# Microinjection into giant vesicles and light microscopy investigation of enzyme-mediated vesicle transformations

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**Background:** 'Giant vesicles' have diameters of several micrometers and can be observed by light microscopy. Their size may allow manipulation of individual vesicles and direct observation of the progress of a chemical reaction in real time. We set out to test this possibility using enzymatic hydrolysis of vesicle components as a model system.

**Results:** We describe a novel micromanipulation technique that allows us to microinject femtoliter amounts of a reagent solution adjacent to or into giant vesicles with diameters ranging from 10 to 60 μm. The vesicle transformations can be monitored directly in real time by light microscopy and recorded by video analysis. Snake venom phospholipase $A_2$ was added to vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine, and the enzymatic hydrolysis of components of the lipid bilayer was observed over time. A specific effect on the targeted giant vesicle was seen and video recorded, while the neighbouring vesicles remained unaffected. Addition of the enzyme to the outside of a vesicle caused it to burst, whereas injection of the enzyme inside a vesicle resulted in a slow and constant decrease in its size, until it eventually disappeared from the resolution power of the light microscope.

**Conclusions:** These results show that it is possible to micromanipulate an individual vesicle, and to follow visually the progress of an enzymatic reaction occurring on the vesicle bilayer over time.

**Chemistry & Biology** February 1996, **3**:105–111

Key words: enzyme, giant liposome, giant vesicle, microinjection, phospholipase

## Introduction

So called 'giant vesicles' (or 'giant liposomes') are spherical, closed molecular bilayers that entrap an aqueous compartment with a diameter in the order of several micrometers [1–15]. Due to their large size, giant vesicles can be observed by light microscopy, permitting the direct visualization of certain aspects of their supramolecular chemistry. We have recently shown an example of this with the self-reproduction of giant vesicles composed of oleic acid and oleate molecules [16], a process that is catalyzed by the membrane bilayer [17]. In this case, the reagent (oleic anhydride) was added externally to a vesicle suspension, as is usual for a chemical reaction.

Due to their size, however, giant vesicles offer a quite different possibility for carrying out chemical reactions — the local, controlled addition of a reagent with the kind of micromanipulation equipment typically used in cell biology [18,19]. In this way, it may be possible to carry out a localized chemical reaction on an individual vesicle and follow its progress in real time by light microscopy. The study of reactions occurring on the lipid membrane would appear to be particularly amenable to this approach. Here we present the results of one such study, using giant vesicles (about 20–40 μm in diameter) prepared from 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC), to which snake venom phospholipase $A_2$ ($PLA_2$) is added by a novel microinjection technique.

Two novel developments are described. The microinjection of water soluble or water insoluble substances into the interior of an individual giant vesicle has not, to our knowledge, been described before, although the idea has been mentioned by Menger [20]. The second new procedure is the real-time light microscopic video monitoring of vesicle transformations after enzymatic modification of the vesicle components.

## Results and discussion

### Formation, stability and micromanipulation of giant vesicles

Giant vesicles of POPC were prepared by the electroformation method developed by Angelova and Dimitrov [6,21]. The method has been applied previously to the preparation of vesicles in salt-free, pure water for bending elasticity measurements [22] and for optical trapping manipulations of giant vesicles [23]. (These papers also discuss the possible mechanism of lipid swelling and liposome formation in the presence of an external alternating electric field.) In the present study, POPC was deposited onto the platinum wire electrodes not from a chloroform:methanol solution as originally proposed [6], but from a diethyl ether:methanol mixture (see Fig. 1 and Materials and methods), allowing more efficient spread of the lipids and leading to formation of considerably larger vesicles. The giant vesicles envelop a far greater volume of liquid than conventional liposomes. A unilamellar POPC liposome with a diameter of 40 μm has an internal aqueous volume of 34 pl, which is separated from the

