaster, AL). Phosphatidylethanolamine (egg yolk, Type III), bovine serum albumin, and nigericin were from Sigma Chemical Co. (St. Louis, MO). Valinomycin and monensin were from Wako Chemical Co. (Tokyo). Octadecyl rhodamine B (R18), di4-ANNEPS, PBFI, and 1,3,6,8-pyrenetetrasulfonic acid (PTS) were obtained from Molecular Probes, Inc. (Eugene, OR). All other chemicals were of analytical grade. Deionized water (Mill-Q system; Millipore, Tokyo) was used in all experiments.

Lipids except cardiolipin were each dissolved at 10 mg/ml in chloroform:methanol (2:1 by volume) dehydrated with Molecular Sieves 3A (Wako Chemical Co.) and stored under a blanket of argon at  $-25^\circ\text{C}$ . Cardiolipin was dissolved at 5 mg/ml in chloroform and stored in the same way. Nigericin, monensin, and valinomycin were dissolved and stored at 10 mM in methanol or ethanol.

Before use, the purity of the lipids was checked by thin-layer chromatography on a precoated plate (SILICAGEL 60; Merck, Darmstadt, Germany) with chloroform:methanol:water (65:25:4) as the developing solvent. Lipids that exhibited contaminant spots, e.g., lyso compounds, were not used. The degree of lipid oxidation was estimated from UV absorbance

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(New, 1990). The absorbance at 233 nm for diene, an intermediate in the oxidation process, was compared with the peak near 200 nm for monoene in a spectrophotometer (UV-3101PC; Shimadzu Co., Tokyo). The ratio between the two peaks was typically  $\sim 0.05$ ; lipids with a ratio of  $>0.1$  were not used.

## Preparation of giant liposomes

Here we describe our standard protocol for the preparation; further below we discuss the key factors that affect the yield (see Discussion).

Phosphatidylcholine and another lipid (phosphatidylglycerol in most experiments) were mixed at 9:1 by weight in chloroform:methanol (2:1 by volume) to make a lipid solution at 10 mg/ml. 100  $\mu$ l of the solution in a 10-ml glass test tube (i.d.  $\sim 1.5$  cm) was dried at 45°C with a rotary evaporator to form a thin lipid film upon the surface of the lower portion of the tube (2–3-cm height). The tube was subsequently placed in vacuo for  $>6$  h to remove the last trace of the organic solvent in the lipid film. The completely dried lipid film was then prehydrated at 45°C with water-saturated nitrogen for 15–25 min. 5–6 ml of an aqueous solution containing 0.1 M sucrose and appropriate salts, which had been  $N_2$  purged, was added gently to the test tube. We refer to this solution as an “internal medium” because it was eventually enclosed in liposomes. The tube was sealed under argon and incubated at 37°C overnight. During the incubation the whole lipid film was gradually stripped off the glass surface and formed an almost transparent bulky white cloud floating in the middle of the solution, which contained giant liposomes.  $\sim 1$  ml of the white cloud was harvested and stored in a plastic tube.

For fluorescence imaging of liposomal membranes, R18 was added to the starting lipid mixture at 0.1% by weight. At this concentration, self-quenching of R18 (Hoekstra et al., 1984; MacDonald, 1990) should be negligible. Liposomes encapsulating PBFI or PTS were prepared in an internal medium containing 20  $\mu$ M PBFI or 50  $\mu$ M PTS.

## Observation of liposomes under a microscope

Liposomes were observed through a 40 $\times$  objective (N.A. 0.85, Fluor Ph3DL; Nikon, Tokyo) or a 20 $\times$  objective (N.A. 0.75, Fluor Ph3DL; Nikon) on an inverted microscope (ICM-405; Carl Zeiss, Inc., Tokyo) in phase-contrast and fluorescence modes. We constructed an observation chamber (1 cm  $\times$  1 cm wide and 3 mm high) on a bottom cover slip by placing on it a U-shaped spacer made of a silicone rubber sheet and then placing another cover slip on the top of the spacer. The rubber adhered to the glass, forming a tight seal. One side of the chamber was left open for manipulation. The chamber was filled with an appropriate salt solution (“external medium”), and the liposome suspension (usually 5–10  $\mu$ l) was introduced from the open side. The external medium contained 0.1 M glucose instead of 0.1 M sucrose. Thus, liposomes settled down on the bottom because of the difference in the density, and their contrast was enhanced as a result of the stepwise difference in the refractive indices of the internal and the external media. The bottom surface of the chamber was coated with bovine serum albumin to prevent adhesion of liposomes. The temperature in the chamber was monitored with a small thermocouple inserted through a pore on a side wall of the spacer.

Phase-contrast images were captured with a charge-coupled-device (CCD) camera (C3077; Hamamatsu Photonics K. K., Hamamatsu, Japan), and fluorescence images with a silicon-intensified-target camera (C1000-12; Hamamatsu Photonics) unless noted otherwise. The video output was fed to an image processor (C2000; Hamamatsu Photonics) for recording and analysis. Long sequences of images were recorded on a video recorder (A-VS-1; Toshiba, Tokyo) and analyzed off line on the image processor.

## Analysis of fluorescence images

Liposomes stained with R18 were excited at 546 nm, and fluorescence between 565 and 615 nm was detected. To assess the lamellarity through fluorescence intensity we integrated the fluorescence image over 16 frames

(0.53 s) and corrected it for background (medium alone) and shading (small unevenness of illumination in an image plane; Kinoshita et al. (1991)). We made the shading correction by taking an image of homogeneous fluorescent solution and dividing by this image the images of liposomes that had been corrected for its background. The peripheries of liposomes appeared bright in the fluorescence images (see Fig. 2 A). Liposomes that exhibited approximately circular periphery were selected for analysis. For each liposome the intensity profile along the circular periphery (“ring profile”) was calculated as described (Hibino et al., 1991). The profile was practically flat (to within 10%), and its average value was taken as the fluorescence intensity of that liposome.

