Giant Vesicles as Biochemical Compartments: The Use of Microinjection Techniques

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Detailed experimental procedures are described for the preparation of thin-walled giant phosphatidylcholine vesicles, which are useful for microinjections. In these microinjection experiments, a target vesicle (typically about 50 to 100 μm in diameter) was punctured by a microneedle and an aqueous solution was injected into the internal volume of the vesicle. The method, which was used for giant vesicle preparation, is a modification of the so-called electroformation method, originally described by Angelova and Dimitrov (*Faraday Discuss. Chem. Soc.* **1986**, *81*, 303−311 and 345−349). With this method, the vesicles grow in an investigation chamber at a platinum wire in an aqueous medium with the help of an alternating electric field, and we have investigated how the experimental parameters (in particular applied voltage and frequency and ionic strength of the aqueous medium) influence the vesicle formation process. Using a specially constructed investigation chamber and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) as lipid, the applied voltage was varied between 0.6 and 10 V, holding the frequency constant at 10 Hz. At voltages <5 V, the giant vesicles formed often appeared under the microscope as nonspherical ("cut spheres") and open, "mushroom-like" structures. Often, however, nonsphericity was only an optical artifact, and closed vesicles could be distinguished from open structures by microinjecting fluorescent dye molecules, which in the case of an open structure immediately leaked out. At voltages >5 V, closed structures were observed. At constant voltage (1.3 V), "cut spheres" and "mushroom" type structures appeared mainly in the frequency range 10−100 Hz. Between 0.2 and 2 Hz, mainly closed structures were formed. Typical conditions for vesicle formation useful for microinjections were 2 V and 10 Hz. Occasionally, giant vesicles with diameters of up to 300 μm formed. The presence of high salt concentrations prevented the formation of giant vesicles; in the case of LiCl, NaCl, or KCl, the limiting concentration was 10 mM, while the maximal concentrations for $MgCl_2$, $CoCl_2$, and $CaCl_2$ were 1.7, 1.0, and 0.2 mM, respectively. The giant vesicles formed were osmotically sensitive. Addition of glucose led to a vesicle shrinkage, the beginning of visible shrinkage being dependent on the glucose concentration, ranging from 6 to 7 min (with 10 mM glucose) to 30 s (with 200 mM glucose). Giant vesicles could also be formed with mixtures of POPC and phospholiponucleosides, such as 5′-(1,2-dioleoyl-*sn*-glycero-3-phospho)uridine (DOP-uridine) or 5′-(*n*-hexadecylphospho)uridine (HDP-uridine); or mixtures of POPC with 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-*rac*-(1-glycerol)] (POPG), 1,2-dipalmitoyl-*rac*-glycero-3-phosphoethanolamine (DPPE), or didodecyldimethylammonium bromide (DDAB). No stable thin-walled vesicles formed with pure POPG, bovine brain phosphatidylserine, or pure phospholiponucleosides. Although apparently limited to a certain class of phospholipids (phosphatidylcholines) or to lipid mixtures containing phosphatidylcholines, and limited to low ionic strength solutions, the electroformation method has proven to be the method of choice for successful microinjections, finally allowing to use the aqueous interior of individual vesicles as microreactors with volumes of about 50−100 pL. Successful microinjections into giant POPC vesicles were demonstrated by using calcein as fluorescent probe or DNA that was stained with YO-PRO-1. In some cases, even multiple puncturing of the same vesicle was possible.

Introduction

In a variety of reports during the last 30 years the formation, characterization, and application of micrometer-sized giant vesicles from naturally occurring lipids as well as from synthetic amphiphiles have been described.[1−17] We are in particular interested in using single giant vesicles as microreactor compartments and as models for protocells, aiming at directly observing chemical processes by (video-enhanced) light microscopy in real time. To this aim microinjection of the reactants into a single vesicle is necessary, and successful microinjections into giant vesicles from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-

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choline (POPC) have been reported during our recent studies with phospholipases[14b,d] and *sn*-glycerol-3-phosphate acyltransferase.[14c] The reason these microinjections were possible at all is largely due to the method used for preparing the giant vesicles, the so-called electroformation method, originally developed by Angelova and Dimitrov.[8b−e,18]

Our currently used experimental setup for the preparation of giant phospholipid vesicle is a modification of the original description of the electroformation method,[8b−e] which mainly used direct current (dc) electric fields and negatively charged mixtures of phospholipids.[19] In our case, we are working almost exclusively with alternating current (ac) electric fields and specially constructed investigation chambers containing two parallel platinum wires (see later on Figure 1). Since the vesicle formation not only depends on the geometry of the investigation chamber but also on other experimental conditions, such as thickness of the lipid film deposited on the wire,[8b] we will first describe in detail under which conditions (including applied voltage and frequency) giant phospholipid vesicles form. Most of the work has been carried out with POPC, although other phospholipids or lipid mixtures were used (see below). In the second part of the paper we will report on different microinjection experiments to illustrate the general applicability of the electroformation method for injections of aqueous solutions into individual giant vesicles.

In the research literature dealing with giant lipid vesicles as microreactors, it is more and more evident that progress in the field is related to the availability of an efficient microinjection technique. We have therefore devoted to this subject a systematic investigation, and this paper describes the main results and conclusions. It is mostly an experimental, technical report that we consider fundamental for further applied studies.

Materials and Methods

Chemicals. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3[phospho-*rac*-(1-glycerol)] (POPG) were from Avanti Polar Lipids (USA). Bovine brain phosphatidylserine (PS) was from Serva, Germany. 5′-(1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho)uridine (POP-uridine), 5′-(1,2-dioleoyl-*sn*-glycero-3-phospho)uridine (DOP-uridine), 5′-(1,2-dioleoyl-*sn*-glycero-3-phospho)cytidine (DOP-cytidine), and 5′-(1,2-dioleoyl-*sn*-glycero-3-phospho)adenosine (DOP-adenosine) were synthesized by G. Barsacchi-Bo.[20a,b] 5′-(*n*-Hexadecylphospho)uridine (HDP-uridine) and 5′-(*n*-hexadecylphospho)cytidine (HDP-cytidine) were synthesized by C. Heiz.[21] *N*-(TexasRed sulfonyl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (TexasRed-DOPE, product T-647)[22a] and YO-PRO-1 iodide (**1**, YO-PRO-1, product Y-3603)[22b] were from Molecular Probes, USA. Didodecyldimethylammonium bromide (DDAB), herring sperm DNA sodium salt, yeast tRNA, D-glucose, calcein disodium salt, LiCl, NaCl, KCl, $CaCl_2$, and $MgCl_2$ were from Fluka (Switzerland); $CoCl_2$ was from Merck (Germany); 1,2-dipalmitoyl-*rac*-glycero-3-phosphoethanolamine (DPPE) and tris(hydroxymethyl)aminomethane were from Sigma (USA).