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 ISSN 1074-5521

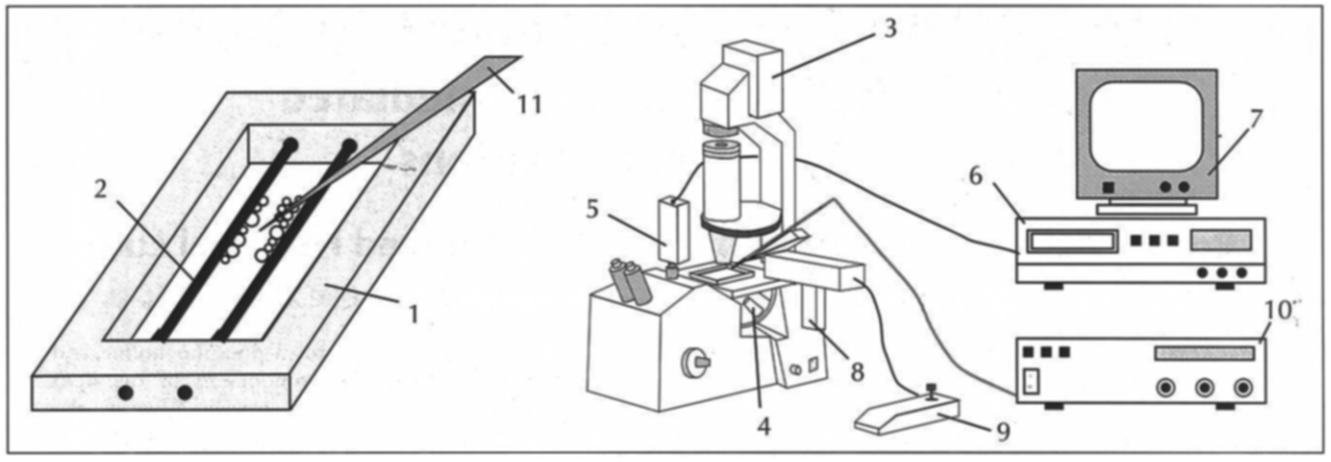


**Fig. 1.** Schematic representation (not to scale) of the experimental setup for the preparation, light-microscopic investigation, and micromanipulation of POPC giant vesicles.The figure shows the polystyrene and glass chamber (1), containing two parallel platinum wire electrodes (2), the phase contrast inverted light microscope (3), equipped with a 40x long working distance objective lens (4) and an additional 1.6x magnification ring. The observations were video recorded by a high resolution video camera (5), connected to a video recorder (6) and a screen (7). For the micromanipulation of the vesicles, a micromanipulator (8), equipped with a joy-stick (9), a microinjector (10) and specially prepared microneedles (11) were used. Further details of the micromanipulation are given in Materials and methods.

external solution by a bilayer composed of $1.4 \times 10^{10}$ (18 pg) POPC molecules (calculated on the basis of a cross-sectional area covered by one POPC molecule of 0.72 $nm^2$ [24] and a bilayer thickness of 0.37 nm [25]). In comparison, a 'conventional' unilamellar POPC liposome with 100 nm diameter has an internal volume of only 0.0000005 pl ($5 \times 10^{-19}$ l) and is composed of $8.1 \times 10^4$ POPC molecules.

The giant vesicles were prepared in dilute buffer solutions as described in the Materials and methods. Under these conditions, unilamellar vesicles with diameters of 10–60 μm were formed after about 1 h on the surface of the two wire electrodes as shown schematically in Figure 1. The vesicles were chemically and physically stable for several days, or several weeks if sterile solutions were used. The microinjection was carried out with special glass needles. We checked that the mechanical treatment with the microneedle did not impair the structural integrity of the bilayer aggregates by injecting a buffer solution (1 mM Tris, pH 8.0, containing up to 20 mM $CaCl_2$).Up to 100 pl of this solution could be injected into the close vicinity of the vesicles without inducing permanent changes in their size (only a transient vesicle deformation was observed due to the pressure caused by the injection). Furthermore, the vesicles could easily be touched by the microneedle without damage, in agreement with the relatively high stretching capacity and low bending elasticity of POPC bilayers [22,26]. Also the penetration of the vesicle membrane by the thinner microneedle ('type II'; see Materials and methods) generally did not damage the vesicle, and the internal vesicle volume could be increased up to 5 % by using this microinjection technique without affecting the vesicle size and shape.

To further explore the possibility of adding reagents into the interior of giant vesicles, and to prove that the injection was into the interior aqueous volume of the vesicle, we injected two substances with refractive indices higher than the aqueous suspension. The water-soluble polysaccharide Ficoll 70 (with an approximate mean molecular mass of 70 000 Da) or the water insoluble oleic anhydride (molecular mass of 547 Da) were microinjected using a 'type II' microneedle. In both cases a darkening of the targeted vesicle was observed, due to the presence of a liquid with a higher refractive index. In addition, no leakage of Ficoll could be observed over a period of at least 12 h, in agreement with the expected permeability properties of phospholipid bilayers [27].

