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Giant vesicles formed by gentle hydration and electroformation: A comparison by fluorescence microscopy

Nicolas Rodriguez [a], Frédéric Pincet [b], Sophie Cribier [a, *]

[a] *Laboratoire de Physico-Chimie Moléculaire des Membranes Biologiques, UMR7099, Institut de Biologie Physico-Chimique, 13 Rue Pierre et Marie Curie, 75005 Paris, France*
[b] *Laboratoire de Physique Statistique, Ecole Normale Supérieure, UMR 8550, 24 Rue Lhomond, 75005 Paris, France*

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Abstract

Giant unilamellar vesicles (diameter of a few tens of micrometers) are commonly produced by hydration of a dried lipidic film. After addition of the aqueous solution, two major protocols are used: (i) the gentle hydration method where the vesicles spontaneously form and (ii) the electroformation method where an ac electric field is applied. Electroformation is known to improve the rate of unilamellarity of the vesicles though it imposes more restricting conditions for the lipidic composition of the vesicles. Here we further characterize these methods by using fluorescence microscopy. It enables not only a sensitive detection of the defects but also an evaluation of the quantity of lipids in these defects. A classification of the defects is proposed and statistics of their relative importance in regard to both methods and lipid composition are presented: it shows for example that 80% of the vesicles obtained by electroformation from 98% 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine are devoid of significant defects against only 40% of the vesicles with the gentle hydration method. It is also shown that the presence of too many negatively charged lipids does not favor the formation of unilamellar vesicles with both methods. For the gentle hydration, we checked if the negatively charged lipids were inserted in the vesicles membrane in the same proportion as that of the lipid mixture from which they are formed. The constant incorporation of a negatively charged labeled lipid despite an increasing presence of negatively charged 1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-L-Serine] tends to confirm that the composition of vesicles is indeed close to that of the initial mixture.

Keywords: Giant vesicle; Electroformation; Lamellarity; Fluorescence microscopy; Phosphatidylserine incorporation

Giant vesicles are often used as membrane model systems because of their size, which enables visualization by optical microscopy and micromanipulation of individual vesicles. For most purposes unilamellar vesicles devoid of any defects such as internal or external lipidic structures are necessary. In the case of a mixture made of different types of lipids a good control of the lipidic composition of the vesicles is important as it is demonstrated by the complex phase diagram of vesicles that can be obtained from simple ternary mixtures of lipids [1]: for instance a variation of a few percents of this composition can control the existence of lipid rafts. Therefore, it is usually important for the composition of the vesicle to be as close as possible to that of the initial lipidic mixture used for the vesicles formation. This article presents a characterization of these two aspects (defects and composition) based on fluorescence microscopy observations.

1. Experimental procedure

Giant vesicles are generally obtained by the hydration of a dried lipid film. This can be achieved in presence of an electric field (electroformation method [2–4]) or not (gentle hydration method [5–7]). These two commonly used methods will be investigated and compared. Such a study of the defects of giant vesicles according to their method of formation has already been conducted by Bagatolli et al. [8] using two-photon fluorescence spectroscopy. The first part of this article completes their study by quantifying the proportion and size

* Corresponding author.
E-mail address: Sophie.cribier@ibpc.fr (S. Cribier).

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of defects. Moreover slight differences in our preparation methods seem to produce noticeable changes in the quality (proportion of vesicles devoid of defects) of the vesicles.

The first stage for making giant vesicles is the preparation of a mixture of lipids in a solution of chloroform:methanol (5:3, v/v). To improve the mixing of lipids the solvent is evaporated and the lipids are dissolved again twice (once under argon, once with a rotary evaporator): this procedure enables the dissociation of possible clusters of lipids. The final concentration is 10 g/l for the gentle hydration method and 0.25 g/l for electroformation. The solution is spread on a rough Teflon disk in the case of the gentle hydration method and on a glass covered with a conducting indium tin oxide (ITO) film in the case of the electroformation. The film is dried under vacuum for 1 h and rehydrated with a 330 mOsm sucrose solution (20 μg lipids/ml for both methods). An ac electric field (progressively increased from 0 to 1.1 V, frequency: 8 Hz) is applied for the electroformation for 15 h at 20 °C. The vesicles obtained by the gentle hydration method grow at 34 °C for 24 h. For observation a drop of the vesicle preparation is injected in a 340 mOsm glucose solution.

