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Giant vesicles as models to study the interactions between membranes and proteins

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Abstract

The interaction between polypeptides and membranes is a fundamental aspect of cell biochemistry. Liposomes have been used in this context as in vitro systems to study such interactions. We present here the case of giant vesicles (GVs), which, due to their size (radius larger than 10 microns), mimic more closely the situation observed in cell membranes and furthermore permit to study protein–membrane interactions by direct optical monitoring. It is shown that GVs formed from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine by electroformation are permeable to certain low molecular weight molecules such as the nucleic acid dye YO-PRO-1 and fluorescein diphosphate whereas conventional liposomes (large or small unilamellar liposomes) are not. In addition, it is shown that non-membrane proteins, such as DNases or RNases, added to the selected GVs from the outside, are able to convert their substrate, which is strictly localized on the internal side of the membrane. This effect is only seen in GVs (also when they are removed from the original electroformation environment) and is absent in conventional liposomes. The fact that these effects are only present in GVs obtained by electroformation and not in conventional small liposomes is taken as an indication that certain physico-chemical properties of the bilayer are affected by the membrane curvature, although the mechanism underlying such differences could not be established as yet.

Keywords: Giant vesicle; Micromanipulation; Microinjection; Permeability; Membrane interaction

1. Introduction

Giant vesicles (GVs) have been the focus of much attention in the last few years. Their large size permits, by the use of special micromanipulation techniques, the injection of chemicals into their interior water pool. This opens the way to the bioengineering of selected bioreactor compartments having the size of a biological cell [1–3]. Furthermore, due to their size, GVs can be observed by light microscopy, while electron microscopy is necessary for conventional liposomes. One should also recognize that due to their very large radius (up to 100 μm), GVs are better models for the planar membranes than the small conventional vesicles with their high curvature. In connection with their radius is also the question whether the large size of GVs brings about novel physico-chemical properties that are not present in conventional small unilamellar or large unilamellar liposomes (SUVs or LUVs). In this respect, permeability to biomolecules and macromolecules is of particular importance. The permeability of SUVs or LUVs to low molecular weight biochemicals is very limited, although there are some reports describing

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the uptake of amino acids [4–6] or even short peptides [7].

The present paper deals with the interaction of proteins as well as low molecular weight compounds with GVs obtained from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) by the electroformation method. The question is whether and under what conditions these molecules, once added to the external aqueous medium, come into contact with the interior compartment of the giant liposomes. In all cases, a comparison with the behavior of LUVs will be presented. In this way, the present work may shed some light both on the question of the interaction between membranes and proteins, and on the question of the difference between small conventional vesicles and GVs.

2. Materials and methods

2.1. Materials

POPC was obtained from Avanti Polar Lipids (Birmingham, AL, USA). Fluorescein diphosphate (FDP) and YO-PRO-1 iodide (4-[(3-methyl-2(3H)-benzoxazolylidene)methyl]-1-[3-(trimethylammonio)-propyl]-quinolinium diiodide) were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Deoxyribonuclease II from porcine spleen, type IV, lyophilized powder in 30% NaCl (EC 3.1.22.1, 2000–5000 Kunitz units mg^{-1}) was obtained from Sigma (St. Louis, MO, USA). Cholesterol from human gallstones, deoxyribonucleic acid sodium salt from herring testes and transfer ribonucleic acid from wheat germ were purchased from Fluka (Buchs, Switzerland). Deoxyribonuclease I from bovine pancreas, grade II, lyophilized powder prepared from aqueous distilled solution (EC 3.1.21.1, approximately 2000 Kunitz units mg^{-1} protein), ribonuclease A from bovine pancreas, dry powder (EC 3.1.27.5, approximately 50 Kunitz units mg^{-1}) and fluorescein-12-UTP tetralithium salt were purchased from Roche Diagnostics (Rotkreuz, Switzerland). Proteinase K from *Tritirachium album* (EC 3.4.21.14, 30 mAnson units mg^{-1}) was obtained from Merck (Darmstadt, Germany). Alkaline phosphatase (APase) from calf intestine (EC 3.1.3.1, 10000 Kunitz units ml^{-1}) was purchased from New England BioLabs, Inc. (Beverly, MA, USA). Plasmid DNA pWMEGFP was isolated and purified as earlier described [8]. MDV RNA [9] was a kind gift of Christof K. Biebricher, Göttingen, Germany. Bio-Gel A-15 m Gel was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were of analytical grade.

2.2. Preparation of GVs

GVs were prepared by the electroformation method as previously described [10]. In brief, POPC or a mixture of POPC and cholesterol (90:10 mol%, 98:2 mol%) was dissolved in diethylether:methanol 9:1 (v/v) at a concentration of 0.2 mg ml^{-1}. Approximately 2 μl of this solution was carefully deposited with a Hamilton syringe on two different areas of each platinum electrode of the investigation chamber. The lipid films were then dried under a nitrogen stream for about 1 min and afterwards stored under reduced pressure (<10 mbar) in a desiccator for at least 15 h. The investigation chamber was then put on the stage of the light microscope (Axiovert 135 TV from Carl Zeiss AG, Zürich, Switzerland) and connected to the frequency generator (Conrad Electronic, Hirschau, Germany) generating an AC field (1 Hz, 1.5 V, peak to peak value). One ml of water was added and the vesicles were formed in the electric field; the overall final concentration of the lipid was approximately 2 μM. After approximately 3 h, some vesicles had reached a size of 60–100 μm in diameter. The alternating electric field was then turned off and micromanipulation experiments were performed.

