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Chemistry

Rapid preparation of giant unilamellar vesicles

(liposomes/encapsulation/proteoliposomes/transport)

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ABSTRACT **We report here a rapid evaporation method that produces in high yield giant unilamellar vesicles up to 50 μm in diameter. The vesicles are obtained after only 2 min and can be prepared from different phospholipids, including L-α-phosphatidylcholine (lecithin), dipalmitoleoyl L-α-phosphatidylcholine, and β-arachidonoyl γ-palmitoyl L-α-phosphatidylcholine. Vesicles can be produced in distilled water and in Hepes, phosphate, and borate buffers in the pH range of 7.0 to 11.5 with ionic strengths up to 50 mM. The short preparation time allows encapsulation of labile molecular targets or enzymes with high catalytic activities. Cell-sized proteoliposomes have been prepared in which γ-glutamyltransferase (EC 2.3.2.2) was functionally incorporated into the membrane wall.**

Liposomes consisting of single phospholipid membranes (unilamellar vesicles) are excellent model systems for studying the dynamics and structural features of many cellular processes, including viral infection, endocytosis, exocytosis, cell fusion, and transport phenomena. In addition to having importance for basic research in biological disciplines, liposomes are used as vehicles for drug application (1), for gene transfer in medical therapy and genetic engineering (2), and as microcapsules for proteolytic enzymes in the food industry (3). Vesicles also open many exciting possibilities for chemical reactions in small confined volumes, 10^{-12} to 10^{-21} liters. If large enough, individual vesicles can be manipulated with glass micropipettes and optical tweezers. For these purposes researchers have developed various methods to prepare vesicles of different sizes, membrane compositions, and layer structures (4).

Unilamellar liposomes can be obtained by reverse-phase evaporation techniques (5, 6). These liposomes are, however, rarely larger than 1 μm in diameter (6), require long preparation times, in excess of 30 min, and involve an extensive mixing between the aqueous and organic phases that possibly can denature proteins (5). A second procedure produces giant unilamellar vesicles (GUVs), up to 300 μm in diameter, by exposing dried crystalline lipid films to aqueous solutions for long periods of time [from 1 (7) to 48 h (8)]. Based on the principles of the second preparation, a modified method that employs low ac fields can produce giant (up to approximately 70-μm) unilamellar liposomes in pure water (9). Limitations of the methods based on film swelling arise in terms of time requirements and/or use of high temperatures of 70°C (7), both of which can lead to extensive degradation or alteration of labile molecules. The technique using ac fields is additionally restricted to low ionic strengths (below, e.g., 10 mM NaCl) and osmolarities (below, e.g., 2.5 mM dextran) (10). Furthermore, the voltage applied cannot exceed ≈2 V, because polarization effects arise from electrochemical reactions at the electrodes, limiting an important control parameter (11). Vesicles can also be produced simply by injecting phospholipids dissolved in an organic solvent into an aqueous phase (12). The drawbacks of this method are that the organic solvent is still contained in the vesicles and surrounding liquid, multilamellar vesicles are produced in high yield, and only small vesicles are formed (<1 μm in diameter).

Taking these facts into consideration, a rapid method to prepare vesicles under nondestructive conditions is desirable for many purposes—especially for work with labile and highly reactive biomolecules. Another important aspect is to obtain GUVs, larger than 2 μm in diameter, that in addition to having high encapsulation efficiencies also can be viewed in a light microscope and be manipulated with ease, using optical tweezers (13) and glass micropipettes. We describe herein a simple method for the preparation of GUVs from several different phospholipids. The procedure takes approximately 2 min and requires only standard laboratory apparatus.

