Matrix Effect of Vesicle Formation As Investigated by Cryotransmission Electron Microscopy

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It has been recently reported that under certain conditions the size distribution of vesicles being formed upon addition of fresh surfactant to an aqueous solution is strongly affected by the presence of preformed and narrowly distributed vesicles. In particular, the final size distribution is strongly biased toward the size distribution of the initial vesicles (the so-called "matrix effect"). On the basis of a novel experimental approach, we present here an investigation of the matrix effect and the corresponding fission processes of oleic acid/oleate vesicles and mixed POPC/oleic acid/oleate vesicles (POPC = 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine). This novel approach is based on two complementary techniques: the use of cryotransmission electron microscopy (cryo-TEM) and the entrapment of ferritin which can be visualized by cryo-TEM in the initial preformed POPC liposomes. Addition of micellar oleate to an equimolar amount of 100 nm extruded POPC liposomes results in the formation of mixed vesicles with a new size and ferritin distribution. This can be determined by cryo-TEM, and two main findings have thus been obtained. On one hand, the matrix effect has been substantiated; i.e., the size distribution of the preformed liposomes strongly affects the final size distribution. However, and surprisingly, the final suspension contains a large amount of vesicles with a diameter between 20 and 40 nm, i.e., significantly smaller than the preformed ones. The fact that these small vesicles were not present in the initial population of preformed vesicles and the fact that some of them contain ferritin molecules, brings one to the conclusion that they have derived from fission processes of larger ferritin-containing vesicles. More generally, this cryo-TEM-based investigation also sheds light on the basic properties of oleic acid/oleate vesicles formed by spontaneous vesiculation, for example, the most probable size (main peak around 40−60 nm) and the surprisingly small unilamellarity (of the order of 1.02).

Introduction

The formation of vesicles in aqueous solution generally affords a rather broad size distribution, with radius ranging typically between 20 nm and 1 μm. This is the case of long chain carboxylic acids, such as oleic acid or caprylic acid at intermediate pH (ca. pH 7.5−9.0)[1−5] or the case of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) when a concentrated methanol solution of this lipid is injected in water (unpublished). It would actually be desirable to regulate the size of the vesicles, as for most applications a relatively narrow size distribution is important. From a theoretical point of view, this does not appear impossible, as vesicles are usually not classic equilibrium systems as, for example, aqueous micelles are. Therefore, it is in principle possible to trap the vesicles into one narrow average dimension range by the proper choice of initial conditions.

Recently, in our group we have made an observation that goes in this direction.[6,7] When a solution of sodium oleate was added to a water solution already containing 100 nm extruded oleic acid/oleate vesicles[7] (oleic acid/oleate vesicles are composed of oleic acid as well as oleate molecules; for the sake of simplicity, we will refer to these vesicles as oleic acid vesicles) or POPC liposomes,[6,8] the final size distribution was again centered around 100 nm: the larger vesicles present in the control experiment (same experimental conditions but without pre-added vesicles in the aqueous solution) were not formed. It is as if the pre-added oleic acid vesicles or POPC liposomes determine the size of the newly formed ones.

Most of these observations[6,7] were made by dynamic light scattering (DLS), which has the advantage of yielding information rather rapidly, thus permitting a large number of experiments in short time. However, the size distribution given by DLS is generally an intensity-weighted one, which tends to overestimate larger aggregates, particularly in polydisperse suspensions (it is not easy to extract number-weighted averages from DLS data). Instead, a cryotransmission electron microscopy (cryo-TEM) analysis—in which the single vesicles are individually counted—permits one to obtain detailed information on the vesicle size distribution and therefore on the matrix effect mechanism.

The problem here lies in the fact that EM analyses are costly and time-consuming; i.e., thousands of single vesicles must be analyzed in order to obtain meaningful statistics. Furthermore, one needs an analytical method to differentiate between preformed and newly formed ("nascent") vesicles.