2. Classification of defects in giant vesicles and their detection by fluorescence microscopy

Defects in vesicles are sometimes detectable by optical microscopy especially if the observation is conducted by phase contrast or differential interference contrast. The fluorescence microscopy enables to noticeably improve this detection. A small fraction (2%, w/w for all our experiments) of the lipids used for the formation of vesicles is fluorescently labeled and excited by an argon laser at 514 nm. The label is Rhodamine bound to the head of a derivative of egg-phosphatidylethanolamin (Rhodamine-PE purchased from Avanti Polar Lipids). The bright field and fluorescence observation of the vesicles will be compared. The vesicles can be observed directly through the ocular or visualized thanks to a Princeton Instruments Micromax camera (a sensitive method with bright field and a mean to quantify the fluorescent signal). The microscope is an IM-35 Zeiss inverted microscope. For bright field or fluorescence we used the same objective: a Zeiss Phaco (magnification 40 times with a numerical aperture of 0.75).

We define a defect as a lipidic structure that makes a giant vesicle differ from an isolated unilamellar vesicle. Defects have to be classified in order to evaluate their possible role in a given experiment; the defects can be bound to the inner or outer leaflet or encapsulated inside the vesicle; they can also vary in shape and size. They can be sorted out in several categories, which are easily distinguished in fluorescence microscopy (Fig. 1).

The first kind of defect is found in a vesicle that contains at least another vesicle whose diameter is more than 80% of the larger one. We call it a “nest of vesicles” (Fig. 1A). This term is more general than multilamellarity, which may

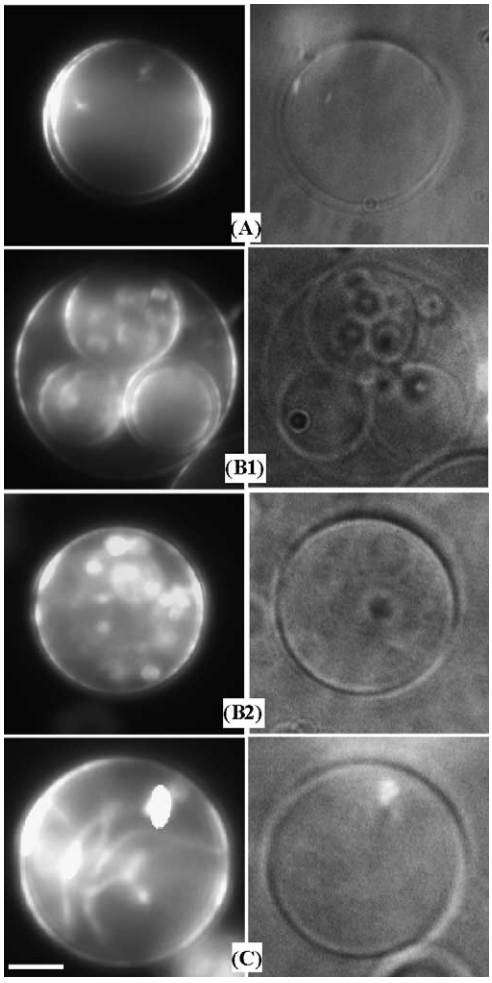

Fig. 1. 98% DOPC, 2% Rhodamine-PE vesicles: comparison between bright field and fluorescence photonic microscopy. (A) nest of vesicles, (B1) giant vesicles inside, easily detectable with bright field, (B2) giant vesicles inside, detectable with fluorescence only and (C) tethers. The bar represents 10 μm. The vesicle diameters are typically 30 μm. These vesicles were formed by the gentle hydration method.

be interpreted as multiple vesicles of almost the same size whose bilayers are in close contact. This defect is well documented and can be detected by various methods [9,10] including fluorescence microscopy [8,11]. Most of the time these vesicles contain only one other vesicle. A few vesicles containing more than one vesicle of significant sizes have been observed. The nest of vesicles are in general detectable with bright field as soon as the size of the inner vesicle is not too close to that of the larger one. Fig. 2 gives a nest of three vesicles. Depending on the position in the pictures 1–3 membranes are observed. The profile shows the density of fluorescence along the white straight line on the picture. The respective contributions of each bilayer extrapolated at a minimal distance of the boundaries to avoid the effects of the laser polarization are about the same for each vesicle. The close proportionality between the fluorescence density and the number of membranes ensures that the fluorescence density is proportional to the amount of membrane involved in