2.3. Microinjection experiments

The first step in all experiments was the injection of the appropriate substance into a selected GV. The time of injection was 1 s, the applied injection pressure was 2000 hPa and the number of injections was 1–4. The micropipettes used for these experiments were prepared as previously described [10]. After injection, the micropipette was removed and the GVs were allowed to stand for 5–10 min. To study the permeability of GVs to low molecular substances, YO-PRO-1 (final overall concentration 1 μM) was added to a GV containing DNA and FDP (final overall concentration 5 μM) was added to a GV

containing APase. To study the interaction of proteins with GVs, the appropriate proteins were added externally to the GVs, once the fluorescence intensity of the YO-PRO-1/nucleic acid complex was constant. For DNase I and DNase II, the final concentrations were 200 μg ml^{-1} and 400 μg ml^{-1}, respectively, for RNase A the final concentration was 70 μg ml^{-1}, for proteinase K it was 320 μg ml^{-1} and for lysozyme it was 200 μg ml^{-1}. The fluorescence intensity of the nucleic acid/YO-PRO-1 complex inside the selected GV was recorded for 2–3 h using a CCD C5810 camera (from Hamamatsu, Japan) and evaluated by a statistical calculation of the mean intensity per pixel using Image-Pro Plus 4.0 (from Altrona AG, Switzerland).

2.4. Investigation of the permeability of GVs detached from the platinum electrode

GVs were formed by electroformation as described above in the presence of 5 mM sucrose, 4 μg ml^{-1} tRNA. The GVs were sucked into a pipette (inner diameter >200 μm) and transferred into an aqueous solution containing 2.5 mM glucose, 3 μM YO-PRO-1, thereby diluting the tRNA concentration about 10 times.

2.5. Permeability experiments with LUVs

LUVs were prepared as described earlier [11] with some minor modifications. To test whether YO-PRO-1 can permeate across LUV membranes, aqueous solution containing crude DNA was added to a lipid film to give a POPC concentration of 120 mM and a DNA concentration of 2 mg ml^{-1}. After sonication, the dispersion was treated by freeze and thaw for 10 times, diluted twice and extruded through two stacked polycarbonate filters with 200 nm pores in diameter. To digest the non-entrapped DNA, 2000 U of DNase I was added and the $MgCl_2$ concentration was adjusted to 5.5 mM. After incubation for 20 h at 37°C, EDTA (final concentration 7 mM) was added and the external medium was separated from the liposomes by spin column gel filtration chromatography [11]. The liposomes were then diluted to a POPC concentration of 3 mM and YO-PRO-1 was added to the external medium (final concentration 1 μM). The signal of the YO-PRO-1/DNA complex was recorded at 545 nm as a function of time with a SPEX-Fluorolog-2 fluorometer (from SPEX Industries, Inc., Edison, NJ, USA), at an excitation wavelength of 491 nm in a quartz cuvette with a path length of 1 cm.

To study whether DNase II interacts with LUV membranes, LUVs with encapsulated YO-PRO-1 and DNA were prepared. An aqueous solution containing crude DNA and YO-PRO-1 was added to a lipid film to obtain a POPC concentration of 120 mM, a DNA concentration of 1.3 mg ml^{-1} and a YO-PRO-1 concentration of 50 μM. The liposomes were prepared by extrusion as described above. To detect a possible protein interaction, the liposomes were diluted to a POPC concentration of 3 mM, DNase II (final overall concentration 10 mg ml^{-1}) was added to the external medium and the fluorescence emission was measured as described above.

To test whether FDP can permeate across LUV membranes, liposomes containing APase were prepared. An aqueous solution containing APase and buffer was added to a lipid film to obtain a POPC concentration of 50 mM, an APase concentration of 5 mU μl^{-1} (in 1.25 mM NaCl, 625 μM Tris–HCl, 125 μM $MgCl_2$ and 12.5 μM dithiothreitol). The liposomes were prepared as described above. The non-encapsulated APase was digested during 30 min with 80 μg (2.4 mAnson units) of proteinase K. To detect a possible permeation of FDP, FDP was added externally to the liposomes (3 mM POPC) at a final concentration of 5 μM and the fluorescence signal at 514 nm was measured after excitation at 490 nm.

In all cases, the control experiments were carried out with liposomes to which the substance to be encapsulated was added after extrusion.

3. Results

3.1. GV permeability to YO-PRO-1 and FDP

Let us consider first the permeability of the GVs formed by electroformation to the low molecular weight compound YO-PRO-1 (Fig. 1, top, and [12,13]). YO-PRO-1 is a highly specific nucleic acid dye and its background fluorescence is very low [14]. After having formed the GVs in an a.c. electric field,

plasmid DNA was injected into a selected GV using the microinjection techniques already described by our laboratory [2,10]. Then YO-PRO-1 was added to the external medium of this DNA-containing GV at a final concentration of 1 μM.

Results are shown in Fig. 2A. In the course of time, the selected GV becomes highly fluorescent. This indicates that YO-PRO-1 diffuses into the selected GV, thus being capable of permeating across an intact phospholipid bilayer membrane. Once the steady state between the dye and the entrapped DNA is established, the fluorescent signal remains constant for more than 1 h (Fig. 2B). The fact that it takes 1 h to arrive at a constant fluorescence level means that the YO-PRO-1 permeability across the POPC GV bilayer is the rate determining step. It is of interest to consider the quantities of material involved in such microinjection experiments. The minimal amount of DNA which can be detected after injection is in the range of 10–100 fg [15]. For the investigations presented in this work, the injected quantities were 10–50 times higher. In Fig. 2A, the local DNA concentration inside the GV corresponds to 1–5 nM (based on a volume of 180 pl for the GV). An overall concentration of 1 μM YO-PRO-1 was added to the external medium of the GVs. According to the known binding constant between DNA and YO-PRO-1 (which is in the 10^{10}–10^{12} M^{-1} range, [16]), and assuming that one YO-PRO-1 molecule binds per one DNA base pair, more than 99.99% of the dye molecules remain free in the external medium.

An analogous experiment was carried out with a GV containing microinjected APase and addition of the substrate FDP (see Fig. 1, bottom) to the external medium. As observed in the case of YO-PRO-1, FDP could permeate across the GV membrane into the interior of the GV where it was dephosphorylated to fluorescein by the APase (see Fig. 2C).