The present work was actually prompted by the fact that a very good solution to this analytical problem could be found.[8] This is based on the entrapment of ferritin[9] inside the liposomes

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and on the use of cryo-TEM. Ferritin, an iron-containing protein, can be easily detected by cryo-TEM due to the presence of the very dense iron core in its center.[10] Addition of fresh surfactant to the preformed and extruded ferritin-containing vesicles results in new vesicles that may or may not contain ferritin molecules, depending on the mechanism of their formation. This work is mostly based on the comparison of the size distribution of vesicles obtained upon addition of fresh surfactant to an aqueous solution in the presence and in the absence of preformed POPC liposomes.

Materials and Methods

Chemicals. Sodium oleate (*cis*-9-octadecenoic acid sodium salt, >99%) and boric acid (>99.5%) were obtained from Fluka, Buchs, Switzerland. Ferritin (from horse spleen) was purchased from Sigma, Buchs, Switzerland. POPC was from Avanti Polar Lipids, Alabaster, AL. Sepharose 4B was from Pharmacia, Uppsala, Sweden.

Cryo-TEM. Copper grids (300 mesh) coated with a perforated carbon film according to a modified procedure described by Fukami and Adachi[11] were used for the preparation of frozen hydrated specimen by the bare-grid technique.[12] The grids were rendered hydrophilic by glow discharg before use. The thin aqueous films were prepared under controlled temperature (RT) and humidity conditions (97−99%)[13] within a custom-built environmental chamber in order to avoid osmotic effects (flattening or shrinkage of vesicles in evaporating fluid layer)[14].

A 10 μL aliquot of the vesicle suspension was put on the grid. The excess liquid was blotted with filter paper for 2−3 s, and the thin aqueous films were rapidly vitrified by plunging them into liquid ethane. The grids were mounted in a Gatan cryoholder and examined in a Zeiss EM 912 OMEGA microscope, equipped with an energy filter, at 120 kV and at a specimen temperature of ca. 105 K. Electron micrographs were digitally recorded using a cooled 1 K × 1 K CCD camera (Proscan). Micrographs were taken from holes with a diameter larger than 2 μm. Many holes (>20) per specimen of at least five different specimens were analyzed in order to reduce sampling errors.

Analysis of the Cryo-TEM Micrographs. The various distributions were established by counting a large number of vesicles omitting undefined aggregates of lipids and ferritin. The vesicle diameter was measured, and the number of ferritin molecules entrapped in each vesicle was counted. Number-weighted and mass-weighted size distributions were determined on the basis of the cryo-TEM micrographs. Number-weighted distributions were usually obtained by plotting the relative number of vesicles (given in percentage of the total number of vesicles) against the vesicle diameter (nanometers). The mass-weighted distribution was obtained by calculating the frequency of each size weighted by the corresponding surface area. The vesicles were assumed to be spherical and unilamellar. In this case the relative mass (given in percentage of the total lipid mass) was plotted against the vesicle diameter (nanometers).

Turbidity Measurements. Turbidity measurements were carried out without stirring at 25 °C and 400 nm with a Cary 1E UV/visible spectrophotometer from Varian, as described previously.[6] The sample volume was usually 1.2 mL, and quartz cells with a path length of 1 cm were used.

Oleate Stock Solutions. The 15−30 mM sodium oleate stock solutions were always prepared freshly before use in desionized water.

Spontaneous Formation of Oleic Acid Vesicles. The vesicle formation was initiated by adding 240 μL of an aqueous oleate solution (15 or 30 mM) to 960 μL of borate buffer (0.1 M, pH 8.5). The pH of the oleate solution was around 10.5 and the pH of the mixed POPC/oleic acid/oleate suspension obtained upon addition of oleate to POPC liposomes was 8.5 (pH of the borate buffer used).