PBFI and PTS were excited at 365 nm, and fluorescence above 397 nm was detected. Raw images were recorded on a video tape and were corrected for background and shading before analysis.

## Measurement of area elastic modulus of a giant liposome

Area elastic moduli of liposomes were measured by the micropipette aspiration technique (Kwok and Evans, 1981). A glass micropipette, drawn from a 1-mm glass tube, was cut perpendicularly to the longitudinal axis by quick fracture to the desired tip diameter, and its tip was slightly fire polished with a microforge without distorting its cylindrical shape. We measured the inner diameter ( $\sim 10$   $\mu$ m) by inserting a glass needle with known dimension. The internal wall of the pipette was coated with bovine serum albumin to prevent adhesion of the liposomal membrane. The micropipette, which was held with a micromanipulator (Narishige, Tokyo), was filled with the external medium. We controlled the aspiration pressure by changing the height of a vertical column of degassed water to which the micropipette was hydraulically connected. The pressure was monitored with a differential pressure transducer with a dynamic range of 100 mm of  $H_2O$  and a precision of 0.1% (DP-15; Validyne, Northridge, CA). Null pressure was determined by the observation of debris flowing into or out of the micropipette tip.

After a liposome was aspirated at a weak negative pressure, the negative pressure  $P$  was increased stepwise, while the diameter  $D$  of the spherical portion of the liposome outside the pipette and the length  $L$  of the portion inside the pipette were observed by phase-contrast microscopy (images were integrated over 16 frames and stored into a frame memory). 10–32 data sets were obtained from a liposome before it ruptured at a high pressure.

The membrane tension  $T$  and the area dilation  $\alpha$  are given approximately by the following equations (Kwok and Evans, 1981):

$$T = P \cdot d[4(1 - d/D)], \quad (1)$$

$$\alpha = d(1 - d/D)(L - L_0)/(D^2 - d^2/4 + dL_0), \quad (2)$$

where  $L_0$  is the length  $L$  for which  $\alpha = 0$  and  $d$  is the inner diameter of the micropipette.  $L_0$  should be  $>d/2$ . For  $T$  above 0.5 dyn/cm, where bending resistance of the membrane is negligible (Helfrich and Servuss, 1984; Evans and Rawicz, 1990), the following linear relation is expected:

$$T = K\alpha, \quad (3)$$

where  $K$  is the area elastic modulus of the membrane. For each liposome, data at  $T > 0.5$  dyn/cm were fitted with the above equations to yield  $K$  and  $L_0$ .

Estimation of  $D$  and  $L$  in the phase image was not straightforward, because the location of the liposome edge was not immediately clear. The edge was better defined in a fluorescence image. We therefore stained several liposomes with 90  $\mu$ M di4-ANEPPS after aspirating the liposomes at the micropipette tip and observed the phase and fluorescence images with one CCD camera to establish the relationship between the edge locations in the phase and the fluorescence image. The concentration of di4-ANEPPS was high to permit fluorescence imaging with the CCD camera. Presumably because of this, the stained liposomes were suscepti-

ble to mechanical perturbation: further aspiration resulted in rupture of the aspirated liposomes.

After the edge location was established, we determined the displacement of the leading edge of the liposome inside the micropipette with subpixel precision by calculating the cross-correlation between two images (Gelles et al., 1988). The estimated precision in this correlation analysis was  $\sim 0.1$  pixel ( $=0.0325$   $\mu\text{m}$ ).