A few applications of the micromanipulation of giant vesicles have already been reported, in particular their use for studying physical properties of the bilayer [4,7,8,12,20,26,28–30]. Here we have demonstrated for the first time a controlled injection of a water-soluble or water-insoluble substance into the interior of a single giant vesicle.

### $PLA_2$ microinjections adjacent to and into POPC giant vesicles

We next tested the effects of the addition of $PLA_2$ (from the venom of the cobra snake *naja naja*) to POPC giant vesicles. This $Ca^{2+}$ ion dependent enzyme catalyzes the hydrolysis of the *sn*-2 ester bond of lecithin, resulting in the formation of lysolecithin and — in the case of POPC — oleic acid. The action of $PLA_2$ on phospholipid bilayers has been the subject of many investigations, (for example, see [31–41]) and its mechanism is still the matter of some controversy.

Three types of experiments were carried out. $PLA_2$ was added either from a distance of about 5 μm from the vesicle surface, or onto the surface of the vesicle, or into the internal aqueous volume of the vesicle.

Let us first consider what the expectations of these three different settings might be. The action of the enzyme

results in the formation of lysolecithin which is not, by itself, able to form liposomes. The transformation of a significant amount of POPC into lysolecithin might therefore cause the local destruction of the bilayer structure, damage which, in principle, could either be 'repaired' by a rearrangement of the remaining POPC, or lead to destruction of the vesicle, if the damage is too extensive. When added from a distance, it is expected that the enzyme, due to diffusion effects, will reach the bilayer in a much more diluted form than when added onto the surface of the vesicle. In the latter case, with the needle touching the membrane, the local concentration of the enzyme on the targeted spot may be very high. In both cases, the enzyme will predominantly attack one region of the vesicle, that is, the reaction will probably be a localized one. When the enzyme is injected, it is also expected to diffuse and be diluted, but in this case, as opposed to the two scenarios above, a much more homogenous distribution of the enzyme is expected to be present at the (inner) membrane layer.

When $PLA_2$ (about 590 fl, containing $2.5 \times 10^7$ enzyme molecules) was added externally and from a distance to a single unilamellar POPC giant vesicle (with a diameter of 30 μm), no obvious changes were evident during the first 10 s, but then remarkable vesicle transformations occurred during the subsequent 10 min (Fig. 2). After the 10 s lag phase, the vesicle started to fluctuate and deviations appeared in the spherical shape until it suddenly burst into dozens of smaller vesicles with diameters of 1–5 μm. Some of these smaller vesicles moved away from the area of observation, while others aggregated into a type of giant 'multivesicular' [42] structure (20 μm), or fused to form larger vesicles (5–10 μm). After an additional 4 min, the vesicles underwent further transformations, until finally they were no longer visible by light microscopy. (Objects ≤ 1 μm can not be detected by light microscopy, so we are not able to describe transformations involving vesicles in this size range.) The initial lag phase depended on the initial size and the lamellar characteristics of the vesicles, and on the amount of added $PLA_2$. Adding more $PLA_2$ or injecting the enzyme at a point closer to the vesicle surface reduced the lag time significantly. These findings may be related to an initial latency period in product formation that has often been observed in $PLA_2$-catalyzed reactions [31,33,36,38,39].

In the second type of experiment, we placed the needle tip as close as possible to the vesicle, adding the $PLA_2$ directly onto the external membrane surface (Fig. 3). Generally, the vesicle transformation processes were the same as those observed in the case of external addition from 5 μm distance described above. The only difference was a drastic decrease in the lag time; the vesicle burst after only one or a few seconds. As shown in Figure 3, this initial burst occurs locally on the side that $PLA_2$ is administered (Fig. 3b), leading to a local destabilization, followed by an immediate reclosure of the vesicle (Fig. 3c). Later (Fig. 3d) the whole vesicle bursts into dozens of smaller vesicles.