Preparation of Ferritin-Containing POPC Liposomes.[8] The POPC liposomes used in the present study were prepared by the reversed phase method as described by Szoka and Papahadjopoulos.[15,16] POPC (66 mg) was dissolved in diethyl ether (10 mL) in a 50 mL round-bottom flask. Then 3 mL of a concentrated ferritin solution (50 mg/mL) in borate buffer (0.1 M, pH 8.5) was added to the POPC solution. The mixture was sonicated for 5 min at 20 °C using a sonication bath to yield a homogeneous red emulsion. Diethyl ether was removed by evaporation using a rotary evaporator (p = 400 mbar, T = 25 °C) until a viscous (gellike) suspension was obtained. The gellike mixture was then vortexed until a low-viscosity suspension was obtained. The obtained vesicles were sized down to approximately 100 nm using a device known as "The Extruder" from Lipex Biomembranes Inc., Vancouver, Canada.[17,18] The suspension was first passed 10 times through two 400 nm Nucleopore polycarbonate membrane filters. The same procedure was then repeated with 200 nm and finally with 100 nm pore size filters. Nonentrapped ferritin molecules were removed by gel permeation chromatography using a Sepharose 4B column (length 53 cm, diameter 1.3 cm) equilibrated with the borate buffer (0.1 M, pH 8.5) prior to use. The vesicle suspension (0.8 mL) was loaded on the column and fractions of ca. 1 mL were collected. The fractions obtained from the gel permeation chromatography were analyzed after destruction of the vesicles with 40 mM cholate. Ferritin was quantified spectrophotometrically (λ = 280 or 440 nm) and the POPC concentration was determined by using the Stewart assay.[19]

Addition of Oleate Micelles to Preformed POPC Vesicles.[6,8] A 240 μL aliquot of an oleate solution (15 mM) was added to 960 μL of ferritin-containing POPC liposomes suspension (3.75 mM). The final oleic acid and POPC concentrations were both 3 mM. The vesicle suspension was examined before and after oleate addition by cryo-TEM.

In the experiments of Figure 5, as well as for Figures 6 and 7, the liposome suspension after the gel permeats as chromatography was 6−1 mM in POPC. This suspension was first investigated by cryo-TEM and then deluted 1:1 with borate buffer to obtain the 3 mM POPC suspension, to which oleate has been added.

Results and Discussion

Operational Setting. Formation of vesicles was generally induced by addition of fresh oleate micelles to an aqueous solution. The POPC liposomes were prepared in borate buffer (0.1 M, pH 8.5) in the presence of ferritin and were sized down to 100 nm diameter by extrusion techniques. Most of these preformed POPC liposomes contained the marker ferritin. Usually, the final concentrations of POPC and of oleic acid + oleate were both 3 mM (final concentrations after oleate addition).

As already mentioned, the operational setting of this work, at variance with a more general paper on growth and fission of vesicles,[8] was aimed at checking by cryo-TEM the so-called matrix effect.[6,7]

To this aim, we first measured the rate of turbidity increase when oleate was added to an aqueous solution that did not contain any preformed vesicles (final oleic acid + oleate concentrations were 3 and 6 mM). As is well-known, the time