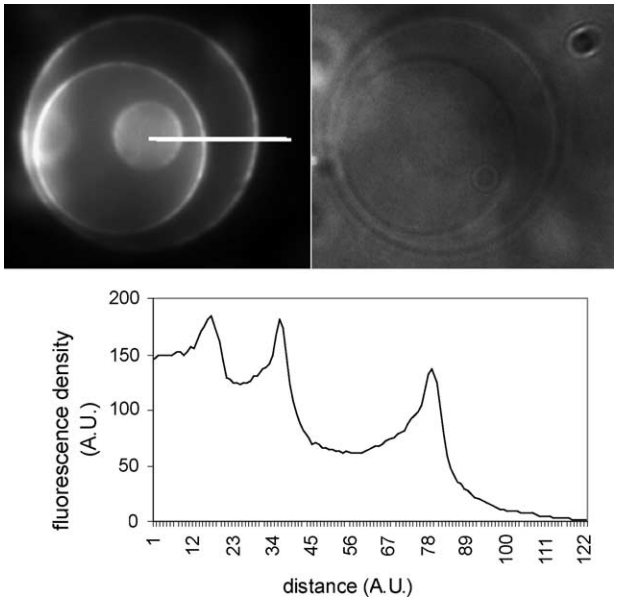

Fig. 2. "Nest" of three vesicles. The fluorescence profile corresponds to the white line on the fluorescent vesicle picture. The contribution of each vesicle to the total fluorescence density extrapolated at the center of the vesicle is about the same.

the defects even if they are encapsulated. Moreover it proves the ability to discriminate a unilamellar from a bilamellar vesicle whose boundaries are not distinguishable. In every experiment presented in this article we noticed that bilamellar vesicles are particularly scarce; actually, we have never observed any bilamellar vesicles. When there was a second vesicle, its diameter was at least 1% lower than the diameter of the outer one.

The second kind of defects is the presence of smaller vesicles or lipids aggregates (Fig. 1B). These defects can be either bound to the outer or inner leaflets or mobile inside the vesicle. The vesicles with another one above the optical resolution that is clearly bound to the exterior are not selected so that most of the defects we study are inside the vesicles. When the vesicles are larger than 1 μm they may be detected with bright field. When they cannot be resolved optically the fluorescence microscopy solely may enable to detect them unless they are numerous. They cannot be distinguished from lipid aggregates. In any case, fluorescence microscopy gives a better evaluation of their number.

Finally the last kind of defect is the presence of tethers (Fig. 1C). These are cylinders whose diameter is usually below 1 μm and whose length can reach 10 μm or more [4,8,12–14]. These mobile and fluctuating structures are connected to the membrane of the vesicle on the inside or on the exterior. They can link several vesicles and are very sensitive to the flows in the chamber and the movements of their vesicles. Their observation is almost impossible with bright field and improved with fluorescence.

3. Comparison between gentle hydration and electroformation methods

The characterization of 98% 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC from Avanti Polar Lipids) and 2% Rhodamine marked-PE lipid vesicles preparations is presented in Fig. 3. The vesicles are preselected in bright field with the following criteria: diameter greater than 10 μm, no adhesion with another giant vesicle and elimination of the most obviously filled vesicles (less than a few percents). The