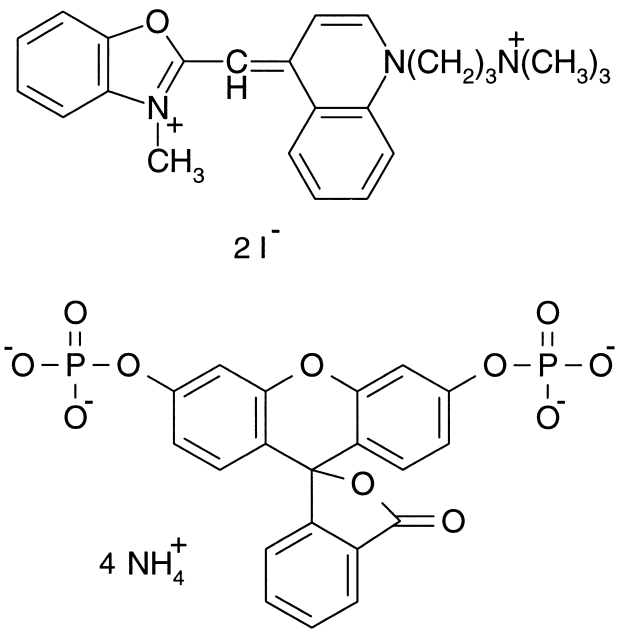

Fig. 1. Structure of YO-PRO-1 (top) and FDP (bottom).

The permeability observed in these experiments was somewhat surprising on the basis of what is known on phospholipid bilayers in the literature. The question is whether the observed permeability is due to the particular behavior of the tested dye molecules, e.g. YO-PRO-1. In this case, such a permeability should be observed also with conventional

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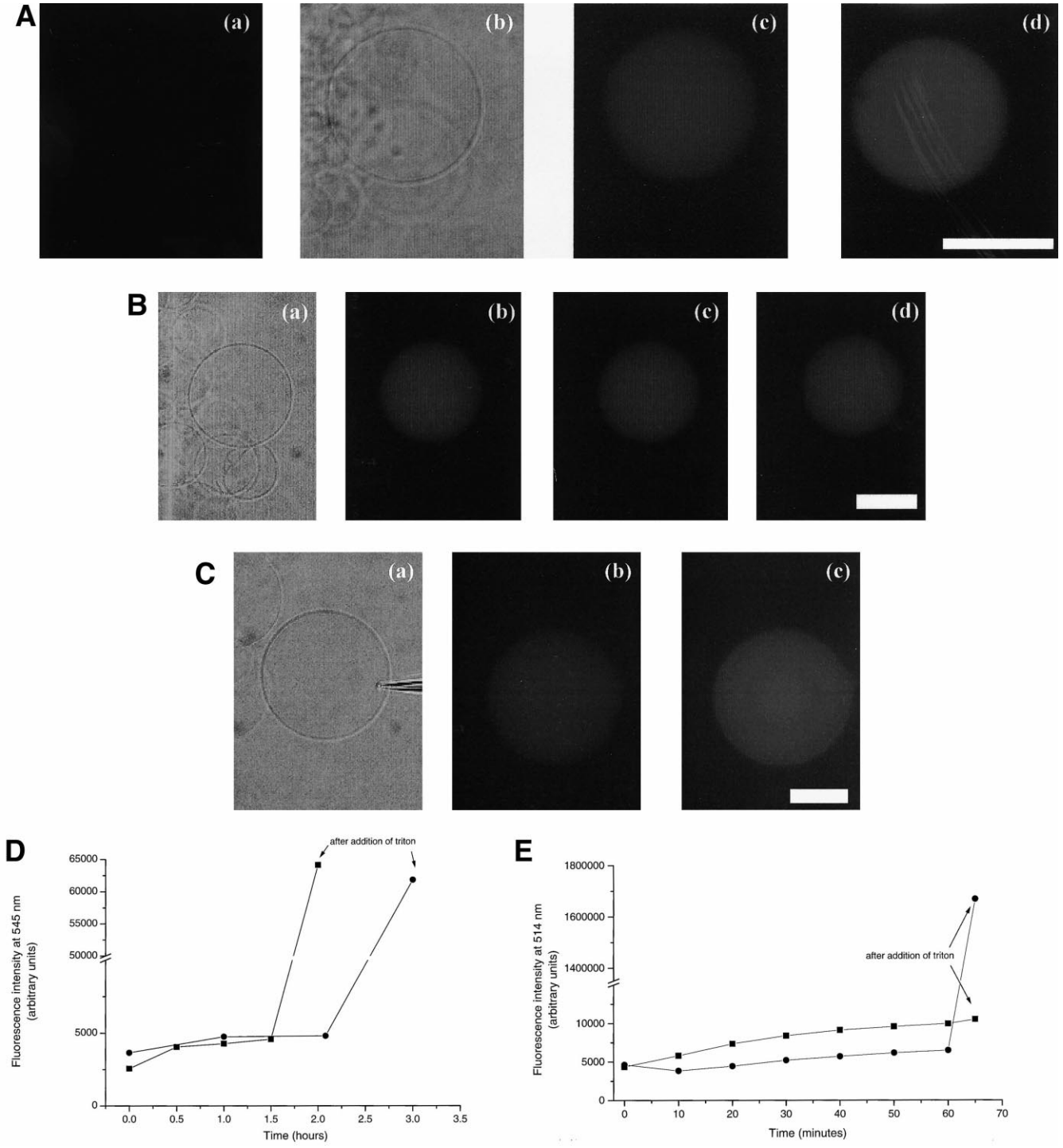
Fig. 2. The nucleic acid dye YO-PRO-1 and FDP permeate across the GV bilayers, but not across the LUV membranes. A: The vesicles were formed in water by the electroformation method, plasmid DNA (78 μg ml^{-1}) was injected and the GV was allowed to stand for 5–10 min. Then YO-PRO-1 was added externally to the vesicles at a final concentration of 1 μM. (a) Before DNA injection, (b, c) 15 min, (d) 50 min after YO-PRO-1 addition. (a, c, d) = fluorescence mode, (b) = DIC mode. Scale bar = 50 μm. B: Fluorescence intensity of DNA/YO-PRO-1 with time. After injection of the plasmid DNA, YO-PRO-1 was added externally to the vesicles. (a, b) 35 min, (c) 53 min, (d) 63 min after YO-PRO-1 addition. (a) = DIC mode, (b–d) = fluorescence mode. Scale bar = 50 μm. C: After injection of a 50 mU ml^{-1} APase solution (in 12.5 mM NaCl, 6.25 mM Tris–HCl, 1.25 mM $MgCl_2$, 125 μM dithiothreitol), FDP was added externally to the vesicles at a final concentration of 5 μM. (a) After injection of APase, (b) 20 min, (c) 27 min after the addition of FDP. (a) = DIC mode, (b,c) = fluorescence mode. Scale bar = 50 μm. D: YO-PRO-1 is not permeable to LUV membranes. YO-PRO-1 (final concentration 1 μM) was added to purified LUVs containing encapsulated crude DNA and the fluorescence intensity was recorded as a function of time. Two typical results are shown. After 2 h (squares) and 3 h (circles), respectively, Triton X-114 was added (final concentration 2% w/v) and the total fluorescence of the solution was determined. E: FDP is not permeable to LUV membranes. FDP (final concentration 5 μM) was added to purified LUVs containing encapsulated APase and the fluorescence was recorded as a function of time (circles). The control experiment (squares) shows the fluorescence intensity without encapsulated APase. After 1 h, the vesicles were destroyed with Triton X-114 (final concentration 2% w/v) and the total fluorescence was measured.