## RESULTS

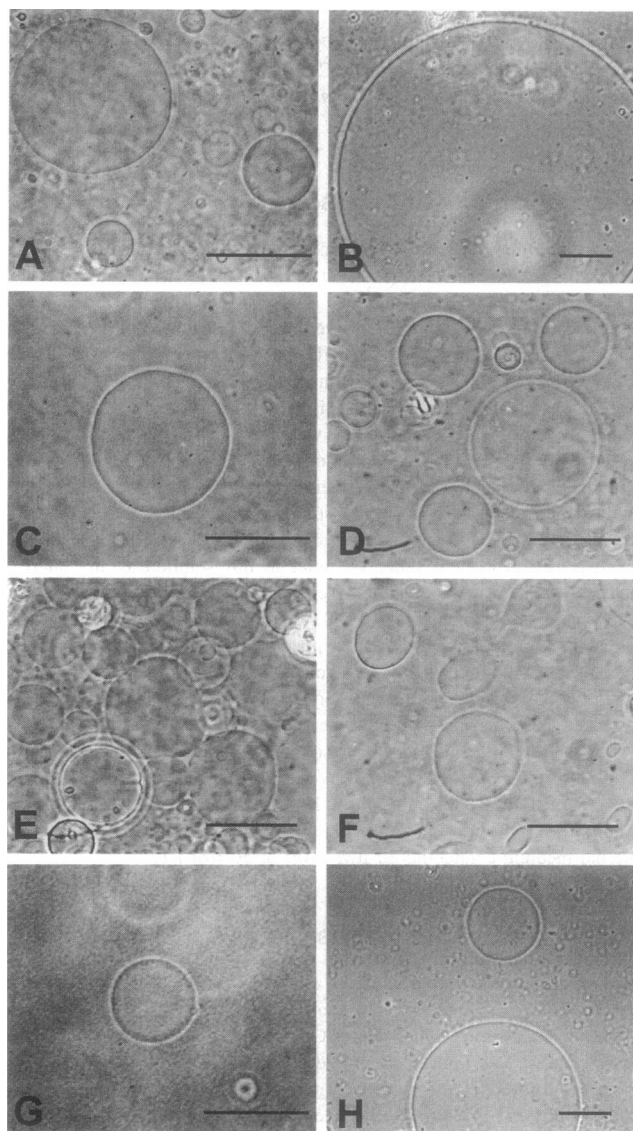
### Observation of giant liposomes

When the liposome suspension was diluted in an external medium and examined by phase-contrast microscopy, giant liposomes with diameters of tens of micrometers were found, as shown in Fig. 1. Most of the giant liposomes appeared circular or slightly off circular, and their peripheries were undulating rapidly owing to thermal fluctuation. The edges of the liposomes appeared as relatively thick, dark bands because of the difference in the refractive indices of the internal and the external media. Direct observation by eye revealed an additional, weak but sharp, contrast at the very edges of liposomes. The edge contrast differed among liposomes. We judged the lowest-contrast and fluctuating liposomes to be unilamellar. The human judgment was borne out by more objective, quantitative fluorometric and aspiration assays, as shown below. Here we call those liposomes that are unilamellar and greater than  $25$   $\mu\text{m}$  in diameter "giant liposomes." The largest giant that we observed had a diameter of  $>300$   $\mu\text{m}$  (Fig. 1 *B*).

Many of the liposomes, though they appeared almost circular in an image plane, were found to be flattened when we observed their outermost edges by changing the focus level (the effect of refraction at the cover slip–solution interface was corrected for). The axial ratio of the oblate liposomes was variable: a ratio as low as 0.56 was found for a large liposome (diameter of  $\sim 90$   $\mu\text{m}$ ). This flattening must have resulted from the difference in the density between the internal and the external media and excess surface area for the volume. The density difference would have created tension in the membrane, which restricted the membrane undulation within a small amplitude.

Starting from 1 mg of lipid in a test tube, our protocol yielded 1 ml of liposome suspension as the final product. When 5–10  $\mu\text{l}$  of the suspension was diluted in an observation chamber, we typically found 10 giant liposomes in the whole chamber. Thus, a total of  $\sim 10^3$  giant liposomes were produced in one tube. This is our criterion for a successful preparation.

In addition to giant liposomes, our protocol produced small vesicles, large but multilamellar liposomes, as well as myelin figures and lipid debris. At the dilution used, however, these extra materials did not seriously interfere with the observation and manipulation of a selected giant liposome (Fig. 1 *C*).



**FIGURE 1** Phase-contrast images of giant liposomes with thin contours. Images were captured through a  $40\times$  objective (for *A*, *C*, and *D–G*) or a  $20\times$  objective (for *B* and *H*) with a CCD or silicon intensified target (for *G* only, the image was integrated over eight frames ( $33$  ms  $\times$   $8$  for noise reduction)). The images were enhanced to clarify the profiles of the liposomes (Inoué and Oldenbourg, 1995). The lipid composition was as follows: *A–C* and *H*, phosphatidylcholine and phosphatidylglycerol (9:1, respectively, by weight); *D*, phosphatidylcholine and phosphatidylserine (9:1); *E*, phosphatidylcholine and phosphatidic acid (9:1); *F*, phosphatidylcholine and cardiolipin (9:1); *G*, phosphatidylethanolamine and phosphatidylglycerol (9:1). For *A–G* the liposomes were prepared in the internal medium containing  $0.1$  M sucrose and  $0.1$  M KCl and diluted in the external medium containing  $0.1$  M glucose and  $0.1$  M KCl ( $0.1$  mM EDTA was included in both media for *A–C*). *H*, Liposomes prepared in  $0.1$  M KCl,  $1.5$  mM  $\text{CaCl}_2$ ,  $0.1$  mM EDTA, and  $0.1$  M sucrose. *A* and *D–G*, Typical views of the field (the suspension was diluted 6–10 times). *B*, The largest liposome found. *C*, A liposome isolated from others and from lipid debris (diluted  $\sim 30$  times). The insides of the liposomes looked slightly darker than the outsides because the slight difference in refractivity between the internal and the external media was enhanced by the phase-contrast optics. When the external medium was the same as the internal medium, only thin contours were observed. Temperature:  $22 \pm 2^\circ\text{C}$ . Scale bar,  $50$   $\mu\text{m}$ .