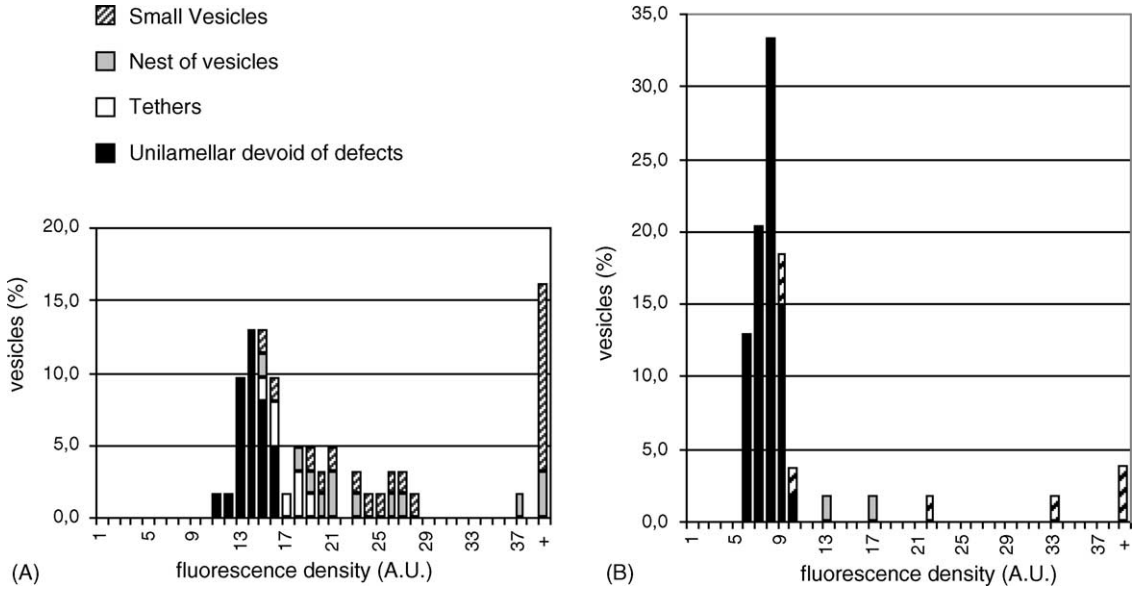

Fig. 3. Defects proportion and quantification by fluorescence in two populations of vesicles (98% DOPC, 2% Rhodamine-PE). (A) formed by gentle hydration and (B) formed by electroformation. The fluorescence density is the mean fluorescence over a disk centered in the middle of the vesicle and that covers about 2/3 of its surface: fluorescence intensity is integrated over the disk and divided by its surface.

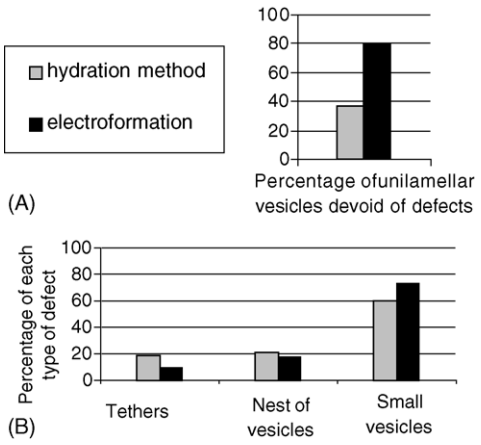

Fig. 4. (A) Mean proportion of clean vesicles (98% DOPC, 2% Rhodamine-PE) for both protocols and (B) relative proportion of the different defects among the vesicles with defects (taken from A).

fluorescence density is the mean fluorescence over a disk centered in the middle of the vesicle and that covers about 2/3 of its surface: fluorescence intensity is integrated over the disk and divided by its surface.

In Fig. 3A, the vesicles have been formed by the gentle hydration method ($N = 62$ vesicles). It was found that 60% of the vesicles have defects. Half of these defects were very difficult to see clearly with bright field.

In Fig. 3B, the vesicles have been formed by electroformation ($N = 54$ vesicles). The vast majority (80%) of these vesicles are unilamellar and devoid of defects (hereinafter called "clean" vesicles) compared to 40% for the vesicles obtained by the gentle hydration method. These rates are representative of what is generally obtained. Fig. 4A summarizes the observations conducted with $N = 114$ electroformed vesicles and $N = 104$ vesicles formed by gentle hydration. The vesicles have been obtained from four separate preparations. The results confirm the high yield of clean unilamellar vesicles produced by the electroformation method. The rate of clean electroformed vesicles that we obtained may be slightly lower than the ones reported before [4,8]. However, these studies focused on the proportion of unilamellar vesicles (shown to be close to 95%, in agreement with our observations) and may have not taken into account a part of the defects that we considered here. In fact small vesicles inside the bigger electroformed one have already been detected by freeze-fracture electron microscopy [4]. A more striking feature is the relatively good quality of the vesicles obtained by gentle hydration method: 40% of clean vesicles, which is much higher than 10% obtained by Bagatolli et al. [8]. The main two differences between our preparation protocols are: (i) the substrate on which the lipidic film is spread (glass and Teflon for Ref. [8] instead of Teflon) and (ii) the presence of sucrose inside and glucose outside the vesicles (pure water in Ref. [8]).