unilamellar vesicles (SUVs or LUVs). To test this possibility, analogous experiments were carried out with conventional LUVs obtained from POPC by extrusion through polycarbonate filters with 200 nm pores. Sonicated crude DNA and APase, respectively, were entrapped in such liposomes as already described [11] and the liposomal preparation was subjected to extensive DNase I and proteinase K digestion in order to eliminate the non-entrapped macromolecules in the outside medium. After purifi-



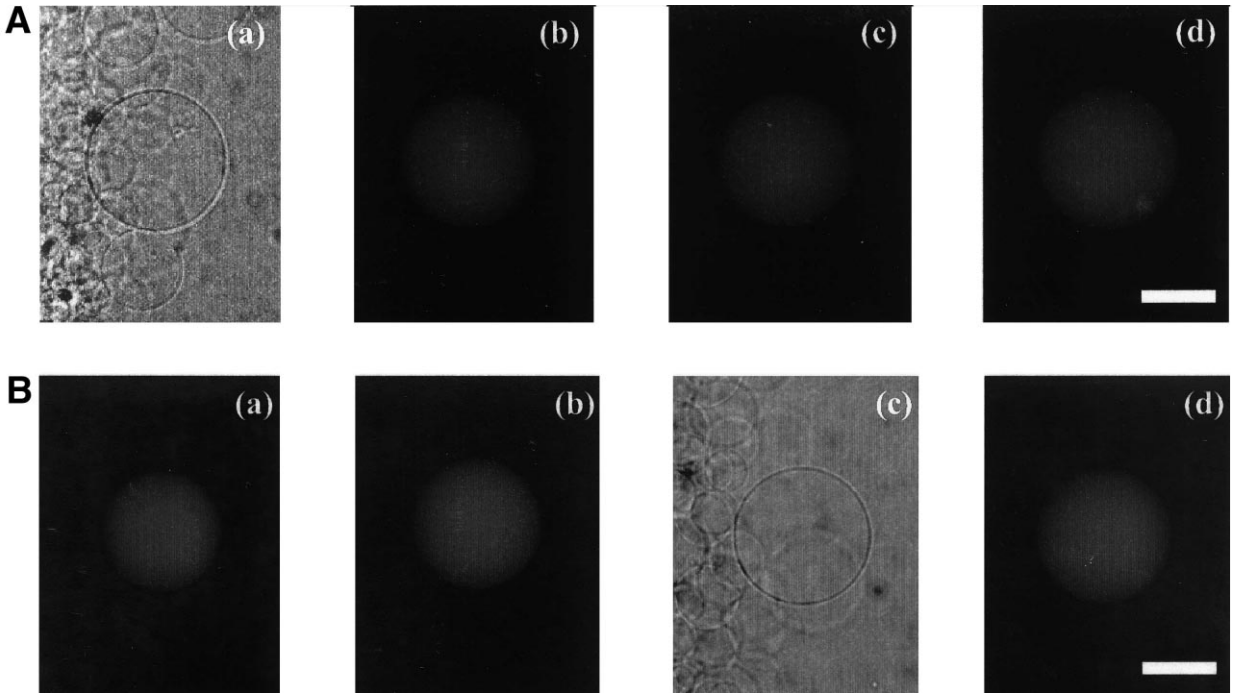


Fig. 3. Small nucleic acids and nucleotides do not leak out of a GV once microinjected into its water pool. A: GVs were formed in water. After injection of tRNA (20 mg ml^{-1}), YO-PRO-1 was added to the external medium. (a, b) 60 min, (c) 80 min, (d) 105 min after injection. (a) = DIC mode, (b–d) = fluorescence mode. Scale bar = 50 μm. B: GVs were formed in water and fluorescein-12-UTP (2.5 mM) was injected. (a) 20 min, (b) 95 min, (c) 115 min, (d) 140 min after injection. (a, b, d) = fluorescence mode, (c) = DIC mode. Scale bar = 50 μm.

cation of the LUVs containing the entrapped material by gel filtration chromatography, YO-PRO-1 or FDP were added externally and the suspensions were incubated for appropriate periods. Fluorescence analysis revealed that the LUV liposomal suspension did not become fluorescent, suggesting that in this case YO-PRO-1 and FDP, respectively, were not able to permeate significantly across the LUV membrane (see Fig. 2D,E).