MATERIALS AND METHODS

Liposome Preparation. The lipids were dissolved in chloroform (0.1 M), and 20 μl of this solution was added to a 50-ml round-bottom flask containing 980 μl of chloroform and 100–200 μl of methanol. The aqueous phase (7 ml of distilled water or buffer) was then carefully added along the flask walls. It should be feasible to scale this procedure up or down, as desired. Liposomes were formed in distilled water, Hepes buffer (10 mM, pH adjusted to 7.4 with NaOH), sodium phosphate buffer (10 mM, with 11 mM $MgCl_2$, pH 7.4), and sodium borate buffer (50 mM, pH 9.2). Molecules to be entrapped in the vesicles or enzymes to be incorporated into the liposome membrane were added to the buffer solution prior to evaporation of the organic solvent. We used 1 mM fluorescein in Hepes buffer, rhodamine 6G (a mixture of 1 ml of 10 μM rhodamine in ethanol, 1 ml of a saturated rhodamine solution in Hepes buffer, and 5 ml of a 10 mM Hepes buffer), and 1 mg of γ-glutamyltransferase (γ-GT; EC 2.3.2.2) in 7 ml of 10 mM phosphate buffer, pH 7.4, with 11 mM $MgCl_2$ and 10 mM glutathione (GSH). The organic solvent was removed in a rotary evaporator (Buechi R-124) under reduced pressure [Cole–Parmer aspirator pump 7049-00, final pressure 10 mmHg (1.3 kPa)] at 40°C (Buechi Waterbath B-481) and 40 rpm. As a consequence of the different boiling points of chloroform (61°C at 100 kPa) and methanol (64°C at 100 kPa), we observed two major boiling events. After evaporation for 2 min, an opalescent fluid was obtained with a volume of approximately 6.5 ml. The resulting aqueous solution contained GUVs in high concentration. Liposomes were characterized by means of brightfield, fluorescence, and electron microscopy to measure the size distribution, the membrane seal, and the detailed membrane structure.

γ-GT Assay. A 1-ml solution of GSH-containing (10 mM) liposomes with incorporated γ-GT was incubated for 2 h with

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Abbreviations: CE–LIF, capillary electrophoresis–laser-induced fluorescence; FDP, fluorescein diphosphate; FMP, fluorescein monophosphate; γ-GC, γ-glutamylcysteine; γ-GT, γ-glutamyltransferase; GUV, giant unilamellar vesicle; GSH, glutathione.

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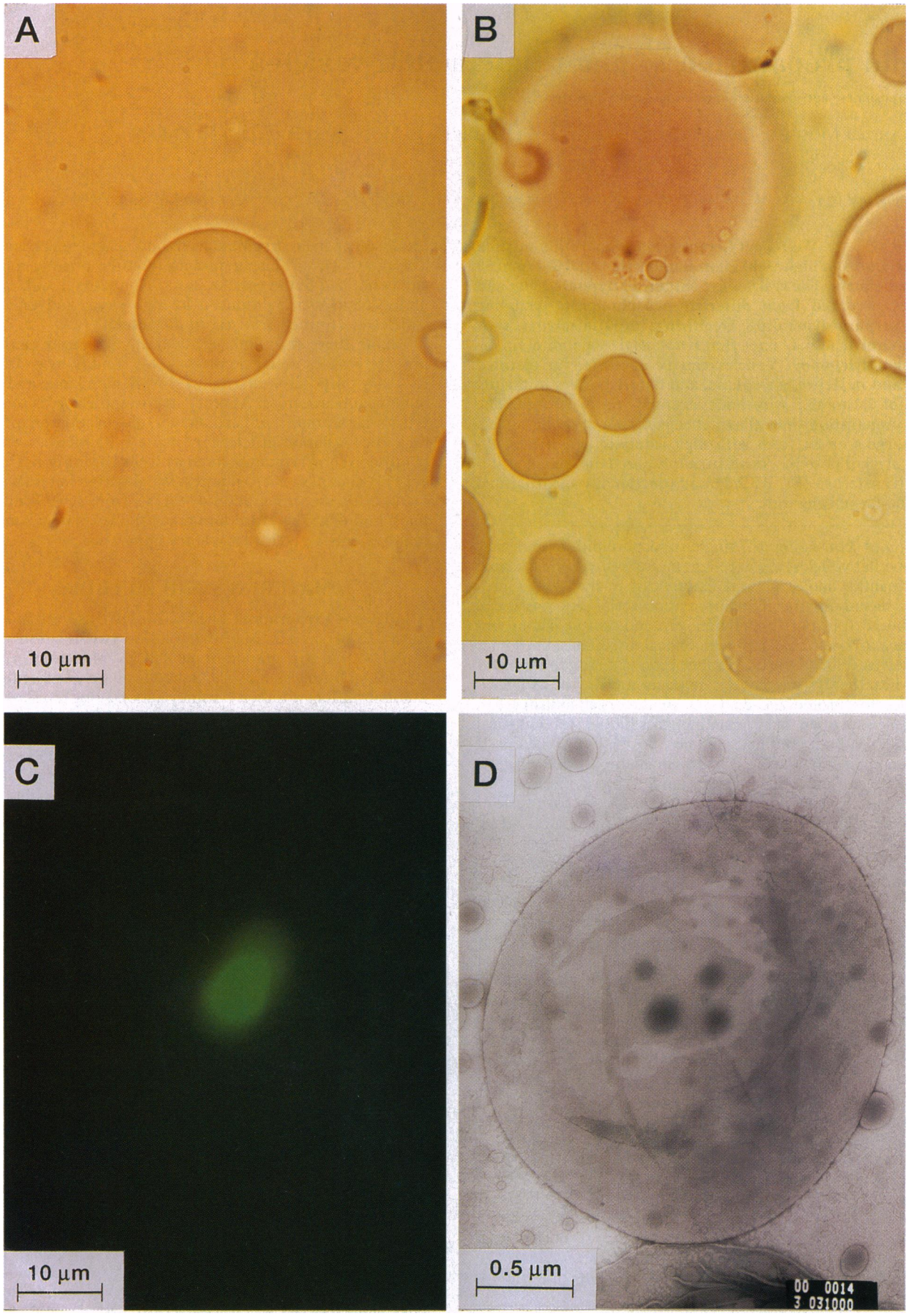