### Conditions for the formation of giant liposomes

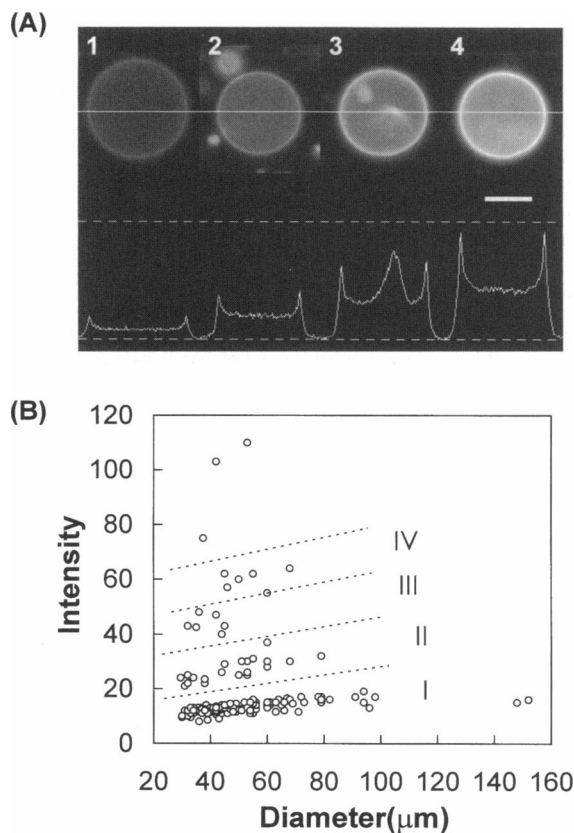
Phosphatidylglycerol could be replaced with another negatively charged lipid, phosphatidylserine (Fig. 1 *D*), phosphatidic acid (Fig. 1 *E*), or cardiolipin (Fig. 1 *F*). The appearance of the giant liposomes did not recognizably differ from when phosphatidylglycerol was used. Doubling phosphatidylglycerol content did not affect the yield of giant liposomes. Instead of phosphatidylcholine, phosphatidylethanolamine (with 10% phosphatidylglycerol) could also form giant liposomes (Fig. 1 *G*), although those liposomes were smaller and the yield lower. Phosphatidylcholine alone, or phosphatidylcholine with 10% phosphatidylethanolamine, failed to form giant liposomes in 0.1 M KCl.

Using the standard lipid composition of 90% phosphatidylcholine and 10% phosphatidylglycerol, we tested whether giant liposomes were formed in a variety of salt solutions (Table 1). In general, the yield was higher at lower ionic strengths. Giant liposomes were successfully produced in an internal medium containing up to 0.1 M of monovalent cations, regardless of anion species including sulfate and phosphate. Addition of  $Mg^{2+}$  or  $Ca^{2+}$  at physiological concentrations did not interfere with the formation of giant liposomes (Fig. 1 *H*). Changing pH between 6 and 8 in 50 mM potassium phosphate did not alter the appearance of giant liposomes. Without a pH buffer, solutions in Table 1 showed a pH of  $\sim 5$ . Addition of HEPES and Tris at 5 mM (pH 7.1) did not affect the yield.

### Fluorometric estimation of the lamellarity

For a quantitative estimation of the number of bilayers in a liposomal membrane we stained the membrane with R18. The staining did not alter the yield or the appearance (phase-contrast images) of the liposomes. Fig. 2 *A* shows fluorescence images of four liposomes prepared in the same test tube. All images were recorded at the same sensitivity. Compared with the least bright liposome at the left, the other three were brighter by factors of  $\sim 2$ ,  $\sim 3$ , and  $\sim 4$ , as the intensity profile at the bottom shows. The liposome at the left was judged unilamellar in the phase-contrast image.

The average intensity of a ring-shaped liposome image was calculated from the ring profile as described in Materials and Methods. In Fig. 2 *B* the average intensity is plotted against the liposome diameter. To obtain this figure we scanned 10 observation chambers and scored all liposomes that appeared circular and were greater than 25  $\mu m$  in diameter. The plots can be divided into several groups, as indicated by the dashed lines. Up to the fourth group the fluorescence intensities were approximately integer multiples of the lowest ones. The distribution in a quantum manner strongly suggests that the liposomes in the lowest-intensity group were unilamellar. These liposomes were in fact the ones that showed the lowest contrast in the phase image. Thus, the fluorescence intensity serves as a reliable indicator of the number of bilayers.



**FIGURE 2** (A) Fluorescence images of giant liposomes stained with R18 (0.1% by weight). Lipid composition and internal and external media were as in Fig. 1 *A* without EDTA. Observation was made in a rectangular open chamber (1 cm  $\times$  1.5 cm  $\times$  3 mm). These images were integrated over 16 frames (33 ms  $\times$  16) and corrected for background and shading. The intensity profile along the horizontal line is shown at the bottom. Liposome 1, at the left, was of the type that has the lowest fluorescence. Scale bar, 25  $\mu m$ . (B) Distribution of fluorescence intensities of liposomes versus liposome diameter. For each liposome the average intensity in the bright periphery was calculated from the ring profile (see Materials and Methods). All liposomes showing circular periphery and having diameters of  $>25 \mu m$  were scored in 10 chambers. The groups I–IV are presumably uni-, bi-, tri-, and tetra-lamellar liposomes.

The fluorometric distinction of lamellarity is facilitated by the relatively weak dependence of the fluorescence intensity on the liposome diameter (Fig. 2 *B*). In the calculation of the ring profile, only the brightest part of the ring-shaped image of a liposome is taken into account (Hibino et al., 1991). The outer edge of a liposome image is brightest because the membrane lies parallel to the optical axis and the fluorescence from that part of the membrane that is within the focal depth is integrated. For liposomes that are much greater than the focal depth (several micrometers) the integrated intensity is expected to be only weakly dependent on the liposome diameter.