One important question is whether these GVs formed by electroformation have physical holes or some other trivial defects. This was tested by a series of leakage experiments, including smaller and larger molecules. Obviously, YO-PRO-1 could enter such a GV, however, the larger DNA, crude DNA sonicated vigorously has an average length of about 1000 bp, did not escape from the GV. Similar experiments with smaller nucleic acids (MDV RNA or transfer RNA) resulted in the same outcome: the fluorescence intensity remained constant for the whole period of the experiment; there was no measurable leakage. Even fluorescent nucleotides remained entrapped inside the GVs once injected into their aqueous pool (see Fig. 3A,B). Therefore, the GVs formed in an a.c. electric field can be considered to have an intact bilayer.

3.2. The case of proteins

Having considered the permeability of YO-PRO-1 and FDP as small molecules, let us turn to the case of proteins. In this work, we will consider in particular enzymes that are able to digest nucleic acids. When these enzymes (DNases or RNases) are added to the fluorescent complex of nucleic acid/YO-PRO-1 in aqueous solution, the fluorescence rapidly disappears (data not shown). This is due to the digestion of the nucleic acid, which in turn indicates the existence of a prerequisite binding between enzyme and nucleic acid.

Having said that, let us consider the case of GVs. We first injected DNA into one selected GV, then we added YO-PRO-1 to the external aqueous medium and approximately 1 h later (when the DNA/YO-

PRO-1 fluorescence intensity was constant) DNase I was added to the bulk medium.

The surprising result is shown in Fig. 4A: one observes a significant decrease of the GV fluorescence, and actually 15 min after DNase I addition, the fluorescence intensity had decreased to about 70% of the initial value. After 40 min, a different fluorescent pattern is apparent: the fluorescence is concentrated on several spots localized on the surface of the vesicle, leaving most of the vesicle non-fluorescent. Typically, 3 h after the appearance of the fluorescent spots, 35% of the fluorescence of the spots was still detected (data not shown).

Similar results were obtained when DNase II instead of DNase I was used (data not shown). It should be noted that DNase I and DNase II are hydrophilic proteins and not membrane proteins, so that no significant hydrophobic interactions with the zwitterionic bilayer are expected. It is also important to note that DNase I and DNase II have different p*I* values ([17–19]), resulting in an overall negative charge of the former enzyme and an overall positive charge of the latter enzyme under the pH of the experiments (5.5–6.5). Or in other words: the appearance of the fluorescent pattern triggered by the interaction of the digestive enzymes with the membrane and their substrate seems to be independent on the net charge of the appropriate enzyme.

Similar experiments were carried out with a GV containing the fluorescent complex tRNA/YO-PRO-1 (or MDV RNA/YO-PRO-1). In these cases, RNase A was added externally, and a decrease in the fluorescence intensity was also detected (see Fig. 4B). RNase A has a p*I* of 9.16, so under the experimental conditions its net charge is positive [20]. Again a 'dotted' fluorescence pattern was observed after 80 min of incubation with externally added RNase A.

The results of these experiments were largely unexpected, and, therefore, it was necessary to perform a series of control experiments to rule out artifacts or trivial effects. First of all, we checked that the addition of relatively high amounts of non-specific protein to the external medium did not change the fluorescence intensity of the GVs. In fact, addition of proteinase K (Fig. 5A) or lysozyme (data not shown) did not change the fluorescence intensity, even if