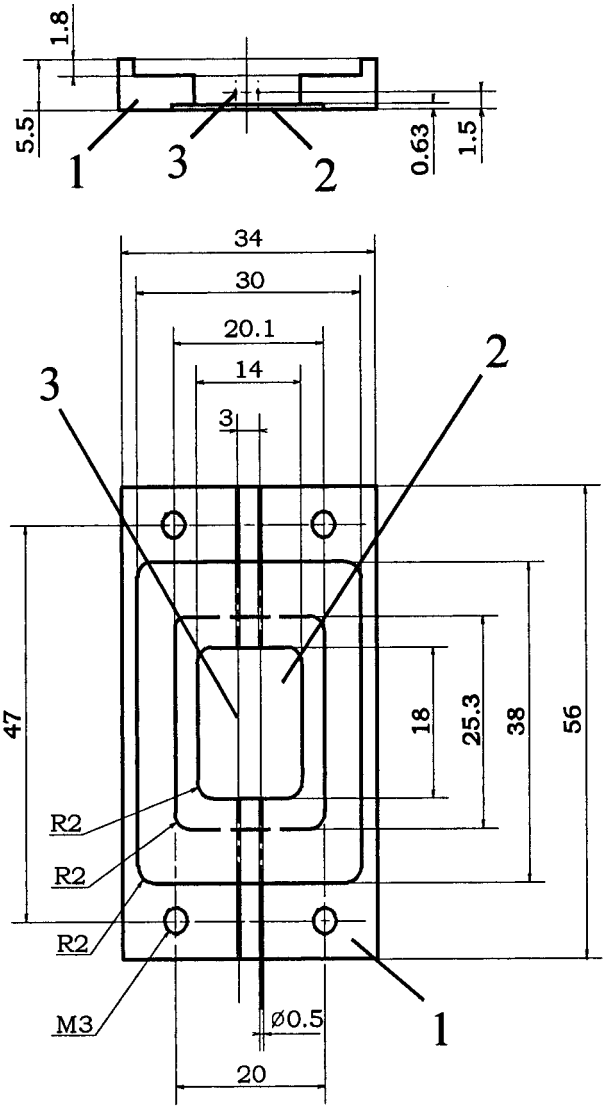

Figure 1. Sketch of the top view and side view of the investigation chamber used, containing the casing (1), a borosilicate glass bottom (2), and two parallel platinum wires (3). All dimensions are given in millimeters.

Microscopic Equipment. The vesicle formation and micromanipulations were observed with an Axiovert 135TV inverted light microscope from Zeiss (Germany), using the differential interference contrast (DIC) or fluorescence detection modes and

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(18) Microinjections into vesicles prepared conventionally are also possible, although it is experimentally more difficult.[13c.]

(19) The experimental setup also differs from the recent extension of the electroformation method by Angeloya et al.[8f] who used ac electric field and ITO-coated glass plates.

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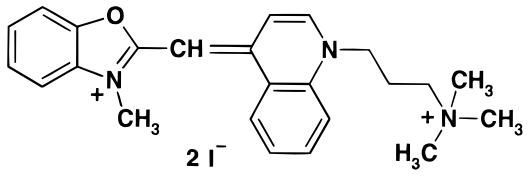

YO-PRO-1

20× long working distance Achroplan lenses. Imaging was recorded with a CCD video camera module XC-75CE from Sony (Japan); pictures were taken by using a PC with LYSP frame grabber and the software Image Access 1.5. Additionally, a S-VHS video recorder AG-7355 from Panasonic (Japan) was used.

Investigation Chamber. A special device in which the giant vesicles were formed by the electroformation method was constructed in the workshop of our institute under the supervision of H. Seinecke. This investigation chamber (see Figure 1) was designed in our laboratory by R. Wick and by M. I. Angelova (Central Laboratory of Biophysics, Bulgarian Academy of Sciences, Sofia, Bulgaria) and represents a minor modification of that used in our earlier investigations.[14b−d] The casing of the chamber was made from poly(vinylidene fluoride) (from Elesterflon, Diessenhofen, Switzerland), and the bottom of the chamber contained a 0.5 mm thick piece of borosilicate glass (from Guinchard SA, Yverdon, Switzerland), glued to the casing. The platinum wires (99.99%, from Möller AG, Zürich, Switzerland) were mounted parallel to each other at a distance of 3 mm, 0.8−1.5 mm above the glass plate. The two wires were fixed to the chamber by metal screws that on one end contained additional flat pieces of metal to make connections to the sweep generator easier. The top of the investigation chamber was open in order to allow the approach of the microneedles.

Lipid Film Formation. During the present work, two different lipid film depositions on the two platinum wires in the investigation chamber were made, in the following named as "method A" and "method B".

Method A. In two different areas of each wire 2 μL of a 263 μM phospholipid solution (0.2 mg of POPC/mL of diethyl ether: methanol, 9:1, v/v), corresponding to 526 pmol of POPC, was deposited with a Hamilton syringe, leading to a total of four regions on the two wires where the formation of vesicles could be investigated. At each spot where the lipids were applied, the solution (containing 3.2×10^{14} POPC molecules) spread over an area of ∼2−3 mm^2. The lipid film was dried by first blowing a stream of nitrogen over the wires for about 1 min, followed by storage under reduced pressure overnight in a desiccator. The dried film was composed of about 30−50 bilayers of POPC molecules, as estimated by taking into account a mean headgroup area of one POPC molecule of 0.6 nm^2.[23]

Method B: In contrast to "method A", 3 μL of a 263 μM phospholipid solution (0.2 mg of POPC/mL of diethyl ether: methanol, 9:1, v/v) was deposited along the entire length of one wire (18 mm). In this case the lipid (789 pmol) was spread over an area of ∼20−30 mm^2, corresponding to about 7−10 POPC layers.

Vesicle Formation Process. After putting the investigation chamber containing the dried lipid films on the stage of the light microscope and connecting the platinum wires with the frequency generator (see below), 1 mL of an aqueous solution was added, and the electric field was switched on. The formation of giant vesicles at the wires at the site of lipid application was then observed by light microscopy.

Frequency Generator. The ac electric field was produced by a frequency generator from Conrad Electronic (Germany) with an adjustable frequency of 0.2 Hz to 200 kHz and a voltage of 0.1−10 V (peak-to-peak value), controlled either by a Tektronix 5115 cathode oscilloscope (Guernsey, Channel Islands) or by a Roline digital multimeter M-3800. The frequency generator was connected to the two platinum wires of the investigation chamber.

Osmotic Vesicle Shrinkage. Osmotic effects were observed after the vesicles were first formed from lipid films prepared by method A (see above) and using ac electric fields with 0.2 Hz and 1.3 V. The vesicles were formed by using 0.8−1.0 mL of water. Afterward 10−200 μL of a 1 M solution of glucose (or 0.64 M NaCl) was added to build up the desired concentration gradient between the aqueous interior of the vesicle and the bulk solution (the total final volume in the investigation chamber was 1.0 mL).

Microneedles and Microinjections. Microneedles were prepared from borosilicate glass microelectrode capillaries from Hilgenberg (Germany), containing a filament and using a Puller P-30 from Sutter Instrumentation Co. Three types of capillaries with different inner diameters (i.d.), outer diameters (o.d.) and filament diameters (f.d.) were used: type I, i.d. = 0.87 mm, o.d. = 1.5 mm, f.d. = 0.20 mm; type II, i.d. = 1.20 mm, o.d. = 1.5 mm, f.d. = 0.15 mm; type III, i.d. = 0.90 mm, o.d. = 1.20 mm, f.d. = 0.12 mm. The quality of the microneedles prepared was characterized by measuring the threshold pressure for nitrogen bubble formation in ethanol, following a method developed by Schnorf et al.[24]

For microinjections, a micromanipulator, connected to a joystick ST20 from Bachofer (Germany) and an Eppendorf microinjector Model 5242 (Germany) were used in connection with a compressor type 6-J from Jun-Air (Denmark).

