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Giant phospholipid vesicles: comparison among the whole lipid sample characteristics using different preparation methods
A two photon fluorescence microscopy study

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Abstract

Several methods for the preparation of giant unilamellar vesicles (GUVs) using synthetic phosphatidylcholine phospholipids were evaluated. We compared the physical characteristics — in terms of lamellarity and morphology — of the whole lipid sample for each different lipid preparation using the sectioning capability of the two-photon excitation fluorescence microscope. From the evaluation of the entire lipid sample we determined that vesicle size, internal shape and shell thickness distributions depend on the vesicle's preparation method. Our results show that the preparation of giant unilamellar vesicles by the application of external electric fields offers several advantages among the other methods tested here. Using this method a high yield ($\sim 95\%$) of giant unilamellar vesicles with a narrow size distribution was obtained. Independently of the preparation method, some lipid structures, which are held together by lipid tethers, were identified and resolved. These particular lipid structures show shell thickness and size heterogeneity. Labeling the lipid samples with 6-lauroyl-2-(*N*,*N*-dimethylamino)naphtalene (LAURDAN) and using the LAURDAN generalized polarization function we show that the lipid packing in these tethers or tubes is similar to those found in the phospholipid vesicles. The fact that both vesicles and tethers are found in the lipid preparations indicates similar stability between these structures.

Keywords: Giant unilamellar vesicles; 6-Lauroyl-2-(*N*,*N*-dimethylamino)naphtalene; Generalized polarization; Unilamellar

Abbreviations: DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimiristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; GP, generalized polarization; GUVs, giant unilamellar vesicles; POPC, 1-palmitoyl 2-oleoyl-*sn*-glycero-3-phosphocholine.

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1. Introduction

Giant unilamellar vesicles (GUVs) are becoming objects of intense scrutiny in diverse areas that focus on membrane behavior (Menger and Keiper, 1998). Due to their size, which is on the same order as the size of cells, single vesicles can be directly observed under the light microscope. The mechanical properties of model membranes have been intensely studied using GUVs (Evans and Kwok, 1982; Needham et al., 1988; Needham and Evans, 1988; Sackmann, 1994; Mathivet et al., 1996; Meléard et al., 1997, 1998, for reviews see Menger and Keiper, 1998). These studies revealed the physical properties of the membranes through the calculation of elementary deformation parameters. A similar study using the same temperature range and experimental approach was reported for GUVs composed of mixtures of phosphatidylcholine phospholipids and cholesterol (Needham et al., 1988). In addition, GUVs were suggested as an ideal system for transport studies and drug delivery (Lassic and Papahadjopoulos, 1995; Moscho et al., 1996; Menger and Keiper, 1998).

GUVs have diameters between 5 and 200 μm (Menger and Keiper, 1998). Different methods are described in the literature to obtain these vesicles. One of the methods to form GUVs is based on the exposure of dried lipid films to aqueous solution for a long period of time (up to 36 h) at temperatures above the lipid phase transition (called 'the gentle hydration method' in our manuscript); (Reeves and Dowben, 1969; Needham and Evans, 1988; Käs and Sackmann, 1991; Akashi et al., 1996; Yang and Glaser, 1996). In this case, giant vesicles with a mean diameter of approximately 15 μm can be obtained. A technique based on organic solvent evaporation in aqueous solution was presented by Moscho et al. (1996) to prepare GUVs (called 'solvent evaporation method' in our manuscript). One of the advantages of this method is the short time used to obtain the vesicles (few minutes). In this case vesicles up to 50 μm in diameter were obtained (Moscho et al., 1996). Another technique for GUVs preparation, introduced by Angelova and Dimitrov in 1986, consist of hydration of the dried lipid films above to the lipid phase transition in presence of electric fields (Angelova and Dimitrov, 1986; Dimitrov and Angelova, 1987, 1988; Angelova et al., 1992; Mathivet et al., 1996). In the later case, a homogeneous population of GUVs, between 30 and 60 μm in diameter, can be obtained (Angelova et al., 1992; Mathivet et al., 1996).

The experimental conditions in the sample preparation, such as ionic strength, pH, lipid composition, substrate on which to dry the lipid film, addition of some sugars, seem to be critical parameters to obtain the giant vesicles (Reeves and Dowben, 1969; Angelova and Dimitrov, 1988; Needham and Evans, 1988; Käs and Sackmann, 1991; Moscho et al., 1996; Akashi et al., 1996). Nevertheless, there is no general agreement about 'unique' conditions to obtain such vesicles. For instance, very low ionic strength (below 10 mM) in the aqueous solution is required to successfully prepare the giant vesicles (Reeves and Dowben, 1969; Needham and Evans, 1988; Angelova and Dimitrov, 1988; Käs and Sackmann, 1991). Alternatively, as reported by Akashi et al., physiological conditions can be used to obtain the giant lipid structures using a percentage of charged lipids in the sample (Akashi et al., 1996). As a general rule two important conditions are required to prepare GUVs: (i) the temperature during the vesicle preparation must be higher than the phase transition temperature of the lipids used to form the GUVs (this also operates in the formation of multilamellar, small unilamellar and large unilamellar vesicles) and (ii) to form the big structures agitation of the samples during the vesicle formation must be prevented.