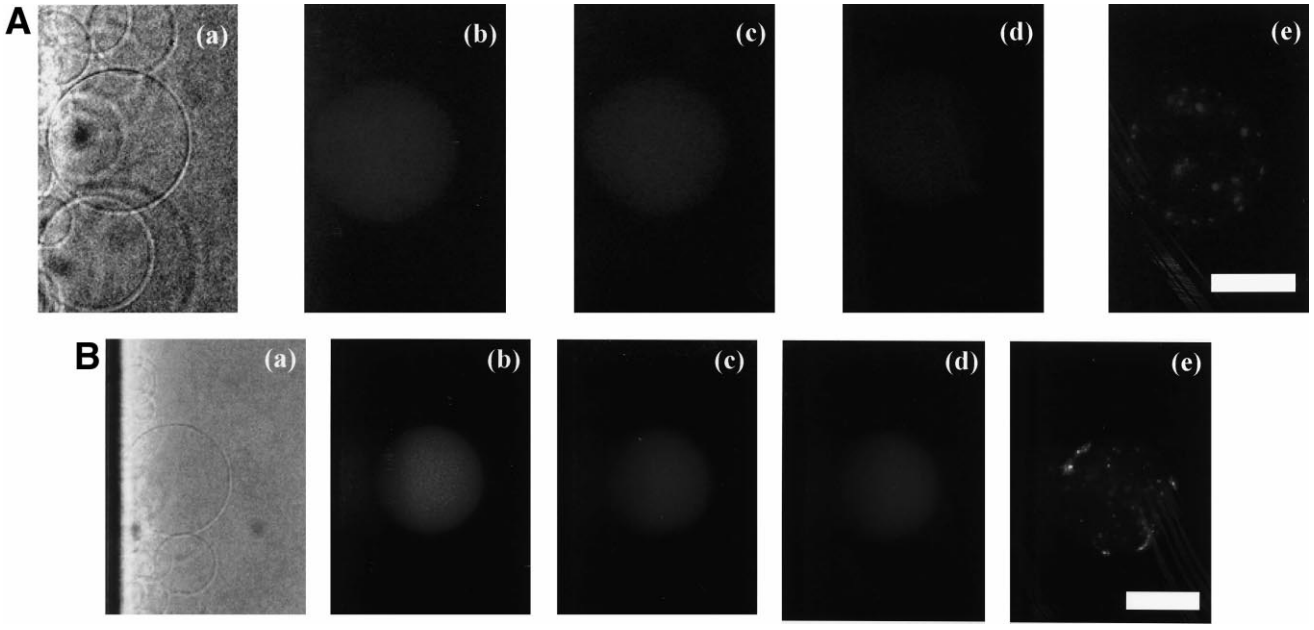


Fig. 4. DNase I and RNase A interact with the GV membrane and induce a decrease in the fluorescence of the nucleic acid/YO-PRO-1 complex. A: After injection of the plasmid DNA (78 μg ml^{-1}) and external addition of YO-PRO-1, DNase I was added externally to the vesicles (final concentration 200 μg ml^{-1}). (a, b) 16 min after DNA injection, (c) 1 min, (d) 14 min, (e) 41 min after DNase I addition. (a) = DIC mode, (b–e) = fluorescence mode. Scale bar = 50 μm. B: After injection of tRNA (20 mg ml^{-1}) and external addition of YO-PRO-1, RNase A was added externally to the vesicles (final concentration 70 μg ml^{-1}). (a, b) 1 min, (c) 16 min, (d) 33 min, (e) 79 min after RNase A addition. (a) = DIC mode, (b–e) = fluorescence mode. Scale bar = 50 μm.

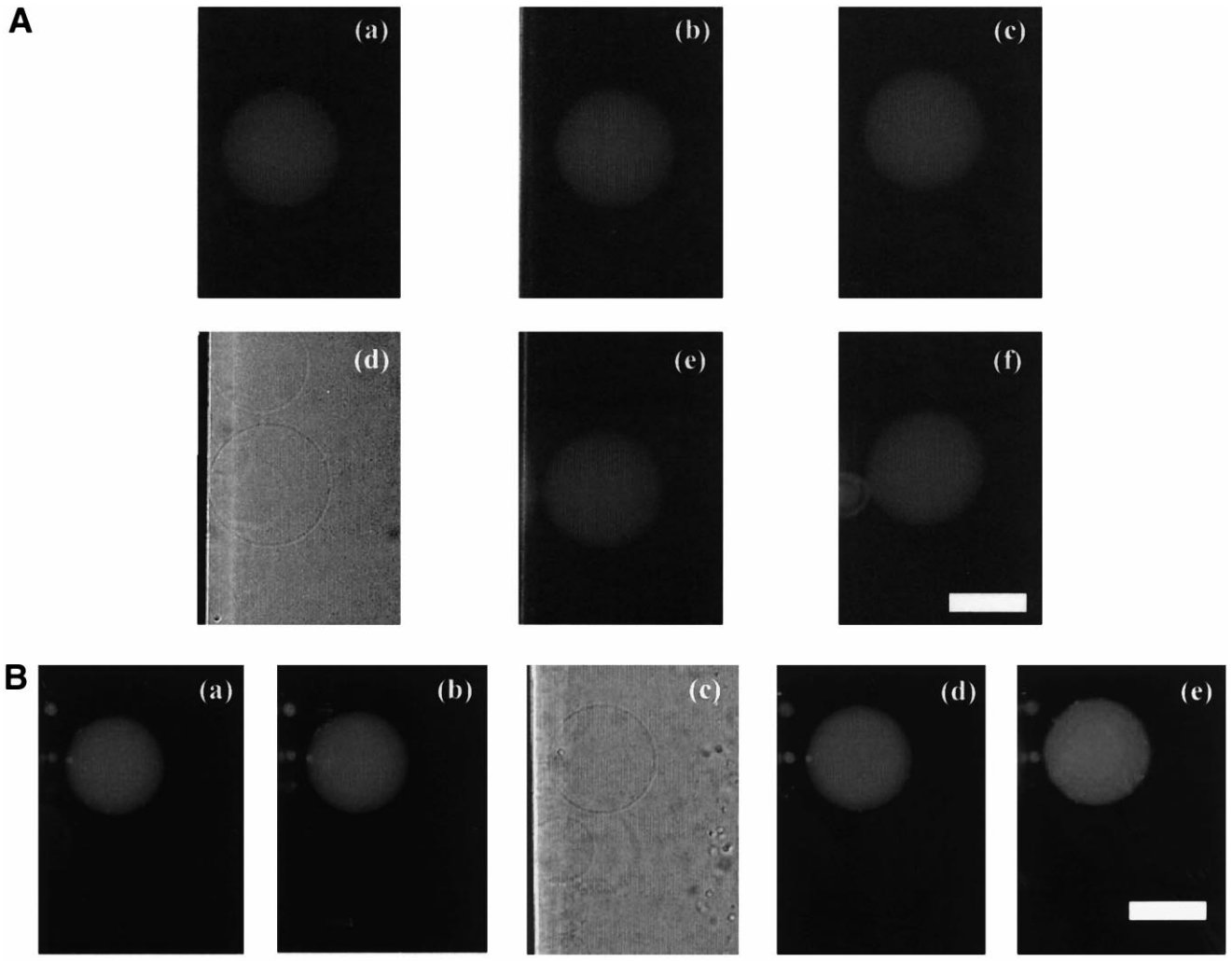


Fig. 5. Effect of non-specific enzymes on the entrapped nucleic acid. A: Proteinase K does not affect the stability of the DNA/YO-PRO-1 fluorescence intensity. After injection of DNA (78 μg ml^{-1}) and external addition of YO-PRO-1, proteinase K was added externally to the GVs. (a) 20 min after DNA injection, (b) 6 min after the first addition of proteinase K (final concentration 160 μg ml^{-1}), (c) 59 min after the first addition, (d, e) 15 min after the second addition of proteinase K (final concentration 320 μg ml^{-1}), (f) 51 min after the second addition. Total observation time: 2 h. (a–c, e, f) = fluorescence mode, (d) = DIC mode. Scale bar = 50 μm. B: DNase I does not affect the stability of the tRNA/YO-PRO-1 complex. DNase I (final concentration 200 μg ml^{-1}) was added externally to the vesicles containing injected tRNA. (a) Before DNase I addition, (b) 19 min, (c, d) 33 min, (e) 46 min after DNase I addition. (a, b, d, e) = fluorescence mode, (c) = DIC mode. Scale bar = 50 μm.