Results and Discussion

Electroformation of Giant POPC Vesicles in Pure Water. The general principles of the electroformation method have been described in the literature.[8b−f] Throughout the present work, we have mainly used POPC, a zwitterionic, well-defined phospholipid with a main phase transition temperature (T_m) in the presence of excess water of −3 °C.[25a] Since all the experiments were carried out at room temperature directly under the microscope, the lipid hydration and vesicle formation occurred in the fluid-analogue (liquid crystalline) state of the phospholipid. Furthermore, most of the experiments were performed in ac electric fields, and vesicle formation occurred on both electrodes indistinguishably.[25b]

Basically two different lipid film depositions were used, named "method A" and "method B" (see Materials and Methods). Method A allows the deposition of different lipids (or lipid mixtures) at different spots at the same wire. With method B, the same lipid (or lipid mixture) is spread along the entire wire. In both cases, the dried films contained in average less than 10 to 50 bilayers of POPC molecules, as estimated from the spreading of the lipid solution (see Materials and Methods). Although the film thickness was never uniform over the entire area of deposition (in particular in the case of method B), a mean thickness of a few, but not too many, double layers was important for the formation of a controlled amount of thin-walled giant vesicles. If the lipid film was too thick, a large population of smaller vesicles formed that could not be utilized for the microinjections described below.[25c]

In a first series of experiments we investigated how the applied voltage and frequency influence the vesicle formation process and the resulting size and morphology of the vesicles. A reproducible formation of giant vesicles

(23) The calculation is based on the assumption that the lipids spread uniformly. The surface area per molecule in dry phosphatidylcholine films is considerably smaller than in that hydrated systems, such as vesicles: Small, D. M. *The Physical Chemistry of Lipids: From Alkanes to Phospholipids*; Plenum Press: New York, 1986.

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(25) (a) Lynch, D. V.; Steponkus, P. L. *Biochim. Biophys. Acta* **1989**, *984*, 267−272. Even at a hydration degree as little as 5 wt %, T_m is below 30 °C. (b) As pointed out by Angelova and Dimitrov[8b] using a mixture of phospholipids, the formation and properties of giant vesicles formed by hydrating a thin lipid film under the influence of an electric field strongly depend on the number of lipid layers originally present in the film. Qualitatively we made the same observations with POPC, and this is the reason in all comparative studies the lipid deposition at the platinum wire was always performed in exactly the same way. (c) A qualitative similar observation was also made by Angelova and Dimitrov[8b] in the case of negatively charged phospholipid mixtures and dc electric fields.

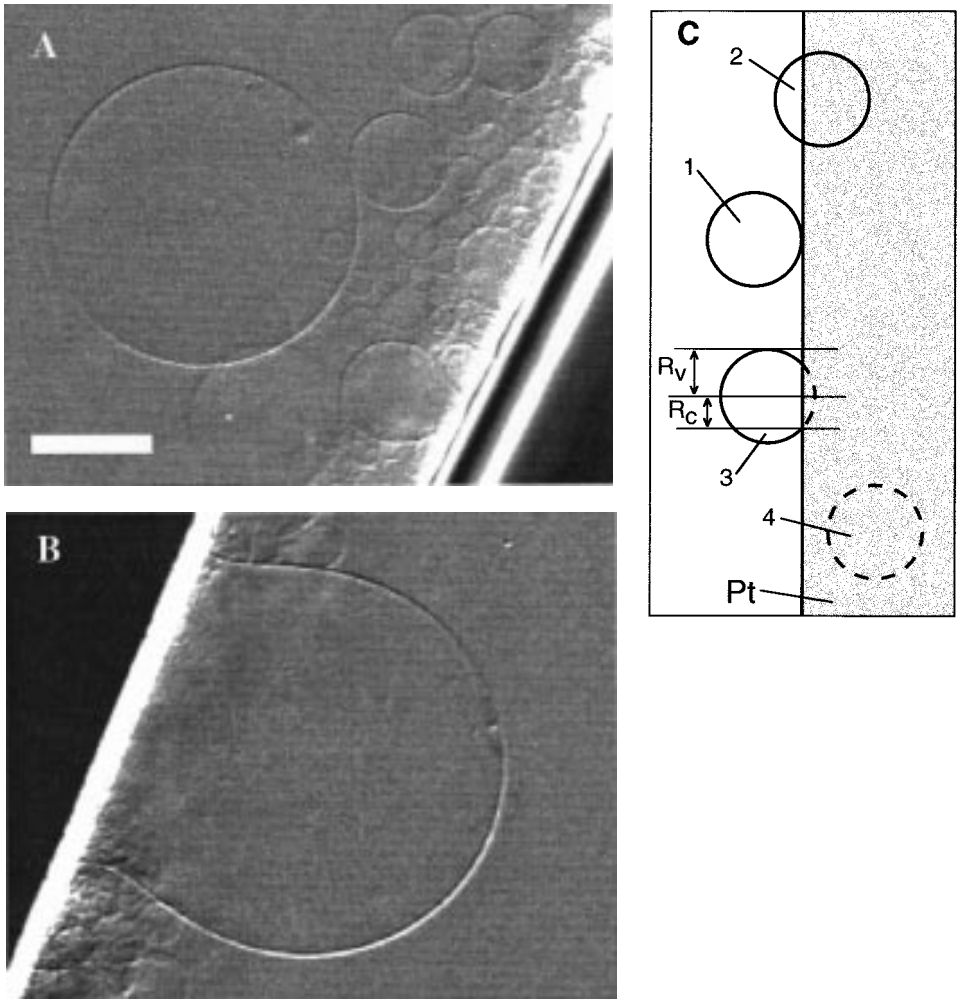

Figure 2. Characteristic appearance of giant vesicles prepared by the electroformation method. The vesicles (radius R_v) grow at the surface of the Pt-wire as schematically illustrated in C (top view). In the light micrographs (DIC mode; length of the bar, 50 μm), a part of the electrode is visible on the right (A) or left-hand side (B). The vesicles may appear as cross sections through spheres (A, vesicle 1 in C) with radius of apparent contact, $R_c = 0$);[8b] as "mushrooms" (B) as cross sections through "cut spheres" (A, vesicle 3 in C) or as flat "dome-type" structure (vesicle 2 in C), all with $R_c \neq 0$.Vesicle 4 in schematic drawing C is behind the wire and would not be visible by the microscope.

is a prerequisite for successful micromanipulations and for the aimed micoinjections.

Depending on the parameters of the applied electric field (such as voltage and frequency) and depending on the time the lipids were exposed to the electric field, the giant vesicles formed at the platinum wires varied in size, (apparent) shape, and distance to the electrode (compare Figure 2A with Figure 2B). Very often, the vesicles formed do not seem to be closed spheres. In some cases, the vesicle may be a true sphere, adhering to the electrode in a way that prevents the photographic observation of the whole structure, as illustrated in Figure 2C. While vesicle 1 appears as a sphere, vesicle 2 lies on top of the wire, and vesicle 3 is partly hidden behind the wire. Both vesicles 2 and 3 in Figure 2C would appear as a "cut sphere". In other words, a part of these two vesicles is made "invisible" by the metal wire. Vesicle 4 is behind the wire and not visible at all. A similar observation was also made by Angelova and Dimitrov[8b] who introduced the parameter R_c (which is an "apparent" radius of contact) as shown in Figure 2C.