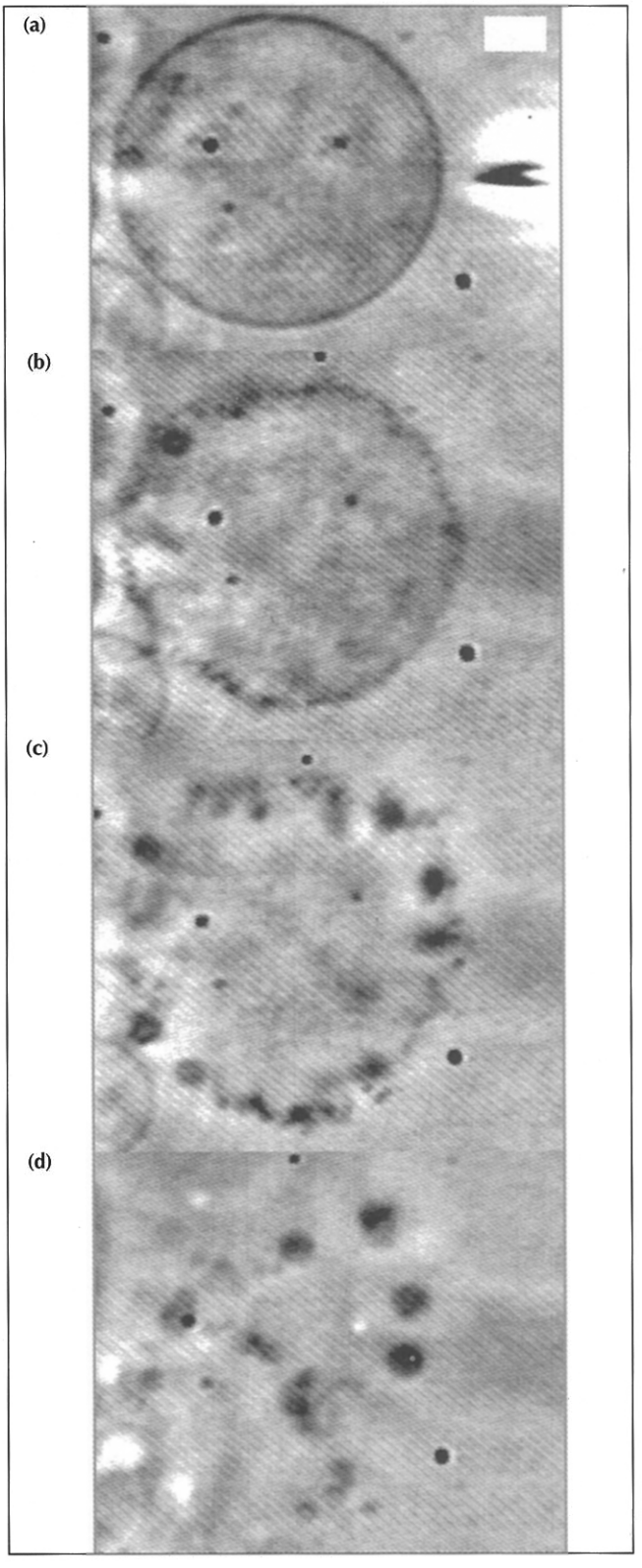


**Fig. 2.** Transformations of a single POPC giant vesicle, induced by external addition of snake venom $PLA_2$ in the vicinity of the vesicle (the distance between the closest vesicle surface and the microneedle tip was ~5 μm). The 'type I' microneedle (see Materials and methods) was used and 590 fl were added. Still photographs from a video recorder taken **(a)** 0 s, **(b)** 12 s, **(c)** 15 s or **(d)** 20 s after addition of $PLA_2$, are shown. The length of the bar corresponds to 5 μm. After a lag phase, the vesicle started to fluctuate and deviate from spherical shape until it burst into smaller vesicles.