The first process in vesicle formation is the hydration of a dried lipid film. Models were proposed for the vesicle formation that emphasize some possible mechanisms after the multilamellar vesicles (MLVs) are obtained (by agitation of hydrated lipid films under excess of water), (Lassic, 1988; Winterhalter and Lassic, 1993). In particular the effect of detergents (detergent depletion method), extrusion or sonication of MLVs to obtain small or large unilamellar vesicles (SUVs and LUVs, respectively) are considered in these models (Lassic, 1988; Winterhalter and Lassic,

1993). In spite of the vast quantity of studies about lipids and liposome formation, the mechanism of GUVs formation has received little attention. Actually, the exact mechanism of GUVs formation is not known. Dimitrov and Angelova (1987) presented some considerations about the GUVs formation (Angelova and Dimitrov, 1988). In particular, these authors pointed out that liposome formation requires layer separation and bending. In the early stage of the vesicle formation the normal forces, that cause repulsion between lipid layers, and the tangential forces, that bend the lipid layers, play a crucial role. Dimitrov and Angelova (1987) concluded that the effect of external electric fields is particularly strong on the normal forces and that electric fields can induce or prevent liposome formation (Angelova and Dimitrov, 1988). Electroosmotic effects are important when AC fields are applied. The mechanical stress induced by the electric field on the bilayer (visualized as a gently agitation of the lipid layers by the alternate low frequency field) can generate rupture of the lipid bilayers and formation of pieces of different sizes. These pieces can bend and form the thin-walled liposomes (Dimitrov and Angelova, 1987).

In this article we point out the similarities and the differences in the overall sample characteristics (such as lamellarity of the vesicles, internal structure, lipid packing properties) among the different preparation methods tested. We use the sectioning capability of the two-photon fluorescence microscope that allows us to resolve and characterize the morphology of large vesicles. In general, all the studies already made on such samples focused only on the GUVs, and did not consider the rest of the sample. The analysis of the entire sample characteristics improves the comparison among different preparation techniques.

2. Material and methods

2.1. Materials

LAURDAN was purchased from Molecular Probes Inc. (Eugene, OR), DPPC, DLPC, POPC and DMPC were supplied by Avanti Polar Lipids (Alabaster, AL). Agarose (Type VII: Low gelling temperature) was purchased from Sigma Chemical Co. (St. Louis MO).

2.2. Methods

2.2.1. Lipid vesicle preparation

2.2.1.1. Gentle hydration method. Stock solutions of LAURDAN and phospholipids were made in chloroform. To prepare the lipid films using the gentle hydration method we follow either the method described by Needham and Evans (1988)or that described by Yang and Glaser (1996). The most important difference between these two methods is the substrate material on which the lipid film is spread (glass or roughened teflon, respectively). Lipid-probe mixtures were made with a lipid:probe ratio of 700:1. The dry lipid:probe mixtures were hydrated in pure Millipore water (17.5 MΩ/cm) above the phospholipid transition temperature (T_m). The T_m for the different lipids are -1.8°C for DLPC, 11°C for POPC, 24.5°C for DMPC and 41.5°C for DPPC. In the case of DLPC and POPC we incubated the samples at 30°C while for DMPC and DPPC we incubated the samples at 36 and 50°C, respectively. A characteristic cloud was observed in these samples after several hours of incubation (12–36 h) as previously described (Needham and Evans, 1988).

2.2.1.2. Solvent evaporation method. To prepare the GUVs, we also used the solvent evaporation method proposed by Moscho et al. (1996). In this method we labeled the lipid sample with LAURDAN after the vesicle preparation, as previously described in cells Parasassi et al. (1993) or premixing the LAURDAN with the lipids (lipid:probe ratio 700:1) mixture in chloroform. We gently added Millipore water to the organic lipid solution and the sample was located in a rotary evaporator (R-124, Büchi In) under reduced pressure as described by Moscho et al. (1996). We prepared the vesicles above the phospholipid T_m using the same temperatures above mentioned for the gentle hydration method. All

samples were prepared and stored in the dark during incubation. Also using this preparation technique, an opalescence solution was observed after solvent evaporation as described previously (Moscho et al., 1996).

2.2.1.3. Electroformation method. To grow the GUVs using the electroformation method (Angelova and Dimitrov, 1986; Dimitrov and Angelova, 1987; Angelova et al., 1992) a special temperature controlled chamber previously described was used (Bagatolli and Gratton, 1999). The following steps were used to prepare the GUVs: (i) 3 μl of the lipid stocks solution (0.2 mg/ml) were spread on each Pt wire under a stream of N_2. To remove the residues of organic solvent the samples were lyophilized for about 2 h; (ii) To add the aqueous solvent inside the chamber (Millipore water 17.5 MΩ/cm), the bottom part of the chamber was sealed with a coverslip. The Millipore water was previously heated at the desired temperature (50°C for DPPC and 30°C for DLPC and POPC) and then sufficient water was added to cover the Pt wires. Just after this step the Pt wires were connected to a function generator (Hewlett-Packard, Santa Clara, CA), and a low frequency AC field (sinusoidal wave function with a frequency of 10 Hz and amplitude of 3 V) was applied for 90 min. After the vesicle formation, the AC field was turned off and the temperature of the chamber was change to the desired temperature for the experiments. A CCD color video camera (CCD-Iris, Sony) on the microscope was used to follow vesicle formation and to select the target vesicle. The temperature was measured inside the sample chamber using a digital thermocouple (model 400B, Omega Inc., Stamford, CT) with a precision of 0.1°C. The LAURDAN labeling procedure was done in one of two ways. Either the fluorescent probe was premixed with the lipids in chloroform or a small amount (less than 1 μl) of LAURDAN in DMSO was added after the vesicle formation (final LAURDAN/lipid ratio 1:500 mol/mol in both cases). The sample characteristics were independent of the labeling procedure in agreement with previous results (Bagatolli and Gratton, 1999).