The question, whether we are dealing with a closed structure or an open one, is operationally very important. Whether true closed structures are present can be detected by a microinjection experiment (see below). In fact, in the case of a closed structure, the injected solute remains trapped inside the vesicle, whereas in the case of an "open structure" (such as the so-called "mushroom" in Figure 2B), the solute generally leaks out along the platinum wire. We checked this routinely by using fluorescent water soluble solute molecules (data not shown). Even in the case of "true" spheres, the giant vesicles formed at the metal wire were often connected through small lipid tubes to the wire, a phenomenon that was observed before[8] and seems to be rather common in the case of lipid vesicles brought into contact with a foreign solid surface (formation of a so-called tether).[7b,26,27] This phenomenon is rather advantageous for the main aim of the present work (microinjections into vesicles) since most of the vesicles do not move away from the place of inspection after mechanical treatment with microneedles.

After these general observations, the influence of the applied ac electric field on the formation of giant POPC vesicles was studied. To this aim, we have investigated the effect of frequency and voltage on the vesicle formation process by using method A for the lipid film formation (see Materials and Methods). If the frequency was kept constant at 10 Hz, giant vesicles formed if voltages between

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Table 1. Preparation of Giant Vesicles by the Electroformation Method (ac Electric Field) Using Phospholipids Different from POPC and Using Phospholipid Mixtures containing POPC (ac electric fields with frequency ν and voltage U)

phospholipids[a]	lipid film[b]	ν (Hz)	U (V)	observations
POPG	A	10	0.01−1.3	within the first 10−60 min vesicles formed ($R_v \sim 10-20\ \mu$m) that contained other smaller vesicles; after 1−2 h the giant vesicles disappeared
			8	$R_v \sim 35-55\ \mu$m, vesicles often contained smaller vesicles
			17	after 1 h 15 min: $R_v \sim 20\ \mu$m ($R_c = 0$), containing smaller vesicles (see Figure 3A)
POPG:POPC (4:1, wt %)	A	10	5	after 1 h 30 min: $R_v \sim 10-20\ \mu$m ($R_c = 0$) and a few vesicles with $R_v = 30-55\ \mu$m ($R_c \neq 0$)
POP-uridine	B	10	8 or 2	no giant vesicles formed; also no giant vesicles with dc electric field (1 V)
DOP-uridine	B	10	4	no giant vesicles formed
	A	10	8	in a few min $R_v \sim 25\ \mu$m ($R_c \neq 0$) and $R_v \sim 10-15\ \mu$m ($R_c = 0$) containing many smaller vesicles; after 1 h 15 min only a few vesicles
	B	10	16	$R_v \sim 10\ \mu$m (see Figure 3B)
DOP-uridine:POPC (50:50)	B	10	16	giant vesicles formed which were stained with YO-PRO-1 (see Figure 3C,D)
DOP-adenosine	A	10	2.5	no giant vesicles formed
DOP-cytidine	A	10	2.5	no giant vesicles formed
HDP-cytidine	B	10	4	no giant vesicles formed
HDP-cytidine:POPC (50:50)	B	10	4	giant vesicles formed quickly (after a few min), comparable with pure POPC (Figure 3E); the vesicles were stained with YO-PRO-1; after several min the thin-walled giant vesicles disappeared and filled aggregates appeared
HDP-uridine:POPC (50:50)	B	10	4	giant vesicles formed quickly, followed by the reorganization to filled giant vesicles which were stained with YO-PRO-1 (see Figure 3F,G)
bovine brain PS	A	10	8	no giant vesicles formed; also no vesicle formation with dc electric field (1 V)
DPPE:POPC (1:400, wt %)	B	10	2	giant vesicles formed as in the case of pure POPC
Texasred-DOPE:POPC (1:50, wt %)	B	10	2	giant vesicles formed with $R_v < 25\ \mu$m
POPC:DDAB (95:5, wt %)	B	10	2	giant vesicles formed; neighbored vesicles often had shared membranes (see Figure 3H)

[a] For the abbreviations used, see Materials and Methods; unless otherwise indicated, the ratio of the lipids in the mixtures is given in mol %. [b] See Materials and Methods.

0.6 and 10 V were applied. The applied voltage only had an influence on the speed of vesicle formation and on the shape of the vesicles (vesicle radius, R_v, and radius of contact, R_c, see Figure 2C). Spheres ($R_c = 0$) were mainly formed between 5 and 10 V, the speed of formation being higher at 10 V than at 5 V. Generally, the higher the applied voltage, the faster is the vesicle formation process. At 3 V, for example, "mushrooms" with $R_v \sim 50\ \mu$m were already present after 20 min.[28] Using a lower voltage, one had to wait for about 2 h to obtain vesicles with a similar size. It is worthwhile to point out that vesicles with diameters of more than 100 μm were formed. Occasionally, the vesicles had diameters of 0.2−0.3 mm, almost the diameter of the electrode wire.

To check the influence of the frequency of the applied ac electric field on the vesicle formation, experiments were performed at a constant voltage of 1.3 V by varying the frequency between 0.2 and 100 Hz. In all cases giant POPC vesicles were formed, and during the growth process the whole vesicle structure oscillated with the applied frequency in the direction of the ac electric field. These movements on the electrodes were particularly visible at low frequency, as already described by Angelova and Dimitrov in their original papers.[8] If the frequency was 0.2 or 2 Hz, most giant vesicles had $R_c = 0$, which means they appeared as true spheres under the microscope (see Figure 2A). With 10, 30, or 100 Hz, giant vesicles appeared as "mushrooms" (see Figure 2B), and in addition, at 30 Hz many "cut sphere" type of vesicles with $R_v \sim 25\ \mu$m (see vesicle 3 in Figure 2C) were formed.[28] The time for the formation of giant vesicles was in all cases about the same.

In conclusion, we made the general observations that the characteristics of the giant POPC vesicles formed depend on the applied voltage and frequency. Low frequencies (2 Hz, 0.2 Hz and 1.3 V) or high voltage (5 V, 10 V and 10 Hz) seem to favor the formation of vesicles with $R_c = 0$, namely, of undoubtly closed, spherical structures (see Figure 2A).

Electroformation of Giant POPC Vesicles in Aqueous Salt Solution. One of the limitations of the electroformation method for the preparation of giant vesicles is the fact that vesicles cannot be prepared in the presence of concentrated salt solutions.[8e] Ions possibly enter the inter-bilayer space of the lipid film and hinder the separation of the lipid bilayers, which is necessary for the formation of the giant vesicles.[8e] This salt limitation is certainly a disadvantage, in particular when applications require the use of buffer solutions. To test which salt concentration can still be used, we have investigated the effect of a variety of salts on the vesicle formation, using POPC, 10 Hz and 1.5 V, and the film formation method A (see Materials and Methods). The salt concentrations with which vesicles could still be formed were in the case of LiCl, NaCl, and KCl 10 mM, for $CaCl_2$ 0.2 mM, for $MgCl_2$ 1.7 mM, for $CoCl_2$ 1 mM, and for Tris/HCl (pH 7.5) 6.6 mM. Tris buffer behaved unusually, since giant vesicles often formed at the wire and then disappeared again. They were generally less stable than those in pure water.