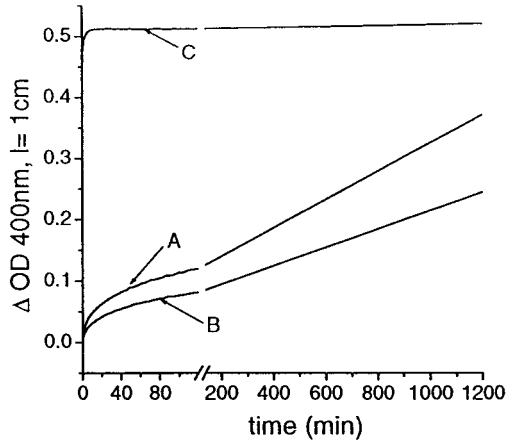

Figure 1. Effect of the presence of preformed 100 nm POPC liposomes on the kinetics of mixed POPC/oleic acid/oleate vesicle formation at 25 °C, as followed turbidimetrically at 400 nm. Curve A: 240 μL of 30 mM aqueous sodium oleate solution was added to 960 μL of borate buffer (0.1 M, pH 8.5); [oleic acid + oleate] after mixing, 6 mM. Curve B: 240 μL of 15 mM aqueous sodium oleate solution was added to 960 μL of borate buffer (0.1 M, pH 8.5); [oleic acid + oleate] after mixing, 3 mM. Curve C: 240 μL of 15 mM aqueous sodium oleate solution was added to 960 μL of 3.75 mM POPC "100 nm liposome"; [POPC] after mixing, 3 mM; [oleic acid + oleate]/[POPC] = 1:1.

progress of the turbidity reflects the formation of vesicles, although the optical density is simply determined not only by the vesicle concentration but also by their volume (actually by the second power of the volume). Results are shown in Figure 1: notice that in both cases (final oleic acid + oleate concentration 6 mM (curve A) and 3 mM (curve B), respectively), a long time is required to reach equilibrium. Furthermore, the final optical density is roughly proportional to the concentration.

Curve C of Figure 1 represents the time progress of the optical change when oleate is added to an aqueous suspension containing 100 nm POPC liposomes (the final oleic acid + oleate and POPC concentration were both 3 mM). In this case, the process is very fast, actually the reaction is almost over within the mixing time. This indicates that the process of vesicle formation is remarkably faster in the presence of pre-added POPC liposomes than in their absence. This must be due to the interaction between oleate and the POPC liposomes and is in keeping with literature data that indicates a fast uptake of oleate by POPC liposomes.[20−24] All this suggests that practically all added oleate has been incorporated in the POPC bilayer membranes. In fact, if a significant part of the added oleate would have formed vesicles by its own accord, i.e., independently from the presence of POPC liposomes, one would have observed the slow process that characterizes the first two traces of Figure 1.

In conclusion, it can be said that under our conditions the presence of pre-added and extruded POPC liposomes affects the process of nascent vesicle formation upon addition of oleate micelles, as far as both the rate and the size distribution are concerned.

Those are, however, qualitative observations and we wish now to establish the vesicle size distribution in more quantitative terms by the combination of cryo-TEM measurements and the use of ferritin as a marker.

To this aim we need first to assess by cryo-TEM the size distribution of oleic acid vesicles obtained by the simple addition of oleate micelles to an aqueous solution. Aside from the question of the matrix effect, such data are important to shed light on the general question of the vesicle size distribution in the case of spontaneous vesiculation of fatty acid surfactants.