2.3. Two-photon microscopy measurements

2.3.1. Preparation of samples

Two different preparations were used to observe the lipid samples, obtained either by the gentle hydration method or solvent evaporation method, under the microscope: (1) a drop of the lipid suspension was deposited on a welled glass slide. Then, a coverslip was mounted on the welled glass slide and the specimen was ready to be observed under the microscope; (2) a drop of the lipid sample with a drop of agarose solution in pure Millipore water (0.5% P/V) was deposited on a flat glass slide. Then, a coverslip was mounted on the microscope slide and the specimen was ready to be observed under the microscope. In the case of GUVs prepared by the electroformation method, the experiments were carried out in the above-mentioned chamber after the vesicle formation as we reported previously (Bagatolli and Gratton, 1999). After vesicle formation and when the electric field is turned off, we observed that the GUVs remains tightly adsorbed to the lipid film that covers the Pt wires. Therefore, we obtained the fluorescent images in the same GUV's formation chamber using the Pt wires as a 'holder'. There are several advantages in using this approach, such as no extra-step on sample manipulation and the possibility to follow a single vesicle during long periods of time (for example to evaluate the effect of the temperature changes on single GUVs; Bagatolli and Gratton, 1999). To visualize the samples we used an inverted microscope (Axiovert 35, Zeiss, Thornwood, NY).

2.3.1.1. GP function. LAURDAN's emission spectrum is blue in the lipid gel phase while in the liquid crystalline phase it moves during the excited state lifetime from the blue to the green (Parasassi et al., 1990, 1991). To quantify the emission spectral changes, the excitation generalized polarization (GP) function was defined analogously to the fluorescence polarization function. This well characterized function is sensitive to the phase-state of lipid aggregates (for reviews see Parasassi and Gratton, 1995; Parasassi et al., 1998; Bagatolli et al., 1999).

2.3.1.2. Experimental apparatus for two-photon excitation microscopy measurements. Two-photon excitation is a nonlinear process in which a fluorophore absorbs two photons simultaneously. Each photon provides half the energy required for excitation. The high photon densities required for two-photon absorption are achieved by focusing a high peak-power laser light source on a diffraction-limited spot through a high numerical aperture objective. Therefore, in the areas above and below the focal plane, two-photon absorption does not occur, because of insufficient photon flux. This phenomenon allows sectioning effect without using emission pinholes like in confocal microscopy. Another advantage of two-photon excitation is the low extent of photobleaching and photodamage above and below the focal plane. For our experiments we used a scanning two-photon fluorescence microscope developed in our laboratory (So et al., 1995, 1996). For the LAURDAN GP measurements we used a procedure previously described (Yu et al., 1996; Parasassi et al., 1997; Bagatolli and Gratton, 1999). We used either a LD-Achroplan 20X long working distance air objective (Zeiss, Homldale, NJ) with a N.A. of 0.4 or a FLUAR 40X oil immersion objective (Zeiss, Homldale, NJ) with a N.A of 1.3. A titanium-sapphire laser (Mira 900, Coherent, Palo Alto, CA) pumped by a frequency-doubled Nd:Vanadate laser (Verdi, Coherent, Palo Alto, CA) was used as the excitation light source. The excitation wavelength was set to 780 nm. The laser was guided by a galvanometer-driven x–y scanner (Cambridge Technology Watertown, MA) to achieve beam scanning in both the x and y directions. The scanning rate was controlled by the input signal from a frequency synthesizer (Hewlett-Packard, Santa Clara, CA) and a frame rate of 25 s was used to acquire the images (256×256 pixels). The laser power was attenuated before the light entered the microscope through a polarizer (40 mW for the $20\times$ objective and 15 mW for the $40\times$ objective). The samples received about one-tenth of the incident power. The fluorescence emission intensity for the LAURDAN GP calculations were obtained using two optical band-pass filters (Ealing electro-optics, New Englander Industrial Park, Holliston, MA) with 46 nm width and centered at 446 and at 499 nm (in the blue and green regions of LAURDAN emission spectrum, respectively). A miniature photomultiplier (R5600-P, Hamamatsu, Brigdewater, NJ) was used for light detection in the photon counting mode. A home-built card in a personal computer acquired the counts. The diameters of the vesicles were measured by using size-calibrated fluorescent spheres (latex FluoSpheres™, polystyrene, blue fluorescent 360/415, diameter 15.5 μm, Molecular Probes Inc., Eugene, OR).

2.4. Transmission electron microscopy measurements

The procedure we used to prepare the sample for transmission electron microscopy was as follows: a drop of the sample was placed on Formvar carbon-coated 200 mesh copper grids and allowed to adhere for 1 min. Then the excess drop was removed from the grid with the edge of a piece of filter paper. The grid was allowed to dry before a drop of a 1% PTA (phosphotungstic acid) solution, pH 6.8, was applied to the grid. After 1 min the excess drop was removed with a piece of filter paper and the grid was allowed to dry for several minutes before being viewed in the Hitachi H-600 transmission electron microscope. The sample was observed at 75 kV.