The first difference emphasizes the role of the roughness of the substrate that can influence the formation of defects in the lipid film and locally determine its thickness. These defects are certainly important to control the hydration and passage of solutes between the layers during the formation process. The size of the vesicles may be strongly influenced by the defects. Moreover each type of substrate (Teflon or glass) induces different van der Waals and electrostatic interactions between the substrate and the lipid layers.

The fact that we used a solution of sucrose instead of pure water changes the osmotic pressure between the layers during the formation. It is well known that osmotic effects are critical for vesicle formation [3]. In general vesicles are formed in the presence of solutes.

The relative distributions of the defects in the two methods of vesicles formation are close (Fig. 4B) in spite of the scarcity of tethers in electroformed vesicles.

As shown in Fig. 2 on the example of a nest of vesicles, the amount of lipid involved in the defects of a vesicle can be directly obtained from the fluorescence density. The same approach can be used on vesicles containing small vesicles or tethers. From the results given in Fig. 3A and B it is possible to infer the mean amount of lipids involved in the defects of vesicles. On average, in a vesicle with defects, it can be expressed as the ratio between the lipids in the defects and the lipids in the main membrane: 1.6 for the electroformation and 1.4 for the gentle hydration method. In summary, taking into account all the vesicles, with and without defects, 25% (resp. 50%) of the lipids are found in defects for the electroformation method (resp. gentle hydration method). These figures are strongly influenced by the presence of vesicles which are completely filled with defects and characterized by a fluorescence density that can reach more than 10 times the fluorescence of a clean vesicle: these vesicles represent about 10% of the vesicles with defects.

The same experiments have been conducted with different lipid mixtures containing negatively charged 1,2-Dioleoyl-*sn*-Glycero-3-[Phospho-L-Serine] (DOPS from Avanti). The aim is to quantify the effect of these charged lipids on the defects formation since charges are known to affect the formation of giant vesicles by electroformation [2,3]. When 10% DOPS (w/w) are included, the defects distribution is close to that of Fig. 4 (data not shown). The proportion of clean vesicles remains the same with vesicles formed by gentle hydration. This proportion is slightly lowered to about 70% in the case of electroformation. It must be noticed that in spite of a close proportion of clean vesicles, the addition of DOPS significantly reduces the yield of vesicles formation by electroformation.

When the proportion of DOPS reaches 20%, giant vesicles cannot be formed by electroformation anymore. This limit can be increased with the gentle hydration method. With 40% DOPS and 58% DOPC it is still possible to get a good yield of giant vesicles formation. However, the proportion of clean vesicles drops down to 15% (Fig. 5) indicating that though it remains possible to work with such a lipidic composition the vesicles should be carefully selected. In spite of the selection of the vesicles in bright field observation, more than 60%

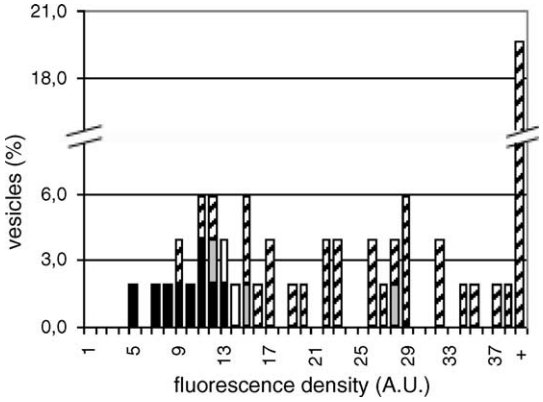

Fig. 5. Defects proportion and quantification by fluorescence in a population ($N = 51$ vesicles) of strongly charged vesicles (58% DOPC, 40% DOPS, 2% Rhodamine-PE) obtained by gentle hydration. Same legend as Fig. 3.

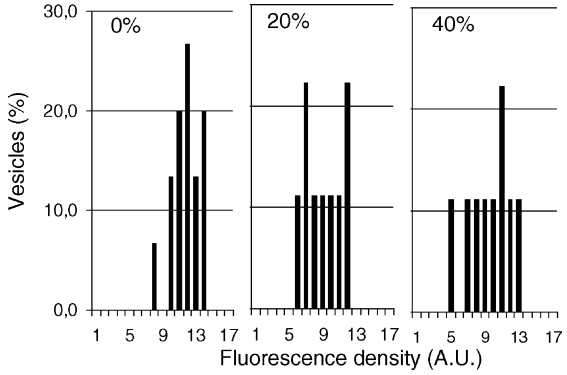

Fig. 6. Fluorescence density distribution of the vesicles devoid of defects taken from three vesicles population whose initial lipid mixtures contain 2% Rhodamine-PE, DOPC and 0, 20 and 40% DOPS, respectively. The mean fluorescence density are 11.8, 9.1 and 9.6 for these three populations of increasing charged lipids concentration.

were filled with defects that count more lipids than their main membrane.