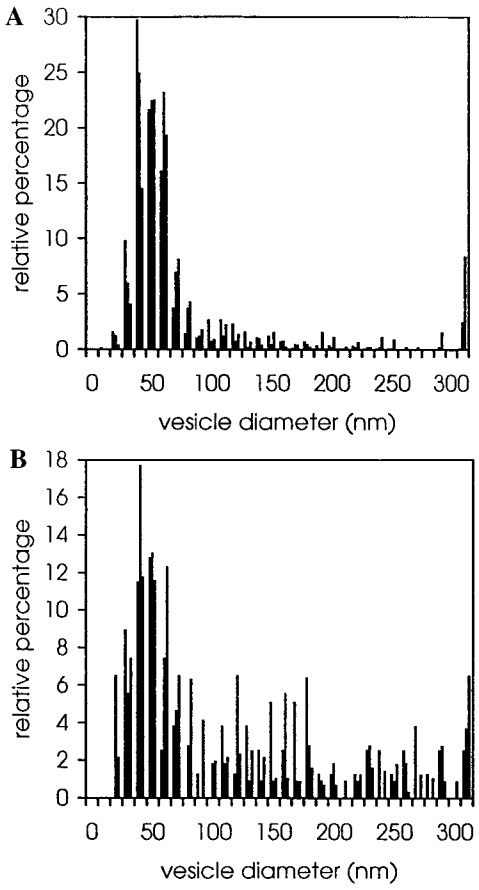

Figure 2. Number-weighted size distributions as obtained by cryo-TEM for the formation of oleic acid vesicles upon addition of oleate micelles to borate buffer (0.1 M, pH 8.5): (A) [oleic acid + oleate] = 6 mM; (B) [oleic acid + oleate] = 3 mM. The last bars correspond to all the vesicles larger than 300 nm. For each set of conditions, three experiments were performed. The samples were examined after ca. 24 h after oleate addition. Data from three different experiments are reported, so as to give an idea of the reproducibility.

Figure 2A shows the size distribution of the oleic acid vesicles (final oleic acid + oleate concentration 6 mM) obtained upon addition of oleate micelles to borate buffer (0.1 M, pH 8.5). Data from three experiments are reported so as to give an example of the reproducibility, which can indeed be considered satisfactorily. The size distribution is rather narrow, the largest number of vesicles having a diameter between 30 and 80 nm (ca. 75% of the total population). Interestingly enough, the size distribution obtained with a lower oleic acid concentration (final oleic acid + oleate concentration 3 mM) is considerably broader, as shown in Figure 2B. In both cases, the concentration of vesicles with size above 300 nm is extremely low. The observation, that a higher concentration under otherwise the same conditions brings about a sharper size distribution is per sé rather interesting and certainly worth of a more systematic study (e.g., with a larger concentration range and possibly different surfactant systems), which was outside the scope of the present investigation.

One other important and somewhat surprising feature is the very low lamellarity degree of oleic acid vesicles. The largest number of vesicles observed in this series of experiments were unilamellar, and actually the average lamellarity degree was around 1.02, which is exceptionally low.

As already mentioned, our initial setting for the experiments is based on the fact that the pre-added POPC liposomes are marked with ferritin. The entrapment of ferritin in liposomes

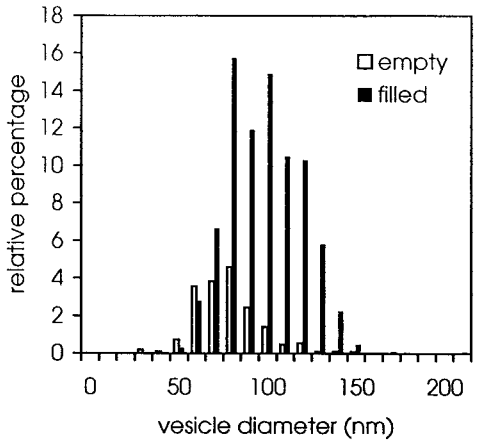

Figure 3. Number-weighted size distribution as obtained by cryo-TEM for the preformed POPC liposomes ([POPC] = 6.1 mM). Empty (empty bars) and ferritin-containing vesicles (black bars) are represented individually in this histogram.