3. Results

3.1. Giant vesicles obtained either by the gentle hydration or solvent evaporation methods

Independently of the method used to prepare the phospholipid vesicles, the lipid samples show high heterogeneity. This heterogeneity is related to the size, shape, shell thickness and internal structure of the different lipid vesicles (Fig. 1). To check the lamellarity of the giant vesicles, we imaged several vesicles of the same lipid preparation (up to 40 vesicles) labeled with LAURDAN using the two-photon excitation microscope. We found that the intensities measured in the border of different vesicles in the liquid crystalline phase

were different independently of the phospholipid composition. Since the existence of multilamelar vesicles would give rise to different intensity images due to the presence of different numbers of LAURDAN labeled lipid bilayers, we concluded that no more than 10% of the vesicles were unilamellar. Typical examples of our observations are shown in Fig. 1A,B. The LAURDAN intensity in the vesicle border in Fig. 1A (that represent a unilamellar vesicle) is four times lower than that found in Fig. 1B. The most common lipid vesicle structure found in the overall sample corresponds to the images show in Fig. 1C–F. However, the peculiar vesicle structure shown in Fig. 1G–I is also often seen. In particular the vesicle structures shown in these last three figures appear to be closed, flattened vesicle structures inside a larger, rounder vesicle. Also the vesicles in Fig. 1G,H show a budding process and fluctuation in the borders. The complex internal structure of the phospholipid vesicles shows location with time-dependent spatial changes but without visible changes of the external appearance (Fig. 2). This internal dynamics affects the large-scale structure of the vesicle and it is not to be confused with the single lipid dynamics as reported by Korlach et al. (1999).

Another frequent structure found in the lipid samples corresponds to lipid tubes or tethers (Fig. 3A). These particular lipid structures connect the vesicles to form a lipid network. The formation of these lipid structures is independent of the phospholipid chain length. To explore the lipid physical state in these different structures (lipid tubes and vesicles) we used the LAURDAN GP function. The LAURDAN GP histogram measured in these different structures are similar, Fig. 3B. In Fig. 4 we present a 3D LAURDAN intensity

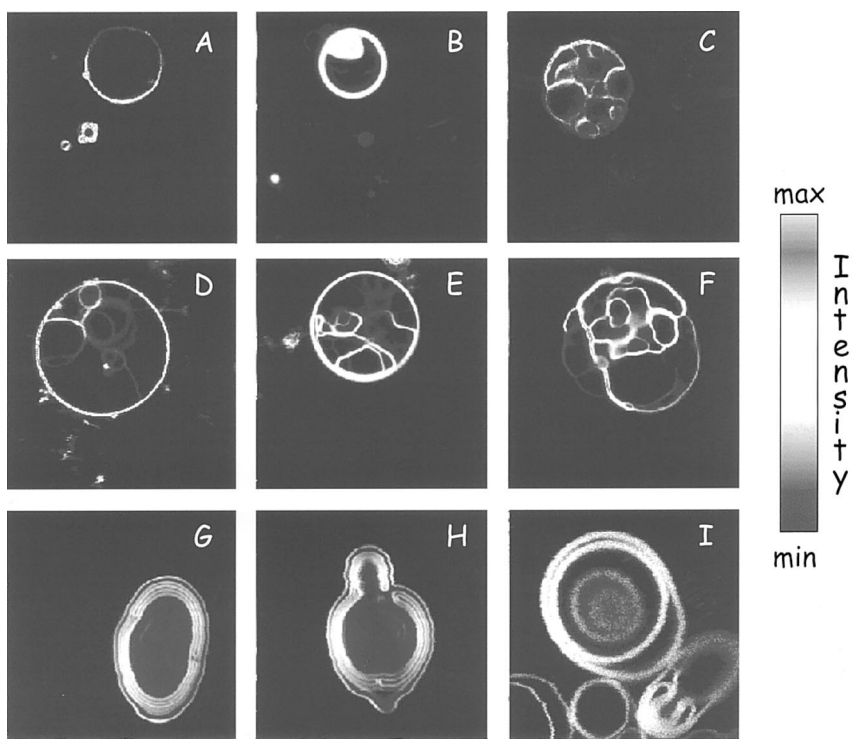

Fig. 1. Two-photon excitation LAURDAN fluorescence intensity images (false color representation) showing the heterogeneity in size, shell thickness and internal structure of giant DLPC (A, B, C, D, E, F) and POPC vesicles (G, H, I). The vesicles were made by hydration of lipid films (A, B, C) or solvent evaporation method (D, E, F, G, H, I). The temperature was 22°C. Frame size is 32 × 32 μm

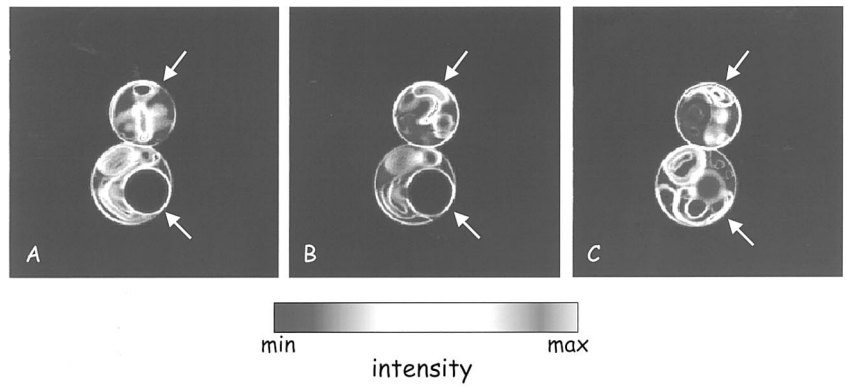

Fig. 2. Two-photon excitation LAURDAN fluorescence intensity images (false color representation) showing the movement of the internal structure of giant DLPC vesicles. Time zero image (A), after 2 (B) and 5 min (C). The temperature was 22°C. Frame size is 32×32 μm.

reconstruction of these tubes. The same heterogeneity of size and lamellarity found in the vesicles is applicable to the lipid tubes or tethers, i.e. unilamellar or multilamellar lipid tubes.