Osmotic Effects of Giant POPC Vesicles. It is well-known that conventional, as well as giant vesicles are sensitive to osmotic pressure differences between the aqueous interior and the bulk solution.[29−35,13c] If for example giant POPC vesicles were first prepared in pure water and then exposed to glucose solution (10−300 mM),[36] the vesicles initially shrank due to a flow of water out of the vesicles. With 10 mM glucose, shrinkage started after 6−7 min, with 50 mM glucose it started after 3 min, with 100 mM after 1 min, and with 200 mM after 30 s.[37]

(28) R_v values given are characteristic mean values for the conditions used. They correspond to typical observations made from several independent experiments.

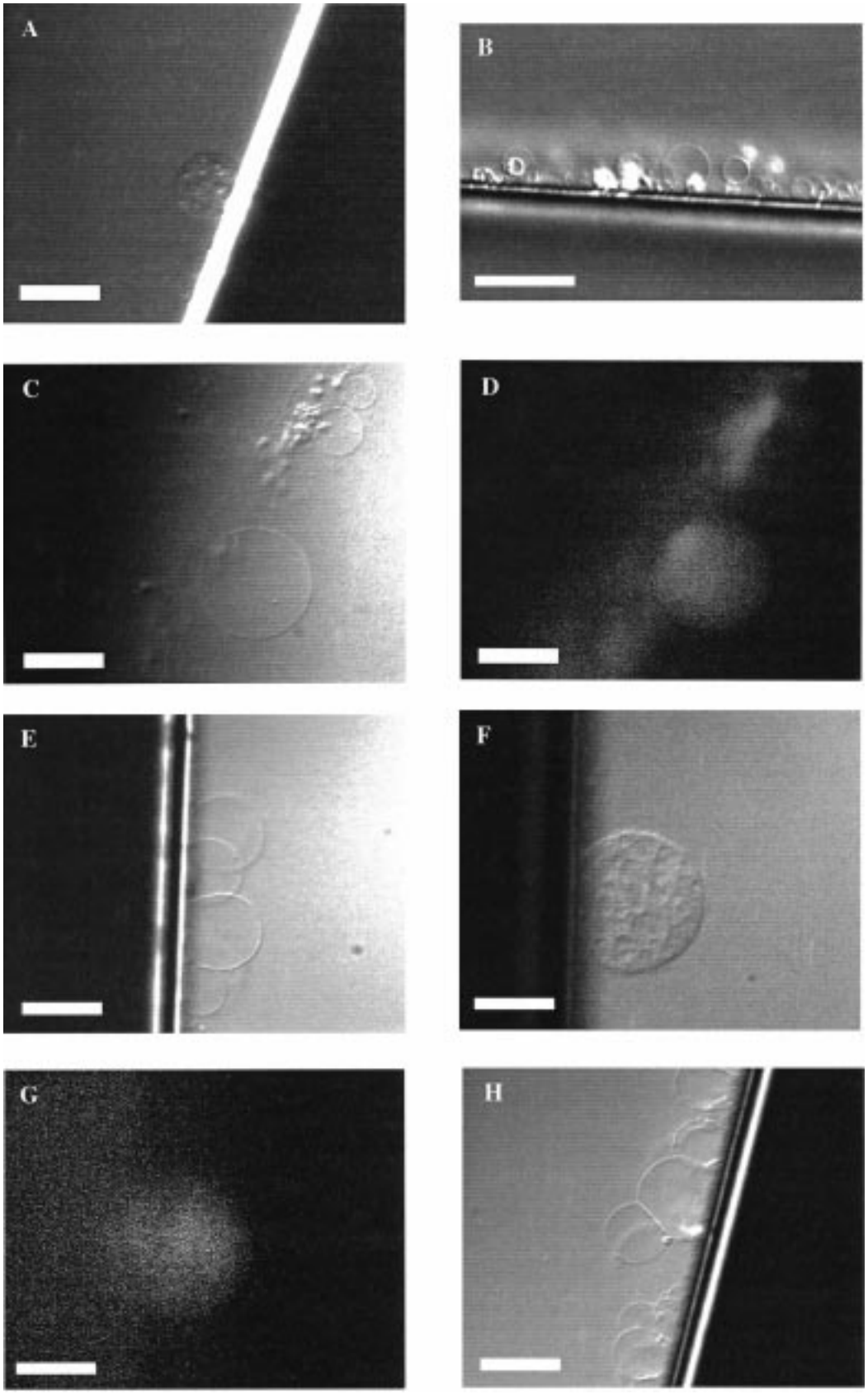

Figure 3. Light micrographs of giant vesicles formed from different phospholipids and phospholipid mixtures, as obtained by the electroformation method in water; DIC (A−C, E, F, H) or fluorescence (D, G) detection mode: A, POPG; B, DOP-uridine; C, D, DOP-uridine:POPC (1:1, molar ratio), after addition of YO-PRO-1; E, HDP-cytidine:POPC (1:1, molar ratio); F, G, HDP-uridine: POPC (1:1, molar ratio), after addition of YO-PRO-1; H, POPC:DDAB (95:5, weight %). Length of the bar represents 50 μm.

Giant Vesicles from Other Phospholipids and from Mixtures with POPC. The electroformation method has been until now restricted to a series of phosphatidylcholines or mixtures with phosphatidylcholines.[8] We have tried to find experimental conditions for the preparation of giant vesicles from a series of other

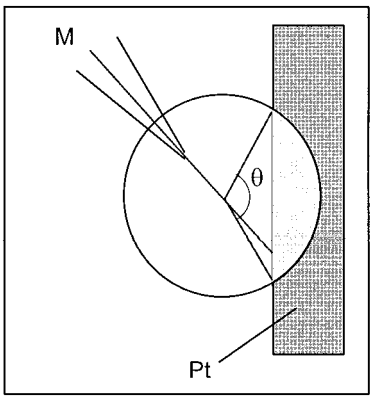

Figure 4. Schematic representation (top view) of the optimal conditions for microinjection experiments, showing the sector angle (Θ) and the direction of microneedle approach: Pt, platinum wire; M, microneedle tip.