### Estimation of lamellarity from the area elastic modulus

To confirm the unilamellarity further we subjected liposomes that were judged unilamellar in the phase-contrast

**TABLE 1** Formation of giant liposomes in various salt solutions

KCl		K <sub>2</sub> SO <sub>4</sub>		K-phosphate		Others	
Medium	Formation*	Medium	Formation*	Medium	Formation*	Medium	Formation*
150 mM (+0 M)	—	100 mM (+0.15 M)	—	100 mM (+0.2 M) <sup>#</sup>	—	100 mM NaCl	+
100 mM	++	50 mM	+	50 mM (+0.2 M) <sup>#</sup>	+	100 mM Choline Cl	+
50 mM	++	25 mM	+	5 mM (+0.2 M) <sup>#</sup>	+	Na phosphate <sup>¶</sup>	++
10 mM	++	10 mM (+0.4 M)	+	50 mM, pH 6.0 <sup>§</sup>	+	100 mM KCl + 1 mM CaCl <sub>2</sub>	+
10 mM (+0.28 M)	+			50 mM, pH 7.2 <sup>§</sup>	++	100 mM KCl + 1.5 mM CaCl <sub>2</sub> <sup>  </sup>	++
1 mM	++			50 mM, pH 8.1 <sup>§</sup>	+	100 mM KCl + 3 mM CaCl <sub>2</sub> <sup>  </sup>	—
0 mM (+0.28 M)	+					100 mM KCl + 1 mM MgCl <sub>2</sub>	+

All solutions included sucrose at 0.1 M or at the concentration indicated in the parentheses.

\* Formation of liposomes: ++, successful preparations (see text) repeated many times; +, one attempt, with success; —, one attempt, with a lower yield and smaller sizes; —, failure (no giant liposome found).

<sup>#</sup> Solutions of K<sub>2</sub>HPO<sub>4</sub> and KH<sub>2</sub>PO<sub>4</sub> of the indicated concentration were mixed to adjust to pH 7.5.

<sup>§</sup> A solution of 50 mM K<sub>2</sub>HPO<sub>4</sub> and a mixture of 50 mM KH<sub>2</sub>PO<sub>4</sub> and 50 mM KCl were mixed to adjust pH to the indicated value ([K<sup>+</sup>] = 100 mM, [phosphate] = 50 mM irrespective of pH).

<sup>¶</sup> A solution of 50 mM Na<sub>2</sub>HPO<sub>4</sub> and a mixture of 50 mM NaH<sub>2</sub>PO<sub>4</sub> and 50 mM NaCl were mixed to adjust to pH 7.2 ([Na<sup>+</sup>] = 100 mM, [phosphate] = 50 mM).

<sup>||</sup> 0.1 mM EDTA was included.

images to the micropipette aspiration test. Fig. 3 A shows a typical plot of membrane tension  $T$  versus area dilation  $\alpha$ . A linear relation was obtained for  $T > 0.5$  dyn/cm, as in other studies (Kwok and Evans, 1981; Evans and Rawicz, 1990). The area elastic modulus  $K$  was calculated from the linear portion and is plotted against liposome diameter in Fig. 3 B. Liposomes that ruptured below  $T = 1.0$  dyn/cm were excluded from this plot because  $K$  could not be determined reliably.

The solid line in Fig. 3B shows the mean value, 163.3 dyn/cm (the one with  $K = 339$  dyn/cm is excluded). This value is within the range of literature values for unilamellar membranes, e.g., 140 dyn/cm for egg phosphatidylcholine (Kwok and Evans, 1981). All points in Fig. 3 B except for one are grouped around the solid line, indicating that these were in fact unilamellar liposomes. The liposome with  $K = 339$  dyn/cm had slightly thicker contrast at its edge; this particular one was probably bilamellar.

Two liposomes stained with R18 and showing the lowest fluorescence intensity were subjected to the micropipette aspiration test, yielding values of  $K$  of 139 and 149 dyn/cm. Both fluorescence and area elastic modulus are thus reliable indicators of lamellarity.

## Ionic transport imaging

To demonstrate the stability of the giant liposomes and their applicability in the biological study we imaged K<sup>+</sup> concentration inside a liposome with the fluorescent K<sup>+</sup>-indicator dye PBFI (Jezek et al., 1990; Venema et al., 1993). A giant unilamellar liposome encapsulating 100 mM Na<sup>+</sup>, 100 mM sucrose, and PBFI (5  $\mu$ l of suspension) was placed in a solution (550  $\mu$ l) containing 100 mM K<sup>+</sup> and 100 mM glucose. As described above, the density difference between the internal and the external media caused flattening of the liposome (Fig. 4 C, left).

The fluorescence intensity of PBFI remained stationary, except for slight photobleaching, for at least several minutes (between  $-473$  and  $0$  s in Fig. 4 B), demonstrating that the liposomal membrane was impermeable to K<sup>+</sup> and that the membrane did not open in spite of vigorous fluctuation. From time 0, nigericin, a K<sup>+</sup>-H<sup>+</sup> exchanger, was delivered to the liposome for  $\sim 20$  s through a micropipette from the bottom side in Fig. 4 A. The fluorescence of PBFI almost doubled, indicating K<sup>+</sup> uptake, and again remained essentially stationary. Calibration using the same media, without liposomes, indicated that the fluorescence intensity of PBFI in a solution containing 100 mM K<sup>+</sup> was approximately twice higher than that in a solution containing 100 mM Na<sup>+</sup>.

During the course of K<sup>+</sup> uptake the liposome changed its morphology. Initially, the liposome appeared as an easily deformable ellipse (cf. the images at 6 and 22 s shown in Fig. 4 A), indicating that the liposome had an excess area for its volume. Later (28 s in Fig. 4 A), its shape became an undeformable circle, indicating that the liposome became a complete sphere. Given that the liposomal membrane area remains constant, the loss of deformability indicates an increase in the liposomal volume (swelling) accompanying the influx of K<sup>+</sup> (Fig. 4 C); the corresponding H<sup>+</sup> efflux would be buffered by the phosphate in the internal and external media. If the membrane had been permeable to other solutes, such an osmotic swelling would not have been observed. In the image plane the diameter of the resultant circle was slightly smaller than the minor axis of the ellipse. The initially flattened liposome grew in height at the expense of the lateral size. Both the swelling and the fluorescence increase were consistently observed in the total of six liposomes tested.