Using transmission electron microscopy we obtain the same sample heterogeneity found by two-photon microscopy (Fig. 5). Similar structures were found using both techniques visualizing the lipid tubes or tethers together with the complex internal structure of the lipid vesicles (compare Fig. 1C,F with 5A,B and 3A). The size range of the lipid vesicles, found by transmission electron microscopy, was very broad. We found small multilamellar vesicles (~ 300 nm diameter, Fig. 5C) together with much bigger ones (up to 10 μm, not shown).

We also examined the time evolution of the sample during the lipid film hydration process. In Fig. 6 we show different sequences during the hydration of DLPC lipid films, using the 3D LAURDAN intensity reconstruction.

3.2. Giant vesicles obtained by the electroformation method

Fig. 7 shows the LAURDAN intensity images of giant vesicles obtained by the electroformation method. For each lipid sample, the LAURDAN fluorescence intensities measured in the border of different vesicles in the liquid crystalline phase were very similar (Fig. 7). As pointed out above, the existence of multilamellar vesicles would give rise to different intensity images due to the presence of different numbers of LAURDAN labeled lipid bilayers. Therefore we concluded that the vesicles are mainly unilamellar (the yield of unilamellar vesicles is higher than 95%) in agreement with previous observations using the electroformation method (Mathivet et al., 1996).

Compared to other techniques, the lipid vesicles obtained using the electroformation method present a more homogeneous distribution in size (Fig. 7). The presence of vesicle internal structure is not observed in this preparation. Just in few cases small vesicles inside the big ones were observed (not shown). Interestingly enough, we also observed some tethers or tubes in this preparation (Fig. 7C,D). However the occurrence of these structures is rare when compared with the other preparation methods.

4. Discussion

As shown in the result section there are differences in vesicle size, internal shape and shell thickness depending of the preparation procedure used. The observation of the entire lipid sample provides important experimental evidences about the effectiveness of each method to prepare the GUVs. Also, more importantly, the comparison among all lipid samples provides important infor-

mation about the mechanism of vesicle formation. As a first conclusion, the samples prepared by the gently hydration or solvent evaporation methods have low yields of unilamellar vesicles with a more heterogeneous size distribution compared with that obtained using the electroformation method. The main disadvantage produced by the low yield of GUVs in these lipid samples is that a considerable amount of time must be used to find the unilamellar vesicles under the microscope. Instead, the preparation and observation of the GUVs, obtained by the electroformation method, in the same chamber without any external manipulation of the sample (Bagatolli and Gratton,