FIG. 1. (*Legend appears on opposite page.*)

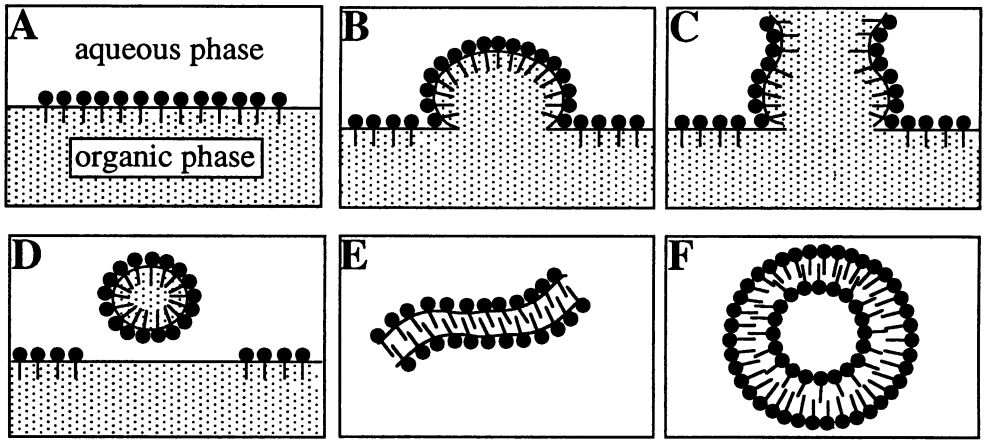

FIG. 2. Diagram of the suggested formation mechanism for GUVs. Initially there is an ordered monolayer of phospholipids at the interface between an aqueous and an organic phase (*A*). During evaporation, bubbles form (*B*) that rupture the phospholipid film into fragments (*C*). The resulting phospholipid monolayer fragments fuse to bilayers (*E*), which spontaneously vesiculate (*F*). An alternative way for the formation of bilayered phospholipid fragments (*D*) involves micellar structures having entrapped liquid organic solvent.

L-cysteine (10 mM) in a phosphate buffer (10 mM, pH 7.4, 11 mM $MgCl_2$). The liposome fraction was separated as a pellet by centrifugation at 15,600 × *g* for 10 min and resuspended in phosphate buffer. A small reaction vial was filled with 20 μl of this solution, and liposomes were lysed for 2 min by addition of 20 μl of acetonitrile and application of shear forces. Then, 20 μl each of borate buffer and a solution of 2,3-naphthalenedicarboxaldehyde (10 mg in 1 ml of acetonitrile) were added. After a 2-min reaction time, an aliquot was analyzed for γ-glutamylcysteine (γ-GC) formation by using a capillary electrophoresis–laser-induced fluorescence (CE–LIF) detection system as described elsewhere (14).

Electron Microscopy. For electron microscopy, a drop of freshly prepared vesicle solution was applied to a Formvar-coated copper grid made hydrophilic by a glow discharge. After 20 s, the excess liquid was absorbed at the periphery of the grid by using filter paper. The remaining sample was air-dried at room temperature for 10 min. Finally, the liposomes were negatively stained with 0.5% uranyl acetate for 20 s, after which most of the staining solution was absorbed at the periphery of the grid by means of filter paper. Samples were examined with a Philips 410 transmission electron microscope at ×31,000 magnification.