Swelling complicates the interpretation of the intensity of fluorescence images. As a control, we prepared liposomes encapsulating PTS, which is insensitive to K<sup>+</sup>, and allowed

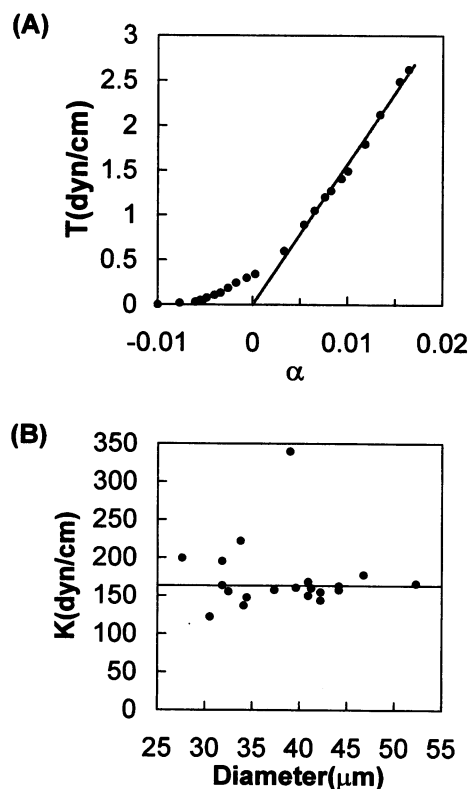


FIGURE 3 (A) Membrane tension  $T$  versus area dilation  $\alpha$  for a liposome measured in the micropipette aspiration test. Lipid composition and media were as in Fig. 1 A without EDTA. The diameter  $D$  of the spherical part of the liposome outside the pipette was  $44.2 \mu\text{m}$ . The pipette's inner diameter  $d$  was  $11.7 \mu\text{m}$ . The area elastic modulus  $K$  was obtained as  $157.5 \text{ dyn/cm}$  by fitting the portion at  $T > 0.5 \text{ dyn/cm}$  with Eq. 3 of the text (solid line). (B) Distribution of area elastic moduli of giant liposomes versus the diameter outside the pipette. Liposomes that were ruptured at  $T < 1.0 \text{ dyn/cm}$  and that had  $L_0 < d/2$  were not scored. The average value, excluding the one with  $K$  of  $339 \text{ dyn/cm}$ , was  $163.3 \text{ dyn/cm}$ . Solid line, the average value. Temperature,  $25 \pm 1^\circ\text{C}$ .

them to swell under hypotonic conditions. The change in the liposome shape that accompanied the swelling (Fig. 4 C) resulted in an apparent increase in the integrated fluorescence intensity, but the increment did not exceed 20%. Most of the fluorescence increase in Fig. 4 B, therefore, represents a genuine response to a rise in  $\text{K}^+$  concentration.

In contrast to nigericin, valinomycin, a  $\text{K}^+$  ionophore, did not increase the PBFI fluorescence. Subsequent addition of nigericin induced a stepwise increase similar to the one in Fig. 4 B. These results are consistent with the expectation that valinomycin alone will immediately establish a membrane potential that opposes further influx of  $\text{K}^+$  (Jezek et al., 1990). A detectable increase in  $\text{K}^+$  concentration inside a liposome requires collapse of the potential, e.g., by the efflux of  $\text{H}^+$  with nigericin. Monensin induced an increase in PBFI fluorescence that was slow compared with the nigericin-induced increase. Monensin thus appears to have some affinity for  $\text{K}^+$ , in addition to its known affinity for  $\text{Na}^+$  and  $\text{H}^+$ .

## DISCUSSION

### Preparation of giant unilamellar liposomes

Successful preparation of giant unilamellar liposomes in physiological ionic solutions requires the following key factors: 1) Inclusion of charged lipids. In our study, giant unilamellar liposomes were formed only when charged lipids were included. This suggests that electrical repulsion between the charged lipid facilitates the formation of unilamellar membranes by opposing the intrinsic adhesive force between the membranes (Rand, 1981; Evans and Needham, 1987). 2)  $37^\circ\text{C}$  incubation, which significantly promoted the liposome formation; when we attempted to prepare liposomes at  $4^\circ\text{C}$  (Mueller et al., 1983) or at room temperature ( $\sim 22^\circ\text{C}$ ), the yield was far lower. 3) Prehydration, which was an absolute requirement for the successful preparation when the ionic strength exceeded  $100 \text{ mM}$  in the internal medium but was not necessary at  $10 \text{ mM}$  or less with respect to KCl. 4) Making of a uniformly thin lipid film. We found that a rotary evaporator was suitable for this purpose and also found that baking the test tube in an oven at  $\sim 260^\circ\text{C}$  for a few hours helped the lipid mixture to spread uniformly upon the glass surface. 5) Freshness of the lipid. Exposure of lipids, especially charged lipids, to air should be minimized: When a lipid containing the oxidized lipid or lyso compounds was used, the number and the size of the liposomes significantly decreased.