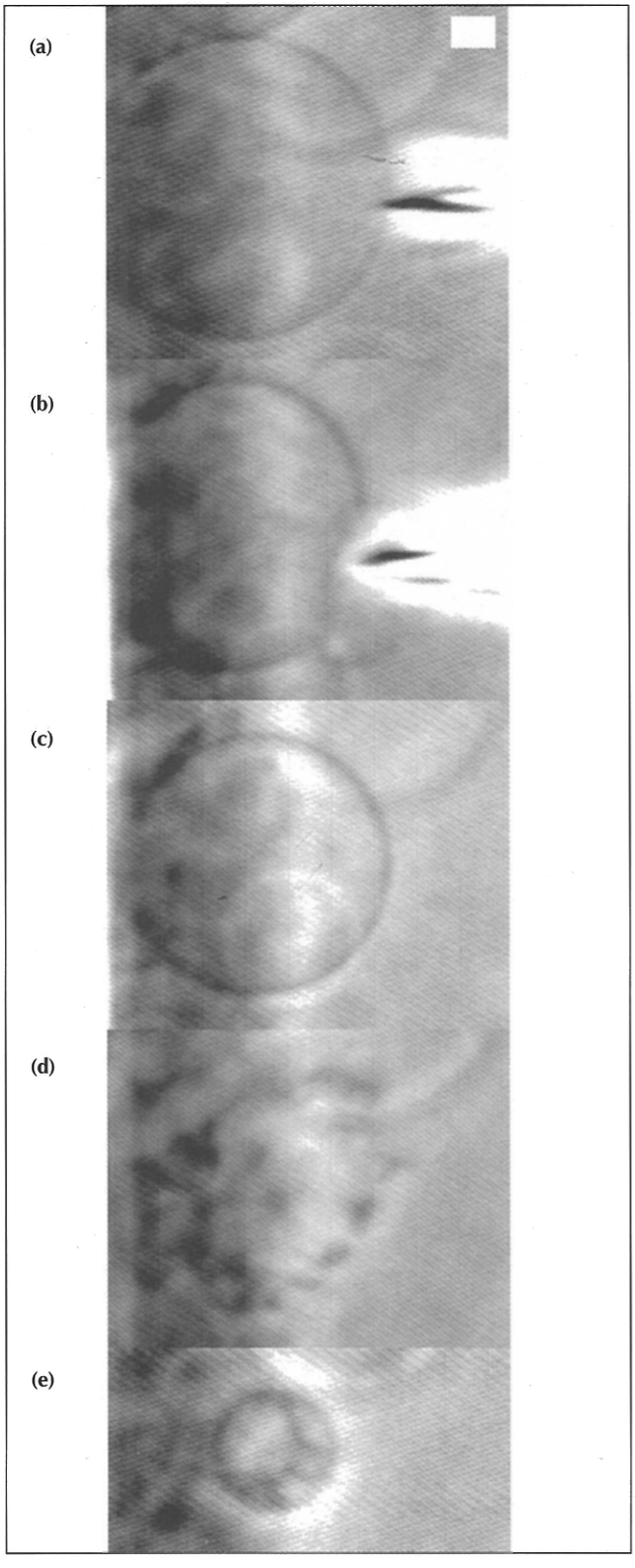


**Fig. 3.** Transformations of a single POPC giant vesicle as induced by external addition of snake venom $PLA_2$ directly onto the surface of the vesicle (the microneedle tip touched the external vesicle surface). The 'type I' microneedle (see Materials and methods) was used and 590 fl were added. Still photographs from a video recorder, taken **(a)** 0 s, **(b)** 2 s, **(c)** 20 s, **(d)** 60 s or **(e)** 75 s after addition of $PLA_2$ are shown. The length of the bar corresponds to 5 μm. After a short lag phase, the vesicle started to fluctuate, immediately followed by a burst into smaller vesicles.