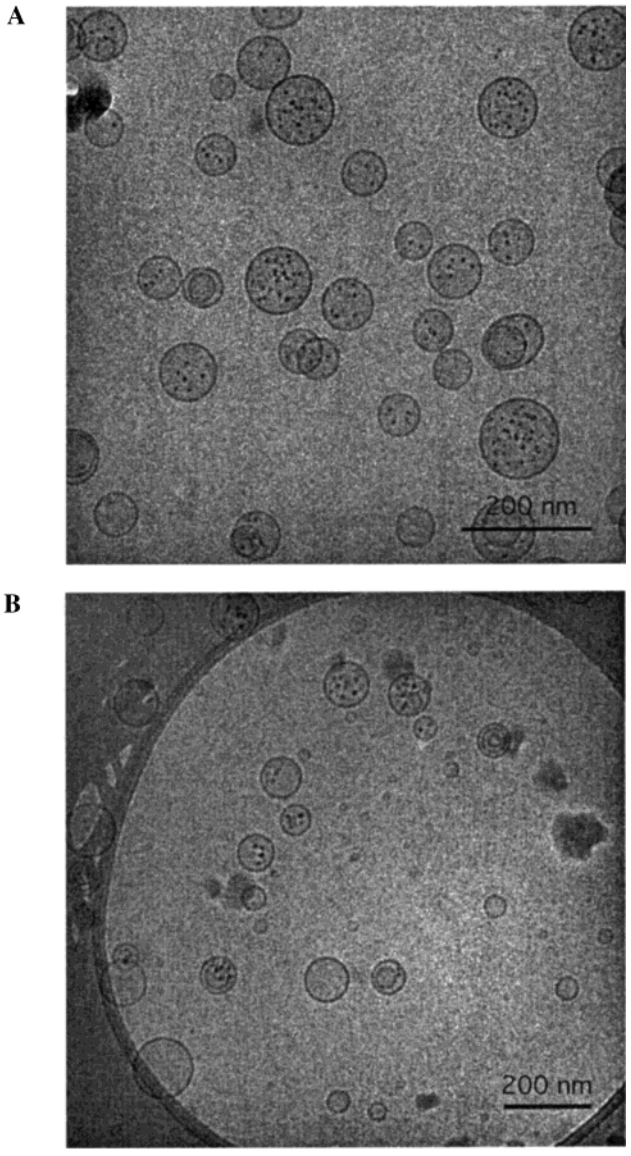

Figure 4. Cryo-TEM micrographs of (A) ferritin -containing POPC liposomes prepared using the reverse phase evaporation method, followed by a sizing down by extrusion through polycarbonate membranes with 100 nm pore diameters ([POPC] = 6.1 mM), and of (B) the vesicle suspension obtained after addition of oleate to preformed POPC liposomes ([POPC] = 3 mM, [oleic acid + oleate] = 3 mM).

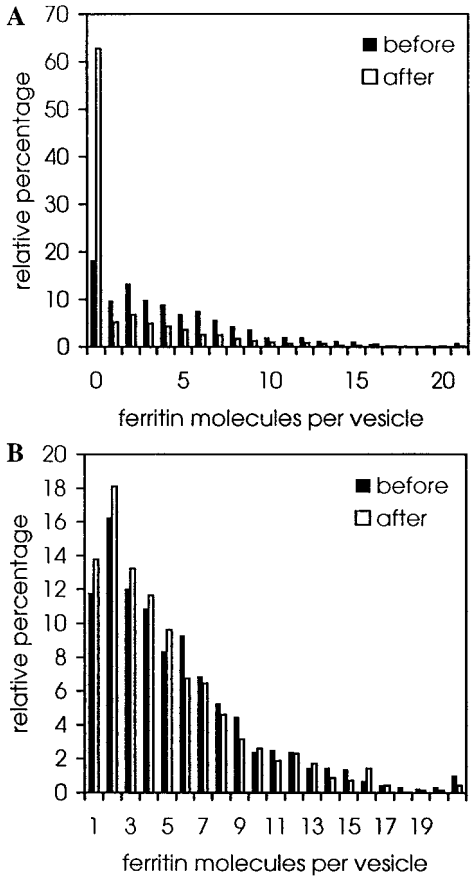

Figure 5. Ferritin distribution (A) among all the vesicles and (B) among the filled vesicles as obtained by cryo-TEM for the preformed ferritin-containing POPC liposomes ([POPC] = 6.1 mM; black bars) and for the vesicle suspension obtained after oleate addition to preformed ferritin-containing POPC liposomes ([POPC] = 3 mM, [oleic acid + oleate] = 3 mM; empty bars). In (B), only the vesicles that contained at least one ferritin molecule have been considered, and the sum of all the filled vesicles was set to 100%.

has been described in a previous paper[8] and it is reported here only in the Experimental Section. Under our conditions not all the POPC liposomes are "filled" (i.e., ferritin-containing), 18% of the liposomes are "empty" (see below Figure 5A). Figure 3 illustrates the size distribution of the filled and of the empty POPC liposomes as determined by cryo-TEM. Notice that the empty liposomes are mainly localized in the region of small diameters, between 50 and 100 nm, whereas most of the vesicles larger than 60 nm are filled.