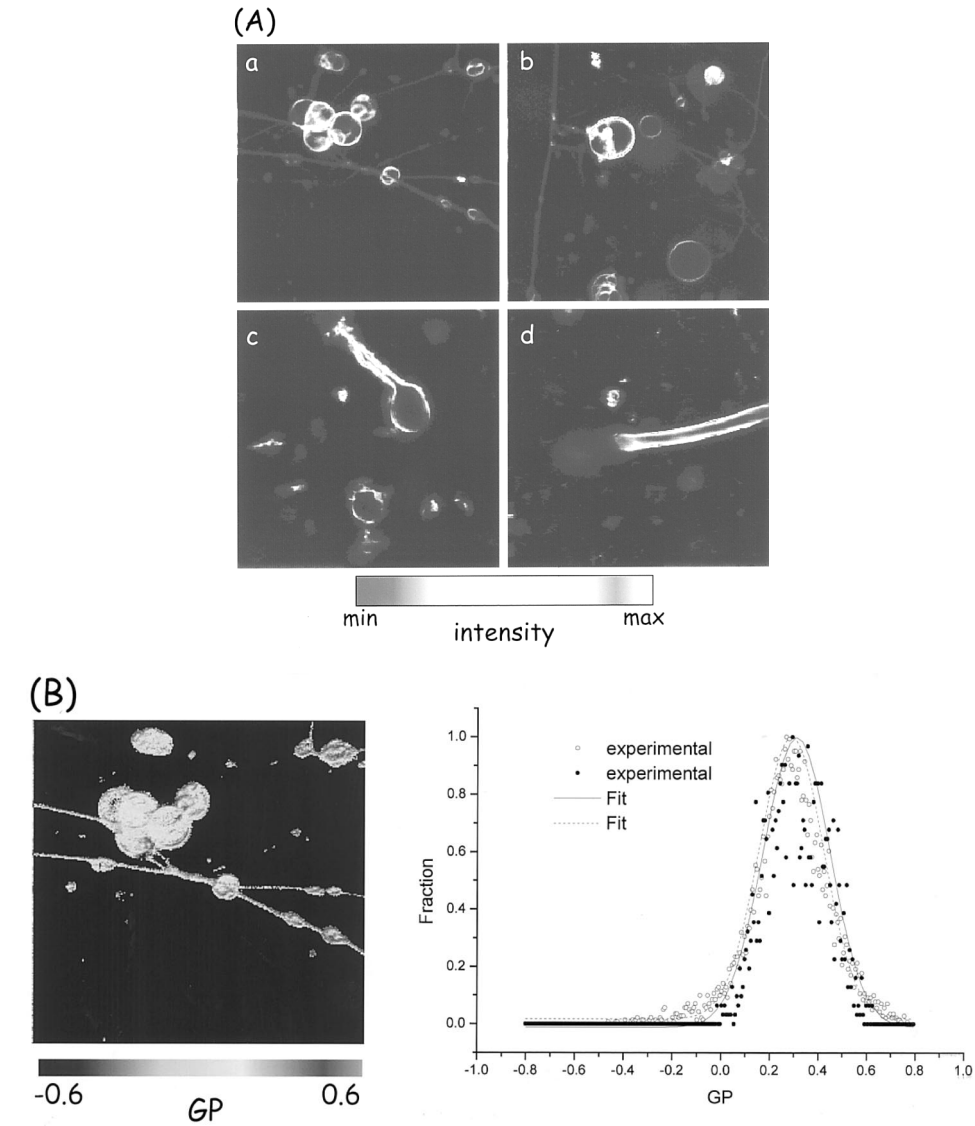

Fig. 3. (A) Two-photon excitation LAURDAN fluorescence intensity images (false color representation) showing the network of DMPC tethers connecting vesicles (A, C) and DPPC tubes (B, D). These particular structures were found independently of the lipid sample composition. (B) Two-photon excitation LAURDAN GP image (false color representation) of DMPC lipid aggregates. The graphic in the right part of the figure shows the normalized GP histogram from different regions of the GP image. Red line and black line corresponds to lipid vesicles and lipid tubes or tethers respectively. Frame size is 32×32 μm. The temperature was 25°C.

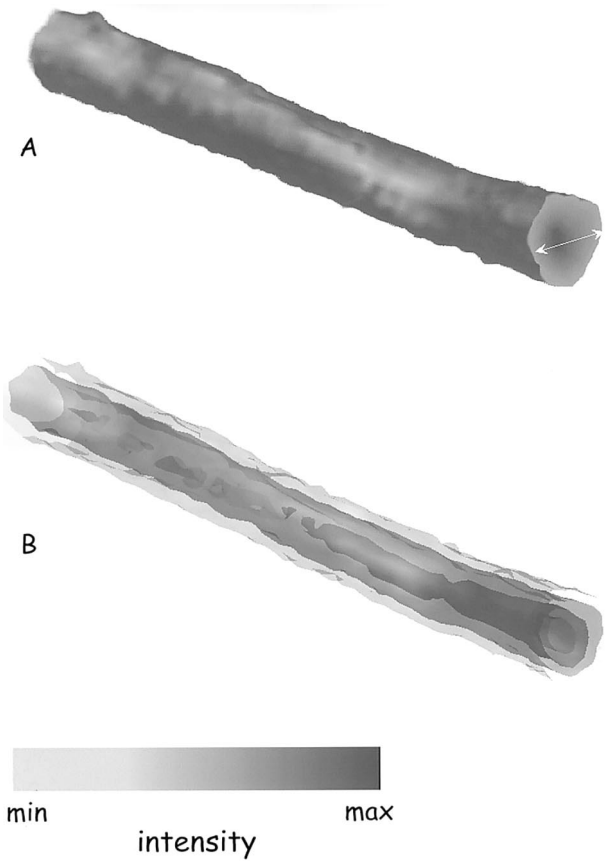

Fig. 4. 3D reconstruction of a DMPC tether using two-photon excitation LAURDAN fluorescence intensity images (false color representation). A complete intensity volume view (A) and four different LAURDAN intensity *iso*-surfaces (B) showing the internal structure of the tubes. The double arrow indicates 4 μm. The 3D figure was obtained using 12 sections every 1 μm and the temperature was 22°C.

1999) provides in a very convenient way to perform the GUVs experiments.

It is interesting to discuss the possible origin of the observed differences among the lipid samples. As we pointed out in the introduction section, the formation of giant vesicles requires lipid layer separation (repulsive normal forces among the lipid bilayers that form the lipid film attached to the solid support) and bending (tangential forces) during the hydration of lipid films (Dimitrov and Angelova, 1987). Lassic, pointed out that water penetrates into the dried lipid films hydrating first the outer layer (Lassic, 1988). The change in the lipid surface area induced by the hydration process affects the topology of the film producing convex bumps (Lassic, 1988). These convex bumps are enlarged in size during the incubation period, forming lipid tubes or tethers. As shown in Fig. 6, there are swelling processes over the entire lipid film using the gentle hydration method. We can clearly observe the lipid convex bumps growing in the z direction, but at the same time we also observe semi-cylindrical structures of lipids growing in the x–y plane (see Fig. 6B,C). These different structures grow simultaneously. Conversely, the shell thickness of these lipid structures shows heterogeneity. The high heterogeneity on the internal structure of the lipid structures obtained using the gentle hydration method (see Figs. 1, 3A and 4) suggest that the repulsive normal forces among the lipid bilayers are weak. We believe that modifications caused by tangential forces on the lipid film dominate the lipid vesicle formation explaining the high incidence of multilamellar structures. This effect is enhanced in principle by defects in the lipid layer. These defects depend on various factors: (i) the concentration of lipid in a particular area of the solid support, i.e. the characteristic of the dried lipid film; (ii) the affinity between the solid support and the lipids; (iii) the topology of the solid interface.

A modification of the gentle hydration method that uses high salt concentration was reported in the literature. Akashi et al. (1996) demonstrated that a percentage of negatively charged lipids (10–20 mol%) helps the formation of GUVs at physiological conditions Akashi et al. (1996). Apparently, the charge repulsion among the lipid bilayers helps layer-separation increasing the contribution of normal forces in vesicle formation. The effect of the charge lipids should compensate the unfavorable effect of the high ionic strength on layer separation found in samples composed of pure zwitterionic lipids (Reeves and Dowben, 1969; Needham and Evans, 1988; Dimitrov and Angelova, 1988; Angelova and Dimitrov, 1988; Käs and Sackmann, 1991). However, as pointed by Akashi et al., this protocol also produces multilamellar structures, myelin figures and lipid debris (Akashi et al., 1996).