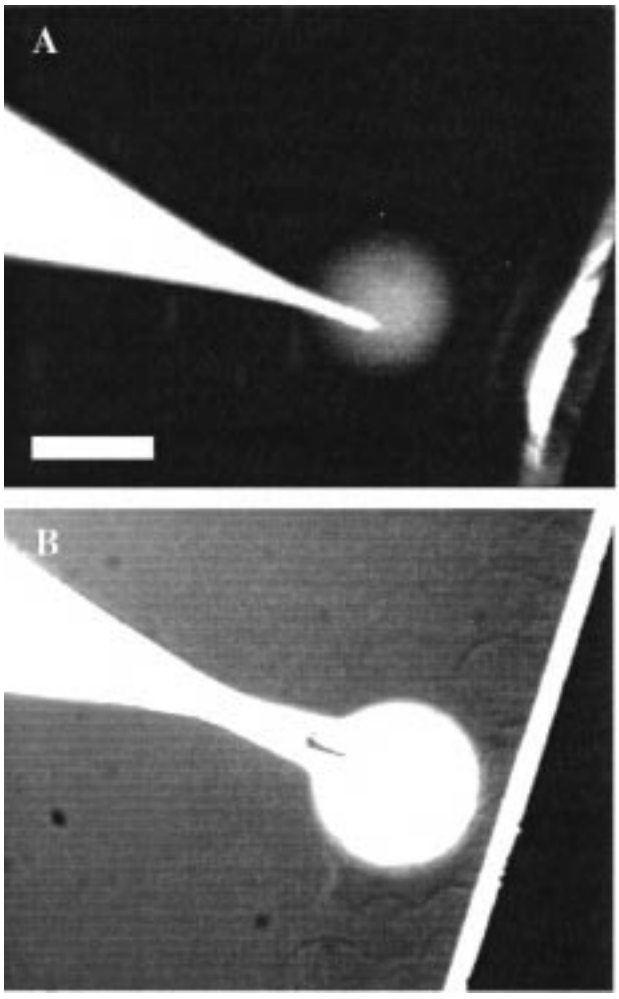

Figure 5. Light micrographs showing microinjections of calcein (5 mM) into a giant POPC vesicle. During the formation process in water, 10 V and 10 Hz were used. After 30 min, the ac electric field was switched off (A) after the first injection and (B) 35 s after the second injection. DIC and fluorescence detection mode. Length of the bar represents 50 μm.

phospholipids and phospholipid mixtures in pure water. Among the phospholipids used, those with nucleoside headgroups were of particular interest, as their potential molecular recognition properties are currently being investigated with conventional vesicles, having diameters below 1 μm.[20b−e] The results are summarized in Table 1. The preparation of giant vesicles from the negatively charged POPG was much more difficult than for POPC. Giant vesicles with $R_c = 0$ only formed at 8 or 17 V with 10 Hz, and the vesicles often contained in the aqueous interior additional smaller vesicles (Figure 3A). Furthermore, the number of giant vesicles in comparison with the case of POPC was much smaller. In the case of mixtures of POPG with POPC, the vesicle formation was more successful (see Table 1). The preparation of giant vesicles from the pure phospholiponucleosides, POP-uridine, DOP-uridine, DOP-adenosine, and DOP-cytidine, was also not easy. For unknown reasons, giant vesicles could only be formed from DOP-uridine (Figure 3B), and depending on the conditions, the giant vesicles appeared to contain smaller vesicles (see Table 1), an observation which was never made with POPC. The chemical structures and properties of the headgroups seem to play an important role for the vesicle formation. A mixture of DOP-uridine with POPC (50:50, mol %) or a mixture of POP-uridine with POPC (50:50, mol %) led to the formation of giant vesicles that could be stained by adding the nucleic acid stain YO-PRO-1 (Figure 3C,D). YO-PRO-1 is a cationic cyanine dye containing a benzoxazole and a quinoline ring, bearing two positive charges. YO-PRO-1 is essentially nonfluorescent, and only when bound to DNA or RNA it is green fluorescent.[22b] We obtained a significant fluorescence with the vesicles containing phospholiponucleosides whereas giant vesicles prepared with pure POPC and exposed to YO-PRO-1 did not fluoresce. These data give two pieces of different and complementary information: on one hand, they clearly show that the giant vesicles formed contained the phospholiponucleosides used; on the other hand, they show that YO-PRO-1 can also bind to single nucleosides different from nucleic acids DNA or RNA.

HDP-cytidine alone is known to form micelles in aqueous solution,[21] and it is not surprising that this amphiphile did not lead to the formation of giant vesicles by using the electroformation method (see Table 1). Giant vesicles formed from mixtures of *n*-hexadecylphosphonucleosides with POPC (Figure 3E). They, however, were unstable and transformed into "filled" giant vesicle aggregates, as shown in Figure 3F. These lipid assemblies seemed to contain many smaller vesicles that could be stained with YO-PRO-1 (see Figure 3G), indicating that the nucleoside-lipid was incorporated into the membranes.

With POPC mixtures of DPPE or Texas Red-DOPE, the formation of giant vesicles occurred as with pure POPC, confirming earlier observations.[14d]

In the case of POPC:DDAB (95:5, wt %), the presence of the positively charged DDAB led to a frequent adhesion of neighbored vesicles (see Figure 3H). It seems that

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(36) Similar shrinkage due to osmotic pressure was also observed by using NaCl (32 mM, which is isoosmotic with ~60 mM glucose).
(37) This initial lag-phase was most likely related to the time needed to equilibrate the bulk solution after addition of the concentrated glucose solution to water, in which the vesicles were formed. A mixing of the solution by gentle shaking was not possible as this type of mechanical treatment would lead to damage of the vesicles. Even with these technical difficulties we observed that the size of all the giant vesicles formed at the electrode decreased with time if exposed to a hyperosmotic solution; often the vesicles moved out of the area of focus during the shrinkage process.