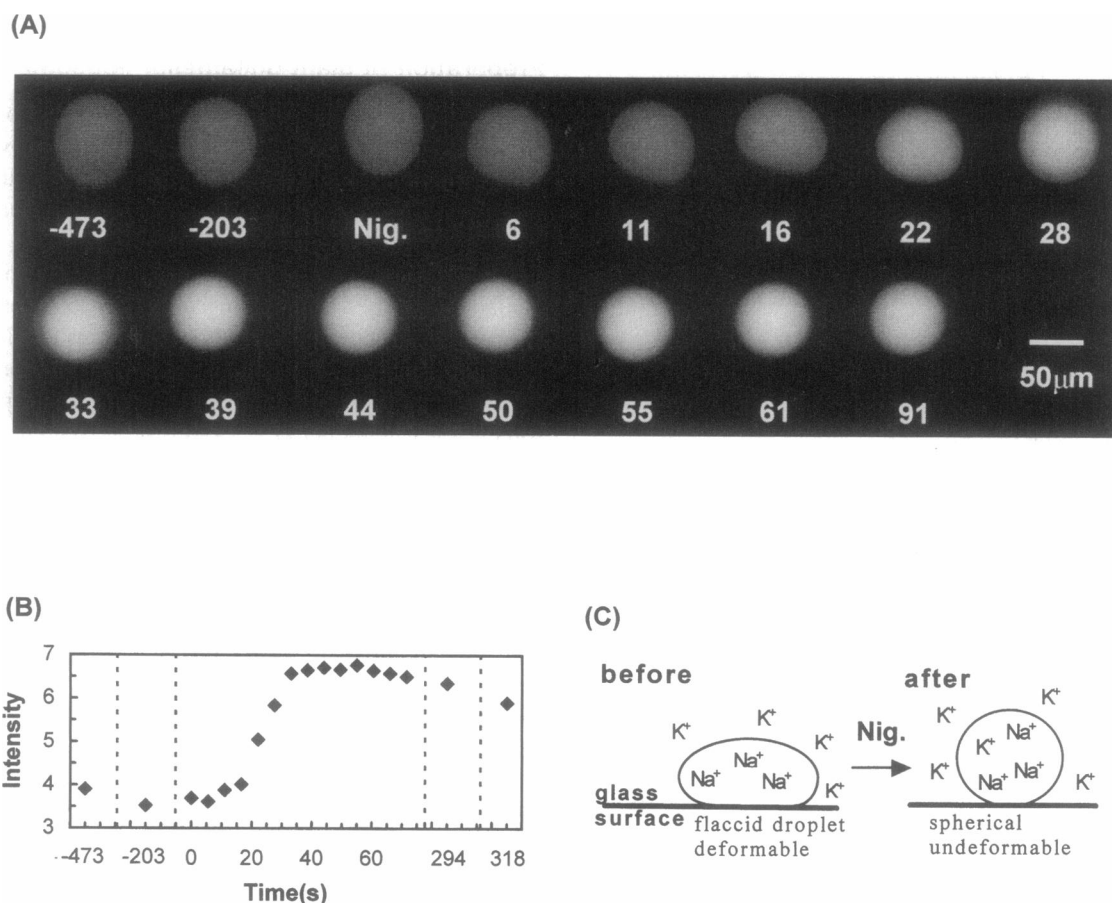
We noticed that inclusion of  $0.1 \text{ mM}$  EDTA in the internal medium often, but not always, increased the yield of the liposomes. Inasmuch as neither  $\text{Mg}^{2+}$  nor  $\text{Ca}^{2+}$  affected the yield, we suspect that contamination of metal ions with higher valence  $\text{Al}^{3+}$ , or divalent but heavy metal ions, such as  $\text{Ni}^{2+}$  and  $\text{Pb}^{2+}$ , interfered with the formation of liposomes. We also noticed that occasional gentle rocking of the test tube during the  $37^\circ\text{C}$  incubation promoted the stripping off of the lipid film. This characteristic may be useful when entrapment of the proteins is intended (see below).

### Relationship to other studies

Giant liposomes containing a charged lipid as an ingredient have been prepared in  $10 \text{ mM}$  NaCl by the hydration method (Needham and Hochmuth (1989)), but we found that the ionic strength could be raised to  $100 \text{ mM}$  with respect to  $\text{K}^+$  and that  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  ions at physiological concentrations could be incorporated into the liposomes.

Neutral lipids have been shown to hydrate and form unilamellar structures even in the presence of salts; thermal fluctuations of membranes are the source of repulsive force between the membranes (Servuss and Helfrich, 1989; Lipowsky, 1991). This, however, has been reported to take much longer time than in pure water (Servuss and Helfrich, 1989). We have been unable to observe, with our preparation method, giant unilamellar liposomes composed of neutral lipids alone at KCl concentrations over  $1 \text{ mM}$ .





**FIGURE 4**  $\text{K}^+$  uptake into a giant liposome induced with nigericin. (A) Snapshots, at indicated times (seconds), of PBFI fluorescence in the liposome. 100  $\mu\text{M}$  nigericin was blown onto the liposome at 0–20 s. The liposome was made of phosphatidylcholine, phosphatidylglycerol, and R18 (9:1:0.01 by weight), and its unilamellarity was confirmed from the fluorescence intensity of R18. The internal medium was a mixture of 50 mM  $\text{Na}_2\text{HPO}_4$  and (50 mM  $\text{NaH}_2\text{PO}_4$  + 50 mM NaCl), pH 7.2, containing, in addition, 0.1 M sucrose and 20  $\mu\text{M}$  PBFI. The external medium was 50 mM  $\text{K}_2\text{HPO}_4$  and (50 mM  $\text{KH}_2\text{PO}_4$  + 50 mM KCl), pH 7.2, containing 0.1 M glucose. (B) Total fluorescence intensity (arbitrary units) integrated over the entire liposome image. Between –470 and –210 s, a solution containing the same amount of methanol as in the nigericin solution was blown on, without noticeable effect. The excitation light was shut off between –210 and 0 s. (C) Schematic illustration of the change in liposome geometry.