CE–LIF Assay for GSH and Fluorescein Diphosphate (FDP) Hydrolysis Products. Thermal degradation of GSH (dimerization) was studied following incubation of GSH in phosphate buffer (pH 7.4) for 2 min at 40°C (comparable to the conditions involved in our method), and for 2 h at 70°C (as used for vesicle productions from swelling of dried phospholipid films). Samples were taken directly after the preparation of the GSH solution (1 mM) and subsequently at 30-min intervals up to 2 h. The extent of hydrolysis of FDP (1 mM in 10 mM Hepes solution, pH 11.5) to fluorescein and fluorescein monophosphate (FMP) after 2 h at 70°C was compared with that observed in samples incubated for 2 min at 40°C. GSH concentrations were measured by using CE–LIF detection (14). FMP and fluorescein concentrations were determined by using the same CE–LIF detection setup used for analysis of GSH under identical experimental conditions (migration times of FMP and fluorescein were 15 and 10 min, respectively).

Chemicals and Materials. L-α-Phosphatidylcholine (lecithin; type XVI-E from fresh egg yolk, approximately 99% pure, lyophilized powder packaged under argon), dipalmitoleoyl L-α-phosphatidylcholine (synthetic, approximately 99% pure, lyophilized powder), and β-arachidonoyl γ-palmitoyl L-α-phosphatidylcholine (approximately 99% pure, approximately 97% positional purity of fatty acids, lyophilized powder packaged under argon), γ-GT (type II from bovine kidney, lyophilized powder containing approximately 85% protein, activity 15–30 units/mg of solid), GSH (reduced form, free acid, 98–100% pure, crystalline), γ-GC (trifluoroacetate salt, minimum 80%, peptide content approximately 70%), L-cysteine (hydrochloride monohydrate, SigmaUltra, >99% pure), and rhodamine 6G (perchlorate) were purchased from Sigma. Fluorescein (F-1300, high-purity reference standard) was obtained from Molecular Probes, and 2,3-naphthalenedicarboxaldehyde (98%) was from Aldrich.

RESULTS AND DISCUSSION

Vesicle Formation. We produced GUVs in high yield with all the buffers used (Fig. 1). The majority of the vesicles were spherical. By means of transmission electron microscopy, we concluded that the liposomes were mainly unilamellar (Fig. 1*D*). We successfully entrapped the fluorescent dyes fluorescein and rhodamine 6G inside the GUVs (Fig. 1 *B* and *C*). Fluorescence microscopy studies of fluorescein and rhodamine 6G-containing vesicles purified by size-exclusion chromatography showed no leakage of dyes into the extravesicular solution.

Although no convincing model exists, in general, for vesicle formation, it has been proposed that liposomes produced by injection methods (12) evolve from phospholipid bilayer structures that precipitate at the boundary of rising bubbles and peel off to form vesicles (15). Liposomes prepared from swelling of dried crystalline lipid films are claimed to bud off vesicles from "myelin figures" (15), and in reverse-phase evaporation techniques an inverted micelle state is thought to precede the formation of vesicles (5).

We believe that vesicle formation in our method proceeds as follows: Initially, an ordered monolayer of phospholipids is

FIG. 1 (*on opposite page.*). (*A* and *B*) Brightfield photomicrographs of liposomes prepared in borate buffer (50 mM, pH 9.3) (*A*) or in Hepes buffer containing rhodamine 6G (*B*). (*C*) Fluorescence photomicrograph of a vesicle with entrapped fluorescein molecules. The fluorescein-containing liposomes were run through a size-exclusion column (Bio-Rad Econo-Pac 10DG column) to retain fluorescein dissolved in the buffer. The image appears blurry because of the long exposure time and the movement of the liposome. (*D*) Electron micrograph of uranyl acetate-stained rhodamine-containing liposomes that shows a single phospholipid bilayer surrounding the aqueous interior. (×24,000.)

formed at the interface between the two phases (Fig. 2*A*). In this interfacial region, the polar head groups are located in the aqueous phase, whereas the fatty acid chains are present in the organic layer. When the organic phase is evaporated under reduced pressure, these ordered structures are ruptured into fragments and forced into the aqueous phase; some fragments are transported by air bubbles (Fig. 2 *B* and *C*). Subsequently, these phospholipid monolayer fragments fuse into bilayer fragments (Fig. 2*E*) [bilayered phospholipid fragments have been identified as precursors for vesicles by electron microscopy studies (16)]. Alternatively, phospholipid bilayer fragments are formed from micellar structures containing the organic solvent (Fig. 2*D*), and as the evaporation progresses, the entrapped liquid (organic phase) will undergo a phase transition into a gas, and the micellar structures will collapse, yielding bilayered phospholipid fragments (Fig. 2*E*). These fragments will then spontaneously undergo self-closure into unilamellar vesicles (Fig. 2*F*), as previously discussed (16). Unilamellar liposomes prepared by our procedure can grow easily to the observed diameters as a consequence of the oriented phospholipid monolayers at phase interfaces and the continuity of the evaporation process.