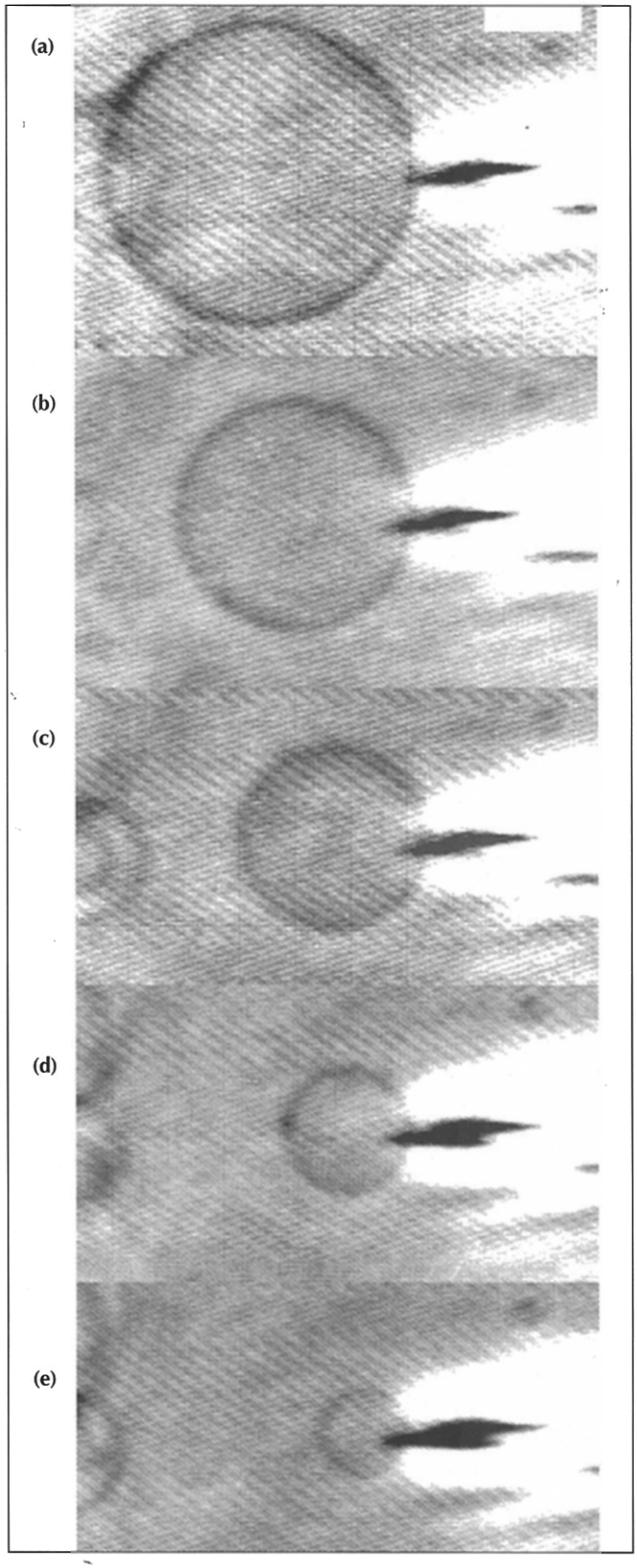


**Fig. 4.** A single POPC giant vesicle remains intact, but shrinks, after internal addition of snake venom $PLA_2$ by penetration of the microneedle through the vesicle bilayer. The 'type II' microneedle (see Materials and methods) was used and 180 fl were added. Still photographs from a video recorder, taken **(a)** just before addition of $PLA_2$, or **(b)** 4 min, **(c)** 6 min, **(d)** 8 min or **(e)** 9 min after addition of $PLA_2$ are shown. After a lag phase, the vesicle started to shrink continuously. The length of the bar corresponds to 5 μm.

In the third case, the $PLA_2$ was injected into the interior of the vesicle after penetration of the 'type II' microneedle through the vesicle membrane (Fig. 4). A volume of 180 fl of $PLA_2$ solution (corresponding to about $7.7 \times 10^6$ enzyme molecules) was added to a vesicle of 18 μm diameter, which was composed of ~$2.8 \times 10^9$ POPC molecules. Internal addition of $PLA_2$ to an individual giant POPC vesicle resulted in a clearly different transformation process than external addition. After an initial lag phase, a continuous, concentric shrinking of the vesicle was observed. The diameter of the vesicle decreased from an initial value of 18 μm to < 1 μm within 10 min after $PLA_2$ addition. The shrinking of the vesicle over time is illustrated in Figure 5.

Note that all these transformations occurred only with the targeted vesicle, leaving neighbouring vesicles intact. It is also important to note that control experiments, which we performed under identical conditions but in the absence of $PLA_2$, did not lead to any vesicle transformations.

To clarify whether the observed vesicle transformations are also partly due to a general protein effect, such as the physical action of the enzyme binding and/or proximity to the lipid bilayer, we have carried out microinjection experiments using lysozyme (which has a mass similar to that of $PLA_2$, but no catalytic activity towards POPC) under conditions that are otherwise the same. In no instance did the addition of lysozyme — even if repeated several times — induce breakage of or damage to the targeted vesicles. Microinjection of lysozyme in the external space between two neighbouring vesicles did, however, induce their reversible adhesion (data not shown). The vesicles stayed in contact for about 15–20 s and then separated again, probably because the lysozyme diffuses out of the region of contact.