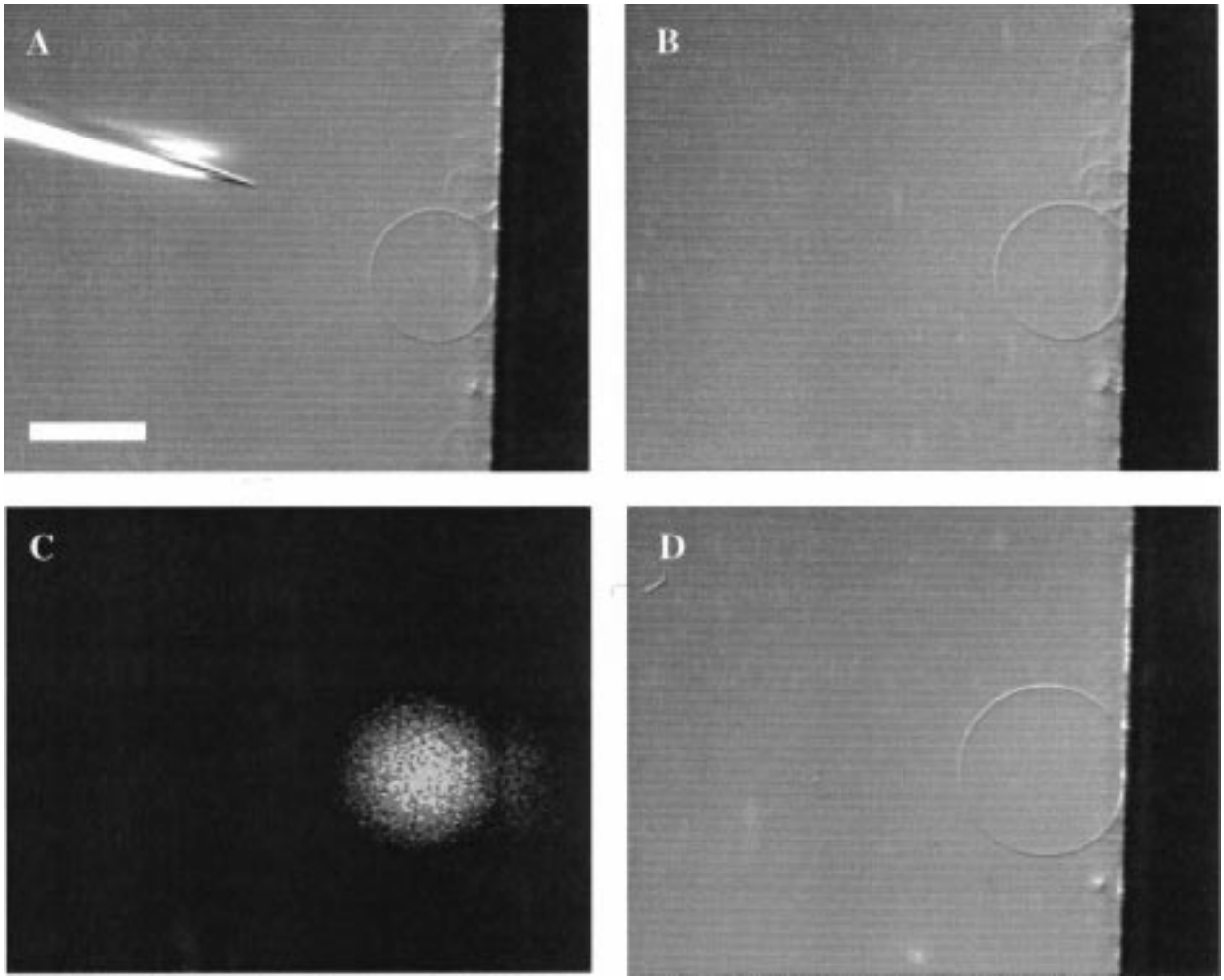

Figure 6. YO-PRO-1 staining of DNA that was previously injected into a giant POPC vesicle. The giant vesicles were first formed in water (2 V, 10 Hz), and a solution of YO-PRO-1 was added externally to the vesicle at a final concentration of 1 μM. (A) Before DNA injection, showing the microneedle on the left-hand side. (B) Immediately after DNA injection into the largest vesicle seen (DNA concentration of the injected solution: 4 mg/mL). (C, D) 90 min after addition of YO-PRO-1, visualized by fluorescence detection (C) or DIC (D). Length of the bar represents 50 μm.

neighbored vesicles share the membrane over a certain contact area.

Concerning PS, there are conflicting reports in the literature.[8b,d,f] Our studies indicate that giant vesicles from pure PS did not form (Table 1).

Microinjections into Giant POPC Vesicles. One of the aims of our investigations is to use giant vesicles as microreactor systems with dimensions comparable to biological cells.[38] The boundary of these microreactors is formed by a bilayer of amphiphilic lipids that is characterized by a rather low permeability for large molecules such as proteins or nucleic acids. If we aim at carrying out chemical and enzymatic reactions inside a single giant vesicle, the reagents have to be transported through the bilayer boundary. This can be done by mechanically adding a desired amount of reagents through microinjections, using microneedles and an appropriate micromanipulator. In this paper, we have focused our aim at microinjections, which allow the controlled addition of a defined amount of reagent into a single target vesicle.

Apart from several drawbacks and limitations indicated in part above, the electroformation method appears to be the method of choice for allowing microinjections into single giant vesicles composed of phosphatidylcholines (such as POPC) or lipid mixtures containing phosphatidylcholines. Since the vesicles formed are in close contact with the metal wire, the use of a holder capillary is not necessary during the injection procedure; by providing a back pressure, the metal wire on which the vesicles form stops the vesicle from escaping when approached by the injection needle.

In the following, we will first report on the experimental conditions that were optimal for successful microinjections, and we will show that it was possible to perform *single puncture repetitive injections* (successive injections during which the microneedle remains inside the target vesicle). Although more difficult, *multiple puncture injections* (up to three times) into the same vesicle were also possible (data not shown). In this case, the target vesicle was punctured twice or even three times. Typical experimental conditions for the whole vesicle formation process were 2 V and 10 Hz.

The microneedles were prepared from borosilicate glass tubes as described above (see Materials and Methods). The inner diameter of the microneedles was determined by scanning electron microscopy and characterized by the bubble formation method.[24] During a typical microinjection, about 5−20 pL (1 pL = 10^{-12} L) was injected into

(38) To illustrate the dimensions of these giant vesicle microreactors: the internal aqueous volume of a single POPC vesicle with a diameter of 50 μM is about 6.5×10^{-11} L (65 pL), separated from the bulk solution by about 2.1×10^{10} POPC molecules.