some proteins, such as lysozyme, led to structural changes of the POPC bilayers. A second type of control experiment was based upon the enzyme specificity. Addition of DNase I to the outside of a GV containing tRNA/YO-PRO-1 (Fig. 5B) had no effect in the sense that no decrease of the fluorescence intensity was observed. More importantly, the tRNA/YO-PRO-1 fluorescence remained homogeneous over 3 h (the same results were also obtained with DNase II instead of DNase I, data not shown). In other words, the fluorescence pattern as described in Fig. 4A was never observed in the case of RNA/YO-PRO-1 with DNases outside. This is a crucial experiment because it demonstrates that the alteration of the fluorescence intensity and the formation of the described fluorescence pattern illustrated in Fig. 4 is a consequence of the specific interaction of the DNase with its substrate DNA and does not occur when RNA (tRNA or MDV RNA) is used instead of DNA. Likewise, addition of RNase A to a GV con-

taining the fluorescent complex DNA/YO-PRO-1 did not cause any decrease in the fluorescence intensity and did not lead to a similar fluorescent pattern as in the case of RNA/YO-PRO-1 and RNase A outside (data not shown).

These cross-experiments with RNase and DNase also rule out an alternative, hypothetical mechanism: that the binding of the enzyme to the bilayer of the GV induces membrane distortions that result in the leakage of the entrapped nucleic acid. Loss of fluorescence in this case would be due to the dilution of nucleic acids in the external medium. This is clearly not so, as the DNA fluorescence does not disappear when RNase A is added instead of DNase, and vice versa.

Again, we asked the question whether the observed phenomenology was characteristic of GVs, or whether it can also be found with conventional liposomes. The lack of a significant interaction between the enzymes and the POPC bilayer could be shown in a control experiment carried out using LUVs obtained by extrusion through filters with 200 nm pores. LUVs containing the fluorescent complex YO-PRO-1/DNA were first isolated by gel filtration chromatography and then subjected to an extensive incubation with DNase II. As shown in Fig. 6, the fluorescence level of the LUV suspension remained constant for several hours, indicating that the enzyme was not able to digest the internalized DNA/YO-PRO-1 complex. This indicates again that GVs differ from the common LUVs or SUVs.

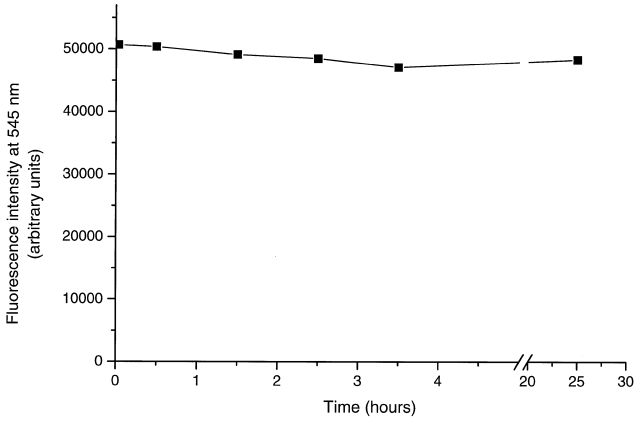


Fig. 6. DNase II is not permeable to LUV membranes. DNase II (final concentration 10 mg ml^{-1}) was added to purified LUVs containing entrapped crude DNA and YO-PRO-1 and the fluorescence intensity was recorded as a function of time.

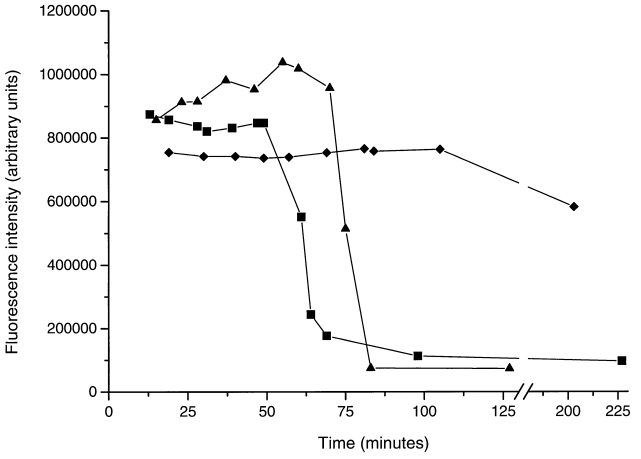


Fig. 7. Effect of cholesterol on the DNase I fluorescence quenching. After injection of DNA (78 μg ml^{-1}) into the selected GV and addition of YO-PRO-1, DNase I was added externally to the vesicles (final concentration 200 μg ml^{-1}) and the fluorescence intensity was recorded as a function of time. Squares = pure POPC; triangles = POPC/cholesterol 98:2 mol%; diamonds = POPC/cholesterol 90:10 mol%.

3.3. The effect of cholesterol

The fluorescence quenching experiments described above suggest a close interaction between the internalized nucleic acid molecules and the enzymes added externally. If this effect is not due to a direct permeability, and not either to trivial defects in the membrane, and because it is not present in LUVs, it must be due to some peculiar properties of the GVs. One such property could be the higher fluidity of the lipid molecules in the GV bilayer in contrast to those in LUVs.