4. Control of the lipidic composition of giant vesicles

The influence of charged lipids on the yield of formation and on the quality of the vesicles shows that these lipids do not favor giant vesicles formation in our conditions (contrarily to the giant vesicles formed at high ionic strengths where more charged lipids are needed to separate the lamellae [11]). It should consequently be checked that the composition of the vesicles is similar to the initial lipid mixture and that there is no decrease in the proportion of charged lipids in the vesicles.

The comparison of the mean fluorescence density of the vesicles devoid of defects obtained by the gentle hydration method and electroformation shows that the fluorescence of the electroformed vesicles is reproducibly 20% lower. This may be explained by the presence of defects whose size prevents their detection even with the fluorescence microscope and that are still present in vesicles considered as clean. But a lower proportion of the negatively charged Rhodamine-PE in the electroformed vesicles cannot be excluded. The dispersion of the fluorescence density among the clean vesicles can be explained by the defects beyond the optical resolution too and by the poor homogeneity of the laser spot in the field that modulate a little the illumination of the fluorescent vesicles. Therefore, we do not interpret this dispersion as an evidence of a dispersion of the amount of labels incorporated in the vesicles.

In the case of the vesicles obtained by gentle hydration, it is possible to check that the negative charges on the lipids do not significantly affect the composition of the lipid mixture in the vesicles in regard to the initial one. Since DOPS and Rhodamine-PE are both negatively charged, the overall concentration of charged lipid is given by the sum of the concentrations of these two lipids. Different mixtures containing an increasing amount of negatively charged lipids (DOPS) were tested by keeping a constant amount of Rhodamine-PE (also negatively charged) in the initial lipid mixtures. If there was an upper limit for the amount of negatively charged lipids incorporated in the vesicles, an increase of the total amount of negatively charged lipids due to an increase of the amount of DOPS would lead to a decrease of the proportion of RhPE in the giant vesicles. The results for 0, 20 and 40% DOPS are shown in Fig. 6. Only the clean vesicles are taken into account to avoid a bias due to variable amount of defects with variable amount of charged lipids. The average density of fluorescence is slightly higher at 0% (11.8 a.u.) than 20% (9.1 a.u.) and 40% (9.6 a.u.) but can be approximately considered as constant without significant decrease of the fluorescence when the proportion of DOPS is increased. This result relies on the hypothesis that the incorporation of a lipid in a giant vesicle depends only of its charge. For example, the different shapes of RhPE and DOPS could infer on the competitive incorporation that we have checked. However, changing the shape of lipids and getting a good yield of vesicle formation (by varying the chain lengths or by adding lipids with non-cylindrical shapes like cholesterol [1]) is commonly achieved whereas we have shown here that the charge has an important influence on the vesicle formation. These observations strengthen our hypothesis. Consequently this experiment tends to confirm the similarity of the compositions of the initial lipid mixture and the vesicles.

5. Conclusion

These experiments emphasize the good quality of electroformed vesicles and confirm the high proportion of clean unilamellar vesicles even if we take into account small defects such as those we have classified. However, the incorporation of charged lipids is a limitation to the electroformation of vesicles. This limitation can be overcome by using the gentle hydration method even if the yield of clean unilamellar vesicles decreases when the amount of charged lipids is

above 10%. Our results combined with those given in Ref. [11] indicate that there seems to be an optimal proportion of charged lipids to form clean unilamellar vesicles at a high yield for a given ionic strength: the electrostatic repulsion must be strong enough to allow the separation of the lamellae in the deposited lipid film but not too strong because the vesicles tend to be then filled with defects. In clean unilamellar vesicles, the charged lipids are effectively inserted in the vesicles in a proportion that seems to be close to that of the lipid mixture.

Consequently both preparation methods are complementary. The electroformation method should be used as soon as the lipidic composition is not an obstacle; otherwise, the gentle hydration method can replace it.

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