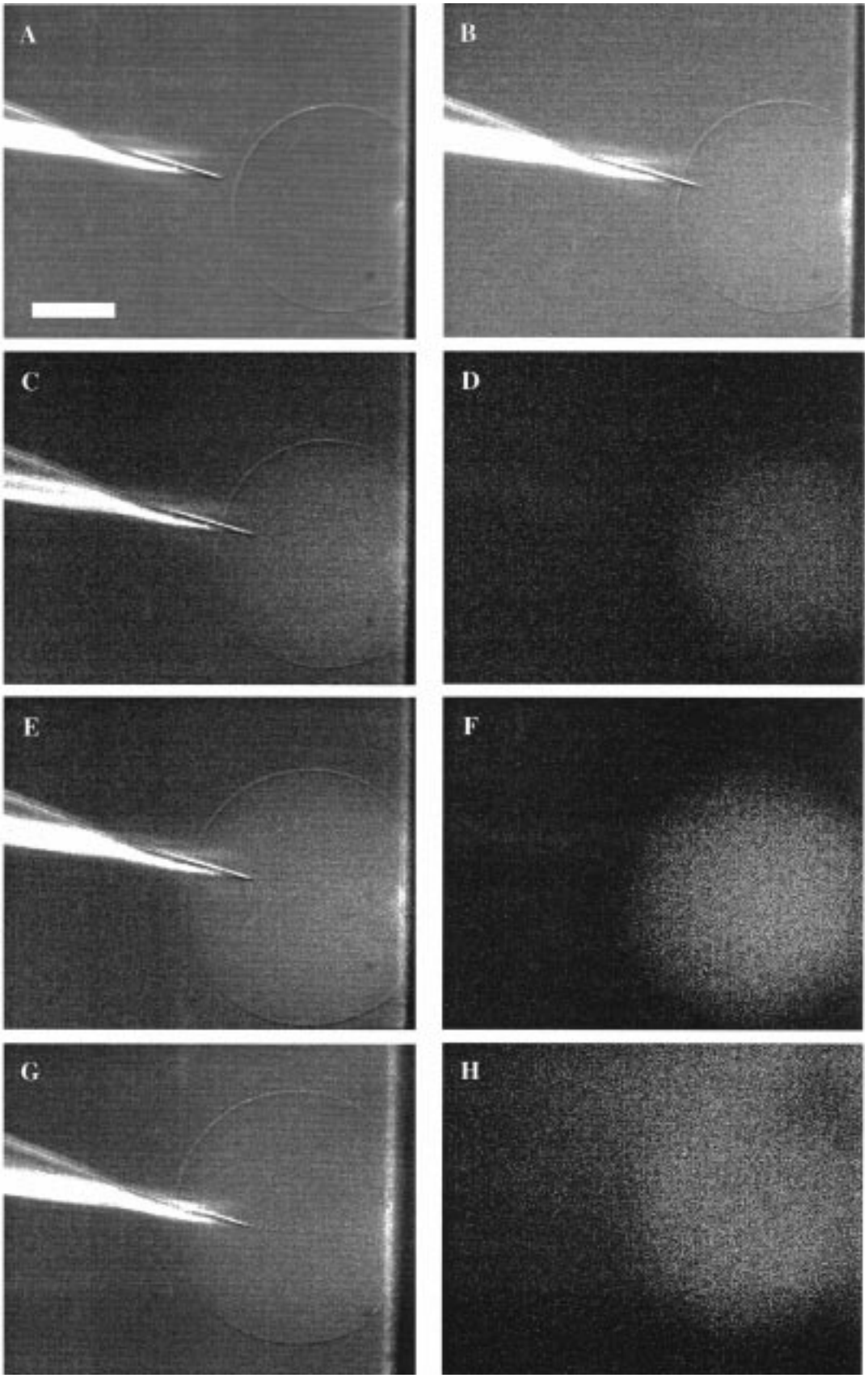

Figure 7. Light micrographs showing the effect of repetitive microinjections into the same giant POPC vesicle: A, B, before injection; C, D, after 15 microinjections; E, F, after 40 microinjections; G, H, after 50 microinjections. The giant vesicles were first formed in water in the presence of YO-PRO-1 (0.5 μM) with 2 V, 10 Hz. A tRNA solution (0.5 mg/mL) was injected into the target vesicle. With each injection ~5−20 pL tRNA solution was added. A, C, E, G: DIC mode. B, D, F, H: fluorescence detection mode. Length of the bar represents 50 μm.

one vesicle. It was rather advantageous to use giant vesicles with $R_c \neq 0$ (see vesicle 3 in Figure 2C) as these vesicles did not move away when approached and touched by the microneedle. Most useful were vesicles that had

a sector angle, Θ, of less than 120° in their microscopic appearance as circle (see Figure 4 for the definition of Θ). The (apparent) radius of contact (R_c) is then given by $R_c = R_v \sin(\Theta/2)$; for $\Theta = 90°$, $R_c = 0.71R_v$. Because the microneedle always had to approach the vesicles from above the cell compartment, the position of the vesicles had to be on the upper half of the platinum wire (vesicle 2 in Figure 2C). Sometimes microinjections into giant vesicles with $R_c = 0$ were also possible as shown in Figure 5. Here, a fluorescent calcein solution was injected into a giant POPC vesicle. As expected, only the vesicle into which calcein was added showed fluorescence. No leakage occurred, indicating that the vesicle was a closed sphere and that the bilayer permeability for calcein was relatively low.

Figure 6 shows another microinjection experiment. In a first step, a DNA solution (4 mg/mL) was injected into the target POPC vesicle. The microneedle was removed from the vesicle, and a solution of YO-PRO-1 was added into the investigation chamber (i.e., externally to the giant vesicles). After some time, the injected vesicle started to fluoresce as shown in Figure 6C, indicating that first of all the DNA injection was successful, and second that YO-PRO-1 could penetrate across the POPC bilayer into the vesicle where it interacted with DNA to give rise to the fluorescence. The observation that POPC bilayers are permeable to YO-PRO-1 can be compared with the behavior of ethidium bromide—another positively charged, well-characterized nucleic acid stain. This stain rapidly leaks out of phosphatidylcholine vesicles.[39,40]

Finally, we would like to report on another microinjection experiment, involving single puncture repetitive injections (see Figure 7). Giant POPC vesicles were first prepared in water in the presence of YO-PRO-1 (0.5 μM). This relatively small amount of stain had no significant effect on the vesicle formation process, and giant POPC vesicles grew at the platinum electrode as usual. After switching off the ac electric field, an aqueous tRNA solution (0.5 mg/mL) was injected into the target vesicle. With each injection about 5−20 pL was added. Parts A and B of Figure 7 show the vesicle before injecting tRNA, and parts C and D of Figure 7 show the same vesicle after 15 injections, during all the injections, the microneedle was left inside the vesicle. The vesicle already showed a weak fluorescence due to the interaction between YO-PRO-1 and tRNA. The fluorescence intensity increased as more tRNA was added. Parts E and F of Figure 7 are light micrographs obtained after 40 injections, and parts G and H of Figure 7 are micrographs obtained after 50 injections, always from the same puncture. At this point, however, leakage of the vesicle—without burst—is evident from the dilution of the fluorescence into the exterior bulk solution. It is interesting to note that these repetitive microinjections led to a remarkable increase of the vesicle volume. The target vesicle had an initial R_v of 64 μm, corresponding to a calculated internal volume of 1.09 nL. The radius of the vesicle increased by about 25% to 80 μm (Figure 7E,G), corresponding to a calculated volume increase of about 90%. This increase was considerably larger than expected on the basis of the amount actually injected (~200−250 pL, corresponding to a volume increase of ~20%). It is most likely that several factors are responsible for this surprising finding. It is unlikely that this increase in volume is solely due to the elasticity of the giant vesicles; the only possible alternative is that the increase is due to additional POPC molecules being added to the giant vesicles—in other words, POPC molecules somehow had to be delivered to the vesicle bilayer during the injection process. This delivery may come from the electrode surface (where there is always a kind of lipid reservoir left) or from the vesicle internal wall, a process that is facilitated if the vesicles are not unilamellar. Another alternative delivery mechanism would be the delivery of POPC molecules through submicroscopic vesicles. With respect to the requirements for water uptake by the growing vesicle, osmotic effects and/or the formation of transient holes probably allowed the influx of water from the external bulk solution.

Concluding Remarks

Since not much is known about the details of the physical processes underlying the vesicle formation under the influence of an electric field, we had to rely on semiempirical, phenomenological investigations. At this point, unfortunately, we cannot provide a mechanistic interpretation of most of our finding. For example, it is not clear why the electroformation method is apparently restricted to a small family of amphiphilic lipids. In fact, the formation of thin-walled vesicles was not successful with all other lipids tested—if not mixed with POPC. Apparently, a change in the chemical structure of the lipid strongly influences the lipid swelling behavior and the whole electro-osmosis-supported vesicle formation process, but the understanding of this fine balance is still out of reach. The same can be said about the influence of the voltage and frequency of the applied ac electric field.

Within these structural limits, giant vesicles appear to be very stable, both in terms of time (one single giant vesicle can be observed without change for at least 1 day) and mechanically (giant vesicles can be punctured or pushed around by a needle without bursting). Care should be taken to avoid optical artifacts, which tend to mask the normal spherical shape under the light microscopy observation. However, structures that are not closed may indeed be present.

A further limitation of the method is related to the use of ions in the aqueous medium in which the vesicles are prepared. Depending on the salt, the maximum salt tolerance may be as low as 1 mM or even lower. If salt is added after the vesicles are formed, osmotic effects may lead to vesicle deformations. This appears to be a serious limitation, particularly for certain molecular biology oriented work, in which the presence of a high concentration of metal ions (e.g., Mg^{2+}) or phosphate ions is needed.

In conclusion, despite its several limitations, the electroformation method is rather useful for preparing giant vesicles that are apt for microinjection experiments. Work is now in progress on applying the microinjection technique for chemical and enzymatic reactions inside single giant vesicles.

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(40) It is worthwhile mentioning that YO-PRO-1 and ethidium bromide are considered to be cell impermeant.[22] From our observation and from literature,[39] it is evident that these biological cell properties cannot directly be adopted to vesicles.