A number of other methods have been utilized to prepare at high ionic strengths giant liposomes entrapping biomacromolecules: ether injection (Deamer and Bangham, 1976), reverse-phase evaporation (Szoka and Papahadjopoulos, 1978; Fraley et al., 1980), and the double-emulsion method (Kim and Martin, 1981). However, the sizes of the liposomes (average  $\sim 10 \mu\text{m}$ ) may not be always sufficient for their manipulation under an optical microscope. A dialysis method (Oku et al., 1982) and a freeze-thaw method (Tank et al., 1982; Oku and MacDonald, 1983a,b; Higashi et al., 1987; Elbaum et al., 1996) have been employed to prepare at high-ionic-strength giant liposomes, the sizes of which, in some cases, exceeded 50  $\mu\text{m}$  in diameter. These methods, however, include step(s) that may be too severe for some proteins to survive. In addition, the unilamellarity of individual liposomes was not always confirmed in many of the above studies.

In some cases the lamellarity for individual liposomes was examined by electron microscopy (Hub et al., 1982; Mathivet et al., 1996) or that for a liposome suspension by

measurement of the trapped volume and total lipid (Oku et al., 1982; Oku and MacDonald, 1983a,b). Obviously, these methods do not allow one to use the assayed individual liposomes in the subsequent experiment. Higashi et al. (1987) measured the membrane electrical capacitance of a giant liposome by means of the patch-clamping technique for evaluation of the unilamellarity; however, no distribution of the capacitance in a quantum manner was exhibited.

In this study, we assessed the lamellarity of the individual liposomes by three independent methods: phase-contrast microscopy, fluorometry, and the micropipette aspiration test. We demonstrated that all three methods gave consistent results, confirming that the giant unilamellar liposomes were indeed prepared under the physiological ionic condition. Of the three, the simplest and most reliable method was fluorometry. Because the fluorescence intensity was relatively insensitive to the liposome size (Fig. 2 B), a simple intensity analysis of a fluorescence image suffices to identify unilamellar liposomes (see the intensity profile in Fig. 2 A).

## Possible applications of giant liposomes

Our method offers a wide range of selection of the ionic species that can be entrapped in the liposomes, thereby allowing entrapment of proteins in the liposome under conditions suitable for individual proteins. The time required for the 37°C incubation can be shortened by occasional, gentle rocking, which would help to reduce the possibility of denaturation. Direct incorporation of membrane proteins in the liposomal membrane would be difficult at the initial preparation stage unless the protein withstood the chloroform-methanol environment. However, one could attempt fusion between small proteoliposomes and preformed giant liposomes, as was done with planar lipid membranes (Cohen et al., 1984).

Most experiments for membrane transport using liposomes were made in cuvettes containing large numbers of small liposomes. Individual behaviors and exact lamellarities of the liposomes were rarely investigated. It should be advantageous, as shown here, that one can observe selected unilamellar liposomes continuously to estimate the volumes and the surface areas of individual liposomes, which are important parameters in the analysis of transport characteristics. In experiments shown in Fig. 4 we occasionally noticed that a liposome underwent lysis after it became fully spherical owing to the influx of  $K^+$ . Such lytic phenomena would be undetected in a cuvette measurement.

The giant liposomes prepared at high ionic strengths by the previous methods were completely spherical and were probably under tension (Oku et al., 1982; Higashi et al., 1987), which would prevent any further deformation. The giant unilamellar liposomes prepared with our method are flaccid and can assume various shapes, as exemplified by Fig. 5. Thus, our liposomes will be suitable for study of the

mechanism of morphological change of cells (Hotani and Miyamoto, 1990; Käs and Sackmann, 1991; Farge and Devaux, 1992; Miyata and Kinosita, 1994) or the effect of ionic strength on mechanical properties of the lipid bilayers (Winterhalter and Helfrich, 1992). Further, inasmuch as the tension in the liposomal membrane can be controlled, by the aspiration method or otherwise, a study of the kinetics of mechanosensitive channels may also be feasible (Sokabe et al., 1991; Opsahl and Webb, 1994).

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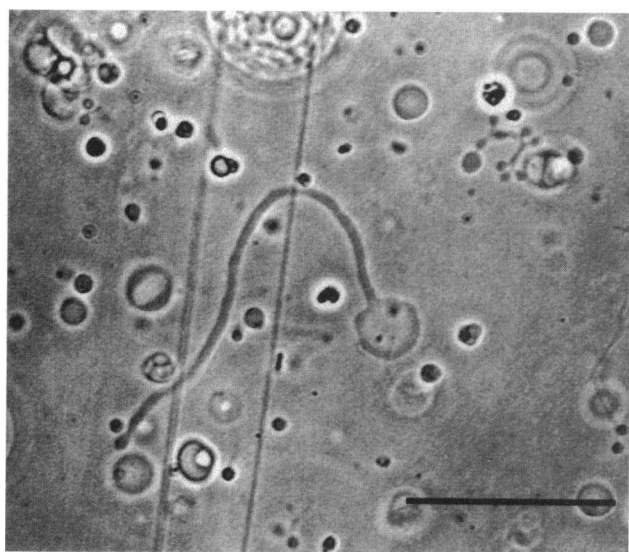


FIGURE 5 Liposome with thin, long tubular structure. The two thin lines running from the top to the bottom in the image are also lipid tubes. The lipid composition and media were the same as for Fig. 1 A. Bar, 50  $\mu$ m. Objective, 40 $\times$ .



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