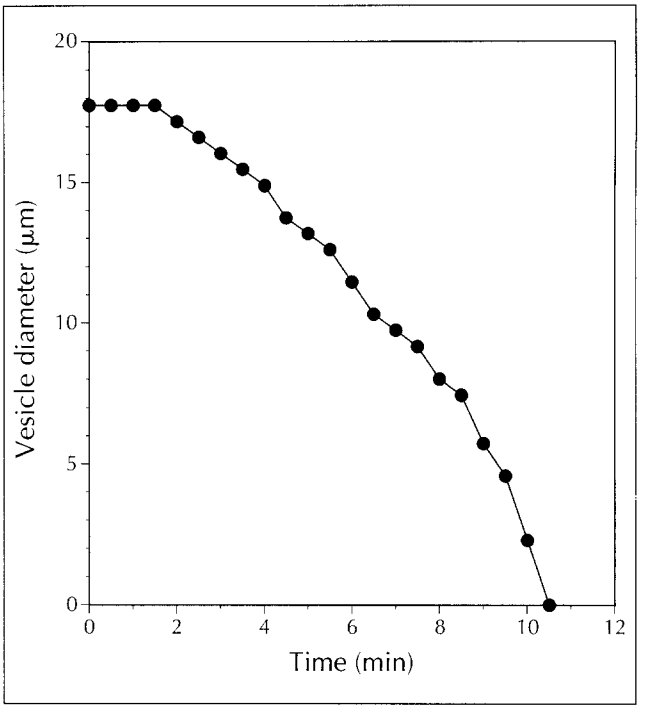


**Fig. 5.** Giant vesicles shrink constantly after internal microinjection of $PLA_2$. Microinjection was performed as detailed in Figure 4 and Materials and methods.

## Significance

**We have shown that it is possible to micromanipulate a giant vesicle and to follow the progress of the ensuing reaction visually. We can thus monitor the processes occurring in an individual structural unit, providing one step toward the realm of cell biology. Application of this technique to the study of membrane proteins appears particularly promising, as these proteins can be easily incorporated into the giant vesicle bilayer membrane. If different membrane proteins, with different enzymatic activities, can be incorporated in this way, it will be possible to engineer semi-synthetic supramolecular structures, whose composition mimics that of living cells, by microinjecting water-soluble enzymes into the internal aqueous space or into the bilayer. The microinjection technique may also be useful to study the effect of compartmentalization on particular chemical reactions. A giant vesicle can be seen as a microreactor that can be filled with various reagents in any order. Fusion of two or more vesicles, filled with different reagents, may allow the study of novel sequential reactions.**

**Our present work does not allow us to draw conclusions regarding the mechanism of action of $PLA_2$, a subject that has proven controversial [35–38,41,43,44]. It does, however, provide a promising starting point, as most of the previous studies have used liposomes characterized by a small radius of curvature, whereas giant vesicles more closely approximate a planar bilayer with an infinite radius of curvature, and are therefore a much better model for biological cells.**

**Can we rationalize the different results when $PLA_2$ is added externally or internally? Both outcomes likely result from the production of lysolecithin by $PLA_2$, as lysolecithin is unable to form liposomes. External microinjection releases the enzyme onto a relatively small region of the surface, and the corresponding high local concentration and high hydrolysis rate may induce a burst of activity that leads to an abrupt disruption of the original vesicle. When the enzyme is microinjected into the interior of the vesicle, the enzyme is partitioned more homogeneously over the internal surface, and the modification of the inner lipid layer may thus take place in a more uniform, continuous way.**

## Materials and methods

### *Preparation, light microscopic investigation, and micromanipulation of giant vesicles*

Giant vesicles were prepared at room temperature by the electroformation method developed by Angelova and Dimitrov [6,21], using a home-made polstyrene and glass chamber (length: 3 cm; width: 4.5 cm) as illustrated in Figure 1. The chamber contained two parallel platinum wire electrodes which were connected to an alternating current field generator, model 164 (from Wavetek, USA). Before applying the field, 1–2 μl of a POPC solution (0.2 mg POPC (from Avanti Polar Lipids, Inc., USA) in 1 ml of diethyl ether:methanol (9:1, v/v)) was deposited onto the center of each of the two wires. After drying under a stream of nitrogen, the electric field (0.1–1.0 V, 10 Hz) was applied and 1 ml of buffer solution (1 mM Tris HCl, pH 8.0) was added to the chamber to cover both wires completely. The electro-osmotically controlled swelling of the lipid deposit [21] led to the formation of vesicles which became larger and larger, until after about 1 h, the electric field was switched off. The resulting vesicles had diameters between about 10 and 60 μm, most of them being unilamellar, as shown by bending elasticity measurements [22]. The vesicles formed on the surface of the wires were observed using a phase-contrast Axiovert 135 inverted light microscope (Zeiss AG, Germany), equipped with a 40x long working distance objective lens (LD Achroplan Ph2) and an additional 1.6x magnification ring. The observations were recorded using a high-resolution video camera (AVT-BC-2) from Zeiss AG, connected to a S-VHS JVC video recorder and a screen. For the micromanipulation of the vesicles, a micromanipulator from Zeiss AG equipped with a joy stick and an Eppendorf microinjector model 5242 was used, in connection with a compressor type 6-J (from Jun-Air, Denmark). The microneedles used were prepared as described below. Between injections, the holding pressure was kept constant at 30 hPa in order to avoid reflux into the needle.