One interesting question is relative to the number of ferritin molecules per individual liposome. This determination is possible in view of the excellent scattering power of ferritin (Figure 4A). Figure 5A shows the ferritin distribution among all the vesicles (filled and empty vesicles together) before and after oleate addition in the experiment in which the final concentrations of both oleic acid + oleate and POPC were 3 mM. Figure 5B shows the ferritin distribution solely among the filled vesicles. The average values (mean vesicle diameter, mean number of ferritin molecules per vesicle, mean number of lamellae per vesicle) are given in numerical form in Table 1. The large number of analyzed vesicles can be taken as an indication of the reliability of our statistical analysis.

Notice from Figure 5A that vesicles containing up to 20 ferritin molecules are present in the initial suspension and that the mean number of entrapped ferritin molecules decreases from 4.5 to 1.9 upon addition of oleate. This means that the number of vesicles in the suspension increased by a factor of ca. 2.4.[25]

TABLE 1: Mean Vesicle Diameter, Mean Number of Ferritin Molecules Per Vesicle and Mean Number of Lamellae Per Vesicle Determined Experimentally for the Preformed Ferritin-Containing POPC Liposomes and for the Vesicle Suspension Obtained upon Addition of Oleate Micelles to the Preformed Ferritin-Containing POPC Liposomes[a]

	POPC liposomes	mixed POPC/oleic acid/oleate vesicles
mean vesicle diameter (nm) ± SD	91 ± 27 (1371)[b]	57 ± 39 (2149)[b]
mean no. of ferritin molecules per vesicle	4.5	1.9
mean no. of lamellae per vesicle	1.20	1.16

[a] All values reported in this table are based on the analysis of micrographs obtained by cryo-TEM. The figures in bracket indicate the number of vesicles counted. [b] We have performed a T-test for the two populations of vesicles. This statistical analysis gives a so-called *P* value, which is a measure of the probability that the difference in the observed mean values between the two populations is due to chance. The unpaired T-test, the paired T-test and the Welch T-test give values less than 0.001. This means a probability less than l:10000 that the difference between the two mean values is due to chance.

This is attended by a very significant increase in the percentage of vesicles containing no ferritin molecules. Furthermore, the average vesicle diameter decreases from 91 to 57 nm. The polydispersity of the suspension also increases, as indicated by the standard deviation (Table 1). All this is consistent with the formation of a large number of new small vesicles which are mostly empty, as we will see more clearly later on. Note also from Table 1 the low average lamellarity degree, with a slight tendency to farther decrease after addition of surfactant.

Figure 5B shows a significant similarity between the two distributions (before and after addition). To better clarify this point, it is important to consider more in detail the size distribution of the mixed POPC/oleic acid/oleate vesicles obtained upon addition of oleate.

Analysis of the Size Distributions after Oleate Addition. Let us consider first an overview of the situation, as given by Figure 6A, which gives the mass-weighted size distribution before and after oleate addition. In Figure 6A, the size distributions before and after oleate addition are both normalized to their own 100%. Notice, however, that the final lipid concentration, i.e., after oleate addition, is twice as large as the initial one. Note the strong similarity of the two distributions: in both cases most of the lipid is confined in the same size range (between 70 and 150 nm diameter). A closer inspection shows, however, some differences. The suspension obtained after oleate addition contains more large vesicles (diameter larger than 200 nm) as well as more small vesicles (diameter smaller 70 nm).