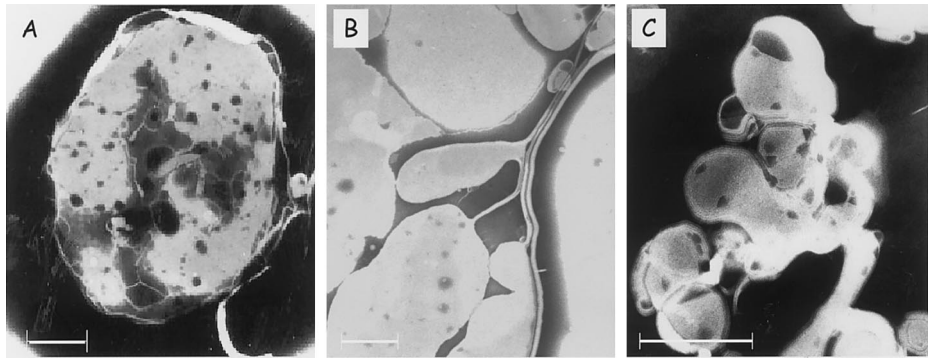

Fig. 5. Transmission electron microscopy images of phospholipid samples. DLPC (A), POPC (B), and DMPC (C). The bars represent 2 μm.

The presence of an external electric field (AC fields) clearly affects the balance between the effects caused by tangential and normal forces on the lipid film attached to the solid surface. As pointed out by Angelova, the effect of the electric field helps to form giant vesicles in samples where normally the gentle hydration method per se does not work (Dimitrov and Angelova, 1987; Angelova et al., 1992). Actually, just few minutes after the application of the AC field, spherical vesicles are observed close to the Pt wires. This contrast with the observations during the gentle hydration method (Fig. 6). It is interesting to remark that there is no detailed information about the physical characteristics of the dried lipid film before the hydration process. The question is if in the very early stage of vesicle formation, the alternate electric field produces any significant change compared with that occurring using the gentle hydration method on the physical properties of the lipid film attached to the solid surface. It is possible that a different mechanism could be operating on the vesicle formation by effect of the AC field compared with that occurring in the gentle hydration method. While a model of the effect of the alternate electric field on the lipid film is beyond the scope of this article, we want to mention that it could be possible that local changes in the lipid layer topology (like pore formation) can occurs by effect of the AC field in the very early stage of vesicle formation favoring the penetration of water.

The other method used in this work was the solvent evaporation method (Moscho et al., 1996). Our results in regards to sample heterogeneity (vesicle size and shell thickness) contrast with those obtained by Moscho et al. (1996) who report a high yield of giant unilamellar vesicles. These authors proposed that vesicle formation is caused by bilayered phospholipid fragments (BPF), which spontaneously vesiculate and form the GUVs. However, there are lipid structures observed in our experiments (on the entire lipid sample) that suggests a more complex mechanism for the vesicle formation than that proposed by Moscho et al. (1996). We can not discard this model but we believe that there are other processes involved in the vesicle formation. Perhaps sheets of lipids can form by the effect of solvent evaporation followed by self-aggregation. This process may explain the complex vesicle internal structure found in these samples.

The complex internal structure of the phospholipid vesicles shows location with time-dependent spatial changes (Fig. 2). We believe that this particular vesicle internal structure may be an intermediate state of a complex equilibrium among different vesicle structures (see Fig. 1). This particular finding can be connected with the conclusions of Gershfeld (1989) that found a critical temperature where unilamellar vesicle formation was observed from multilamellar vesicles.

Finally, an interesting observation is the coexistence of stable lipid tethers or tubes with lipid vesicles. Apparently, the observed lipid network (Fig. 3A, a,c) is the main lipid structure for the