### *Preparation and geometrical characteristics of the microneedles used*

Two types of microneedles ('type I' and 'type II') were prepared from borosilicate tubes (Hildenberg, Germany, length: 10 cm, outer diameter: 1.5 mm, inner diameter: 0.87 mm, omega dot: 0.2 mm) by a vertical, two-step puller instrument, type ZAU/Getra (Bachofer, Germany) at maximum pull strength and variable melting heat intensity. After preparing the microneedles, the inner diameter was determined by a Hitachi S 700 scanning electron microscope at 20 kV acceleration voltage (15 determinations). For this determination, the microneedles were cut with a glass cutter and coated with a 5 nm thick layer of platinum in a sputterer model MED010 (Balzers, Liechtenstein). For the calibration, a grating with a defined period of 577.4 nm was used. The characteristics of the two needle types (diameter and injection volume) are given in Table 1.

The 'type I' microneedle was used when reagents were added externally to the vesicles. The 'type II' microneedle was used for injections into the interior of the vesicles. The pressure-dependent injection volume was determined with the help of the light microscope. The microneedle was filled with distilled water, and a water droplet was injected during 1 s at a pressure of 2000 hPa into immersion oil. The volume of the injected water droplet was determined under the light microscope by assuming that the droplet was spherical.

**Table 1.** Characteristics of the microinjection needles used.

| | 'Type I' | 'Type II' |
|---|---|---|
| Inner diameter (nm) | 135 ± 5 | 100 ± 5 |
| Outer diameter (nm) | 245 ± 10 | 205 ± 6 |
| Injection volume (2000 hPa, 1 s) (fl) | 590 ± 30 | 180 ± 10 |
| (1 fl = $10^{-15}$ l) | | |

### *Microinjection of Ficoll 70 and oleic anhydride*

An 8 % solution (w/v, in 1 mM Tris, 5 mM $CaCl_2$, pH 8.0) of water-soluble Ficoll 70 (Pharmacia, Sweden) or neat water-insoluble oleic anhydride (Sigma, USA) were added into the aqueous interior of a single POPC giant vesicle. The 'type II' microneedle was used with an injection pressure of 2000 hPa over 10 s (for Ficoll 70) or 1 s (for oleic anhydride).

### *Microinjection of $PLA_2$*

Transformations of single POPC giant vesicles were studied after addition of $PLA_2$ from the venom of the cobra snake *naja naja* (Sigma). As determined by Sigma, the $PLA_2$ had a specific hydrolytic activity of 1000 units $mg^{-1}$ (where one unit catalyzes the hydrolysis of 1 μmole of phosphatidylcholine (as a 2 % (w/v) soybean phospholipid emulsion) to lysophosphatidylcholine and a fatty acid at pH 8 and 37 °C). Analytical SDS-PAGE with a 10–15 % polyacrylamide Phast gel on a PhastSystem (Pharmacia, Sweden) showed one single Coomasie-staining band, corresponding to a molecular mass of 14 kDa. All experiments were carried out at room temperature using a concentrated $PLA_2$ solution (1 mg $PLA_2$ $ml^{-1}$, 1 mM Tris, 5 mM $CaCl_2$, pH 8.0), which was added adjacent to (or into) a particular vesicle

### *Microinjection of lysozyme*

Hen-egg lysozyme (Fluka, Switzerland) was added externally in the vicinity of giant POPC vesicles (the distance between the closest vesicle surface and the microneedle tip was about 5 μm). The 'type I' microneedle was used and 590 fl of a concentrated lysozyme solution (1 mg lysozyme $ml^{-1}$, 1 mM Tris, 5 mM $CaCl_2$, pH 8.0) was added at room temperature.

*Acknowledgements:* We are grateful to Gunther Neuhaus and Ingo Potrykus of the Institute of Plant Sciences at the ETH for their very valuable advice and for allowing us to use their microscope and micromanipulation device. We thank Stephan Ballmer from Carl Zeiss AG (Switzerland) for the temporary use of the video camera. We also thank Ernst Wehrli of the Institute of Cell Biology at the ETH for performing the electron microscopy measurements, and Demetrios Papahadjopoulos of the Cancer Research Institute at the University of California at San Francisco for discussion and suggestions. This work was supported by the Swiss National Science Foundation (7BUPJ038679) and by the Bulgarian Science Foundation (Project No. 437/1994).

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Received: **1 Nov 1995**; revisions requested: **24 Jan 1996**; revisions received: **29 Jan 1996**. Accepted: **5 Feb 1996**.