Storability of GUVs. The liposomes were stable for several weeks when stored at room temperature. The only exception was the rhodamine-containing vesicles; they spontaneously disintegrated after 2–3 days. A reasonable explanation for the reduced lifetime of the rhodamine-containing liposomes could be a destabilization of the membrane by integration of this positively charged and visible-light-excitable dye. We do not expect our liposomes to be storable for longer or even equally long periods of time as small unilamellar vesicles (SUVs). Large unilamellar vesicles and GUVs occupy only slightly higher energy levels than multilamellar vesicles (MLVs) and therefore aggregate and fuse more rapidly to MLVs than SUVs (17).

Encapsulation of Labile Molecules. Because many techniques for preparation of vesicles require long periods of time, often at elevated temperatures, or involve extensive exposure of biomolecules to organic solvents, severe limitations apply to what kind of molecules can be entrapped with maintained structural identity and biological activity. It is quite feasible not only that molecules prone to, for example, isomerization reactions (such as *Z–E/E–Z* or L–D/D–L conversions), redox reactions, or hydrolysis lose their biological activity but also that the resulting products have adverse or opposite pharmacological effects. For example, GSH, which interacts with glutamate neuroreceptors, can spontaneously oxidize to a disulfide dimer that has different pharmacological effects on the very same receptors.

We compared the extent of degradation of two labile molecules, GSH and FDP, observed with our method (incubation at 40°C for 2 min) and with previously reported procedures for GUV production (incubation at 70°C for 2 h) (7). GSH spontaneously forms the glutathione disulfide dimer through a two-electron oxidation, and FDP spontaneously hydrolyzes to fluorescein through FMP as an intermediate product. Whereas our method retained 96.5% (calculated by using an apparent first-order degradation rate constant of $0.018 \pm 0.002\ \text{min}^{-1}$, $n = 12$) and 100% ± 1% (mean ± SD, $n = 3$) of the structural identity of GSH and FDP, respectively, the figures obtained after incubation for 2 h at 70°C were 11.5% for GSH and <1% of FDP (estimated from the combined concentration of FMP and fluorescein after incubation).

Incorporation of γ-GT into GUVs. γ-GT is a membrane-bound enzyme that catalyzes, among other related reactions, a transpeptidation reaction in which the γ-glutamyl moiety of GSH is transferred to an extracellular acceptor amino acid (18) according to:

L-amino acid + GSH → γ-glutamyl-L-amino acid + cysteinylglycine.

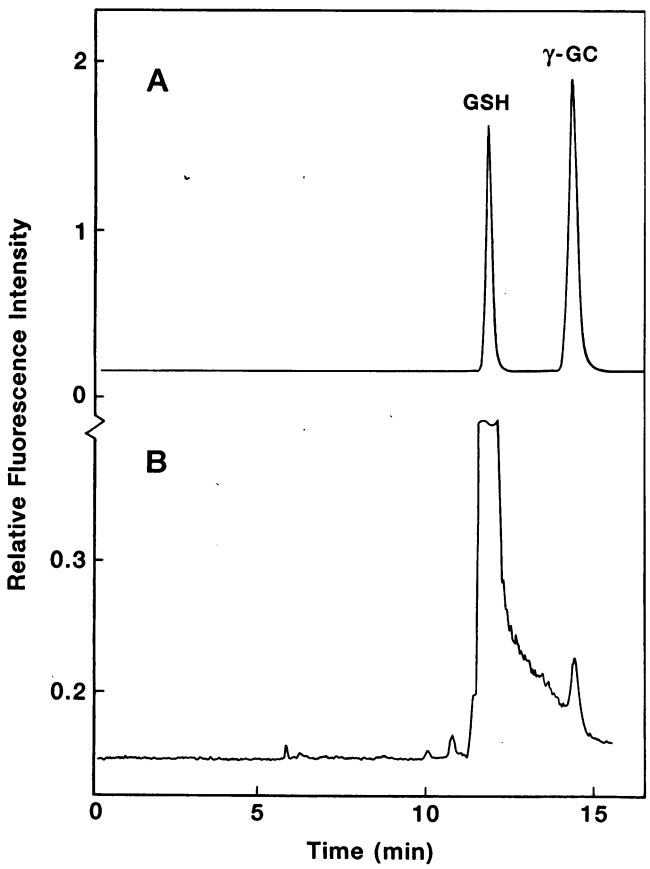

FIG. 3. Electropherograms showing detection of a mixed standard of 10 mM GSH and 10 mM γ-GC (*A*) and formation of γ-GC by γ-GT activity after addition of cysteine (10 mM) to an extravesicular solution containing GUVs with γ-GT incorporated into the membrane (*B*).