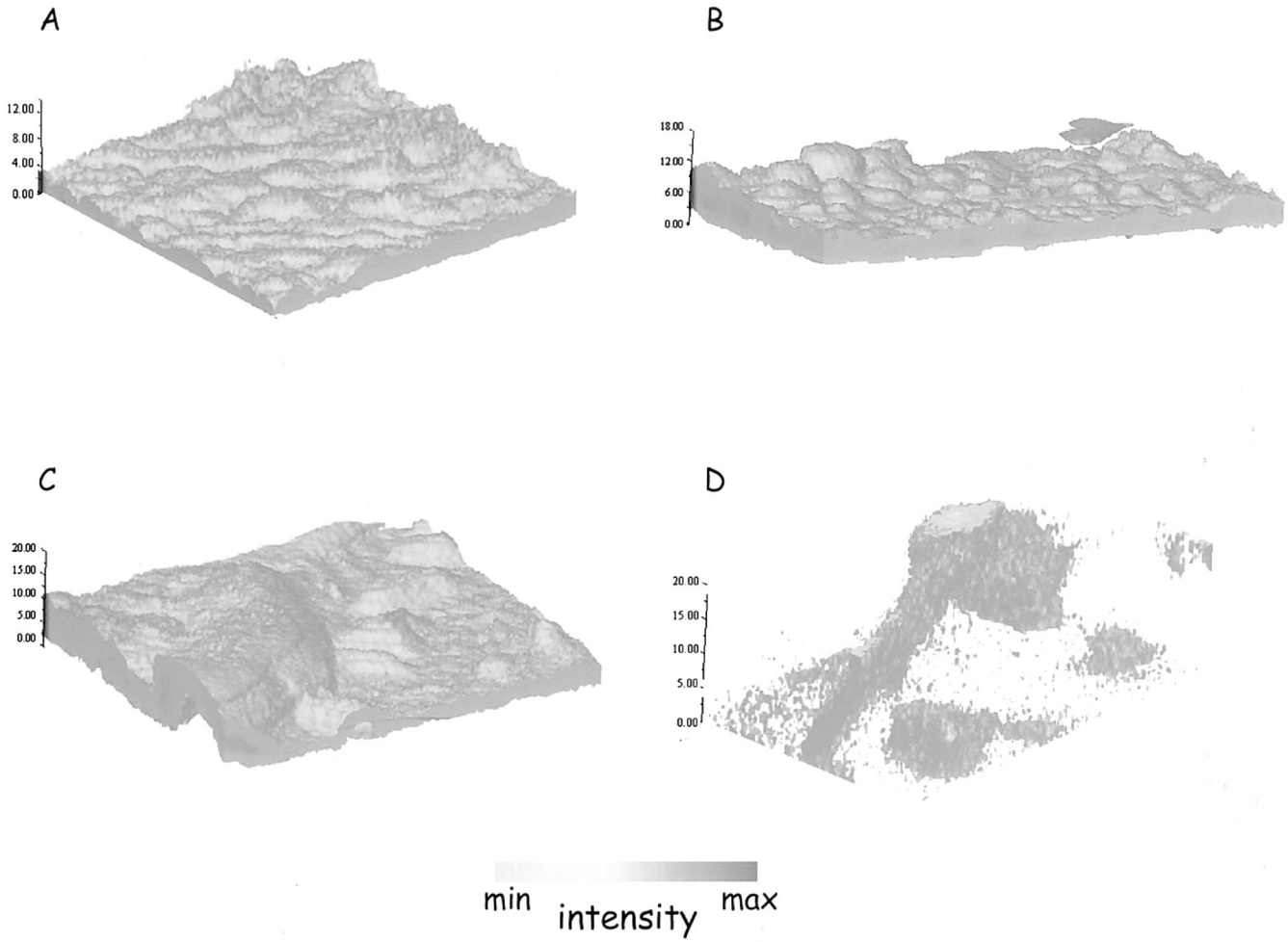

Fig. 6. 3D reconstruction of a DLPC film attached on a glass surface during the gentle hydration method; using two-photon excitation LAURDAN fluorescence intensity images (false color representation). 8 (A), 12 (B), 18 (C) and 22 (D) h after to starting the hydration. The 3D figure was obtained using 22 sections every 1 μm and the temperature was 22°C. The Z scale is in μm.

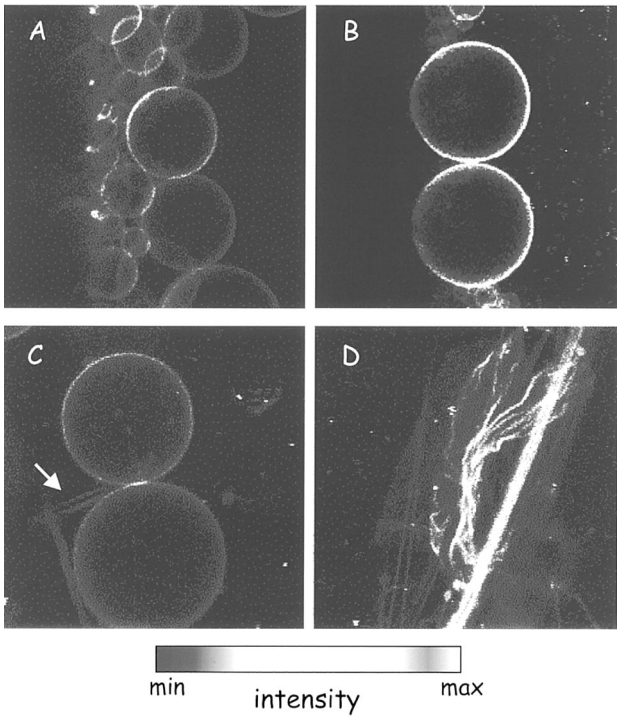

Fig. 7. Two photon excitation LAURDAN intensity images of lipid samples prepared using the electroformation method (false color representation). (A) DLPC; (B) DPPC; (C) and (D) POPC. All images were taken at temperatures corresponding to the fluid phase (50°C for DPPC and 25°C for DLPC and POPC). The orange arrow in (C) show lipid tubes coexisting with the GUVs.

characteristic 'cloud' that is observed in the lipid samples after preparation (using the gentle hydration or the solvent evaporation methods). There are some reports about lipid tubes or tethers in the literature (Bar-Ziv and Moses, 1994; Mathivet et al., 1996; Akashi et al., 1996; Bar-Ziv et al., 1998). However, the structure of these elongated lipid tubes is unknown Mathivet et al. (1996). A model was proposed suggesting that unilamellar membranes make a tubular structure connecting vesicles and also the possible biological relevance of these tubes was discussed (Mathivet et al., 1996). We want to remark two important conclusions from our experiments. The heterogeneity in the shell thickness of the elongated lipid structures, i.e. unilamellar and multilamellar lipid tubes, and the similar lipid packing between lipid tubes and vesicles.

In conclusion, this article basically attempts to show the advantages and disadvantages of the GUVs preparation methods reported in the literature. We demonstrated the importance of analyzing the whole lipid sample characteristics to obtain information about mechanism and efficiency of vesicle formation using different methods. On the other hand, the use of the sectioning capability of the two-photon excitation microscope offers a unique tool to observe the characteristics of free bilayer systems. Using two-photon excitation microscopy, novel information about phospholipid phase transition and lipid domain coexistence was recently reported from our group studying the effect of temperature on GUVs composed of pure phospholipid and phospholipid binary mixtures (Bagatolli and Gratton, 1999, 2000).

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