It is well known that cholesterol has the capability to increase the stiffness of the bilayer membrane and, correspondingly, to decrease the kinetics of solute permeation across the bilayer [21,22]. Recognizing this, we had the idea of repeating the experiments with DNase I with GVs obtained from POPC/cholesterol 98:2 and 90:10 mol%. As shown in Fig. 7, the presence of cholesterol remarkably affected the time process of the fluorescence quenching. In the presence of 10% cholesterol, the reaction velocity was decreased by an estimated factor of 3–4. Actually, the effect was also appreciable with 2% cholesterol. The conclusion is that the described protein effect on the GV membrane can be inhibited by increasing the stiffness of the membrane. Again, these results con-

firm the hypothesis that the observed fluorescence quenching is not due to trivial membrane defects.

3.4. Detachment of the GVs formed by electroformation from the electrode and measurement of YO-PRO-1 permeability

To rule out other artifacts in connection with the electroformation method, we also detached the GVs (formed by the electroformation method in the presence of nucleic acid) from the platinum electrode and transferred them into another solution containing the dye YO-PRO-1. As it can easily be seen (see Fig. 8), YO-PRO-1 was also able to permeate across the GV bilayer thus demonstrating that the described permeability phenomena are also possible with detached GVs and do not have to take place in the proximity of the platinum electrode.

4. Discussion

First, it is important to summarize again all observations that permit to rule out that holes in the membrane or trivial effects may be responsible for the observed permeability phenomena.

(i) The described effect is specific for some low molecular weight compounds (YO-PRO-1 and FDP) and does not take place with nucleotides (see Fig. 3) or Ca^{2+} ions (data not shown). (ii) In the case of enzymes, the effect is seen only using a specific substrate/enzyme combination (DNase/DNA, RNase/RNA, and not with DNase/RNA). (iii) Cholesterol when mixed to POPC inhibits GV membrane permeability. (iv) These permeability effects as demonstrated in the case of YO-PRO-1 are also observed when GVs obtained by electroformation are brought into a new environment.

All this seems to indicate that effects observed with GVs are real permeability phenomena and/or surface effects between membrane and protein leading to permeability, although it is not clear why this should be so. We also have to consider that the enzymes are not membrane proteins, so that there should be no strong hydrophobic interactions with the POPC bilayer. Moreover, also the electrostatic interactions should not be significant, since the two DNases used in our experiments are oppositely charged under our experimental conditions.

Another important consideration is that these bilayer/enzyme effects are not detectable with conventional vesicles. As a consequence, the observed effects must be due to some specific properties of the GVs. The chemical structure of the LUVs and GVs is the same (POPC, zwitterionic molecule). Based on this consideration, one may argue that the different behavior of GVs with respect to LUVs lies in a somewhat different physical structure of the GV membrane, more notably in the much larger curvature radius. We cannot exclude at this point that these effects are typical of GVs obtained by electroformation, although it appears difficult to rationalize this further, once it is clear that holes and trivial defects can be ruled out.

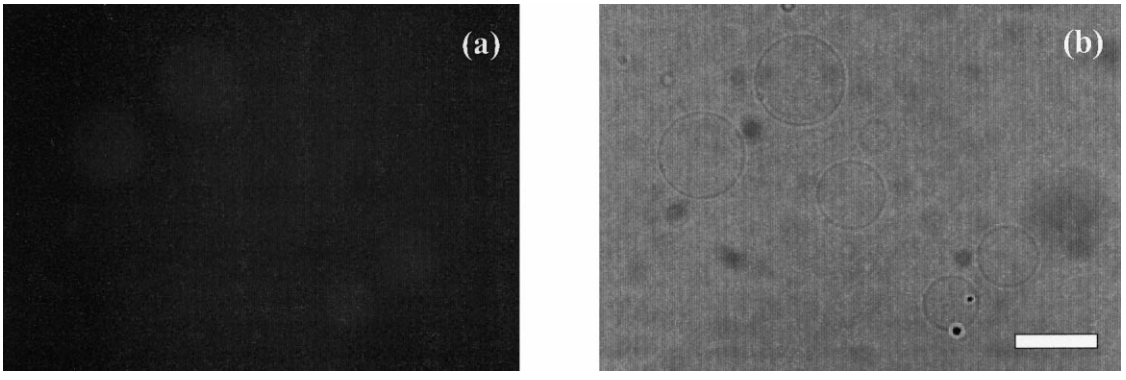


Fig. 8. YO-PRO-1 permeates across GV bilayers that were transferred into a new medium. The vesicles were formed by electroformation in an aqueous solution containing tRNA (4 μg ml^{-1}) and 5 mM sucrose. Then they were sucked into a large pipette and transferred into a medium containing 2.5 mM glucose and 5 μM YO-PRO-1. (a, b) 1 h after transferring the GVs into the medium containing YO-PRO-1. (a) = DIC mode, (b) = fluorescence mode. Scale bar = 50 μm.

In this regard, the key question to consider, also in view of the simple YO-PRO-1 experiments, is the possible structural difference between LUVs and GVs. This question cannot be answered satisfactorily until now due to the lack of knowledge of the structural details of GVs. On the basis of a few sparse observations, it seems however reasonable to assume that, whereas the LUVs have a compact structure, GVs tend to have undulated surfaces with bulges and valleys creating a dynamic behavior, especially when kept under unsealed conditions [23]. In other words, whereas the relatively small radius of LUVs gives rise to relatively stable rigid spherical aggregates, a radius which is 2–3 orders of magnitude larger can lead to a less compact, more fluid and more dynamic structure. This situation may permit the formation of a complex between chemical partners that are initially separated by the bilayer. And again we have to make reference to the fact that what we observe might be specific for GVs obtained from electroformation, and not necessarily for all kinds of GVs.

Clearly, more detailed studies are necessary in order to tackle and answer all these questions. This is particular important in view of the possibility that GVs are a better model than conventional small liposomes for biological membranes.

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