This reaction is independent of ATP, takes place on the external surface of cell membranes, and results in the transport of the γ-glutamyl dipeptide into the cell (19). γ-GT thus functions as a transporter for extracellular amino acids. Incorporation of γ-GT into liposomes has been previously demonstrated in experiments in which glutamate was used as the extravesicular acceptor amino acid (20).

Here we demonstrate uptake of extravesicular L-cysteine by γ-GT incorporaed into liposomes. The electropherograms in Fig. 3 show a mixed standard of GSH and γ-GC, and γ-GC formation resulting from γ-GT-mediated uptake of L-cysteine. The uptake experiments were performed in triplicate, and always yielded detectable quantities of γ-GC. No attempts were made here to optimize this procedure. Nevertheless, it appears safe to conclude that γ-GT was functionally reconstituted in the membrane walls of the vesicles.

CONCLUSIONS

We have described a simple method that produces cell-sized unilamellar liposomes under mild conditions. This procedure is ideally suited for work with labile and highly reactive molecules. Because the biological activity of encapsulated molecules is largely maintained during the preparation, this technique might be useful in liposome-based drug delivery schemes. The ability to produce GUVs rapidly and easily opens for investigation biochemical reactions in small, confined biomimetic compartments.

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1. Ostro, M. J. & Cullis, P. R. (1989) *Am. J. Hosp. Pharm.* **46,** 1576–1587.
2. Dimitriadis, G. J. (1978) *Nature (London)* **274,** 923–925.
3. Skeie, S. (1994) *Int. Dairy J.* **4,** 573–595.
4. Lasic, D. D. (1995) *Liposomes: From Physics to Applications* (Elsevier, Amsterdam), 2nd Ed., pp. 63–107.
5. Szoka, F. & Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4194–4198.
6. Timofeev, B. A., Bolotin, I. M., Stepanova, L. P., Bogdanov, A. A., Georgiu, Kh., Malyshev, S. N., Petrovsky, V. V., Klibanov, A. L. & Torchilin, V. P. J. (1994) *Microencapsulation* **11,** 627–632.
7. Hub, H. H., Zimmermann, U. & Ringsdorf, H. (1982) *FEBS Lett.* **140,** 254–256.
8. Mueller, P., Chien, T. F. & Rudy, B. (1983) *Biophys. J.* **44,** 375–381.
9. Angelova, M. I., Soleau, S., Meleard, P., Faucon, J. F. & Bothorel, P. (1992) *Prog. Colloid Polym. Sci.* **89,** 127–131.
10. Angelova, M. I. & Dimitrov, D. S. (1988) *Prog. Colloid. Polym. Sci.* **76,** 59–67.
11. Dimitrov, D. S. & Angelova, M. I. (1988) *Bioelectrochem. Bioenerg.* **19,** 323–336.
12. Kremer, J. M. H., v.d. Esker, M. W. J., Pathmamanoharan, C. & Wiersema, P. H. (1977) *Biochemistry* **16,** 3932–3935.
13. Chiu, D. T. & Zare, R. N. (1996) *J. Am. Chem. Soc.* **118,** 6512–6513.
14. Orwar, O., Fishman, H. A., Ziv, N. E., Scheller, R. H. & Zare, R. N. (1995) *Anal. Chem.* **67,** 4261–4268.
15. Lasic, D. D. (1988) *Biochem. J.* **256,** 1–11.
16. Fromherz, P. & Rueppel, D. (1985) *FEBS Lett.* **179,** 155–159.
17. Lasic, D. D. (1990) *J. Colloid Interface Sci.* **140,** 302–304.
18. Hanes, E. S., Hird, F. J. R. & Isherwood, E. A. (1952) *Biochem. J.* **51,** 25–35.
19. Meister, A. & Tate, S. S. (1976) *Annu. Rev. Biochem.* **45,** 559–604.
20. Sikka, S. C. & Kalra, V. K. (1980) *J. Biol. Chem.* **255,** 4399–4402.