These differences appear more clearly if we examine Figure 6B, which shows the "absolute"[25] number-weighted size distribution: most of the newly formed vesicles have a diameter smaller than 60 nm. In other words, while the "matrix effect" feature of the reproduction of the initial distribution is respected in the main central region (ca. 70−150 nm), we observe something additional and unexpected: the presence of very many small vesicles.

Figure 6C also shows the number-weighted size distribution of the vesicles obtained after oleate addition. Empty and filled vesicles are represented individually in this figure. Notice that the empty vesicles are mostly found in the left-hand side.

These observations raise the question whether these new small vesicles are mixed POPC/oleic acid/oleate vesicles or whether

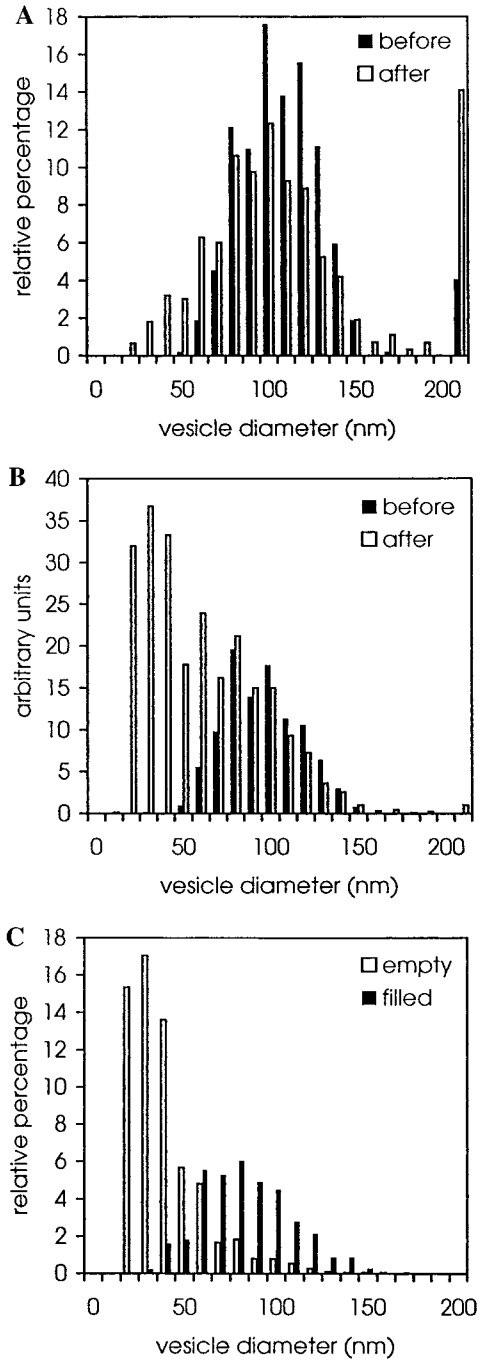

Figure 6. (A) Mass-weighted and (B) "absolute"[25] number-weighted size distributions as obtained by cryo-TEM for the preformed POPC liposomes ([POPC] = 6.1 mM; black bars) and for the vesicle suspension obtained after addition of oleate to preformed POPC liposomes ([POPC] = 3 mM, [oleic acid + oleate] = 3 mM; empty bars). For the calculation of the mass-weighted size distribution, the vesicles were assumed to be spherical and unilamellar. (C) Number-weighted size distribution as obtained by cryo-TEM for the vesicles obtained after addition of oleate to preformed POPC liposomes ([POPC] = 3 mM, [oleic acid + oleate] = 3 mM). Empty (empty bars) and ferritin-containing vesicles (black bars) are represented individually in this histogram.

instead they are 100% oleic acid vesicles (de novo formation of vesicles). In the first case, the very small mixed vesicles should have derived from the original POPC liposomes after uptake of added oleate; i.e., they should have originated from a process of fission. In the second case, they should instead have formed spontaneously and regardless of the presence of POPC liposomes. As already discussed in relation to Figure 1,

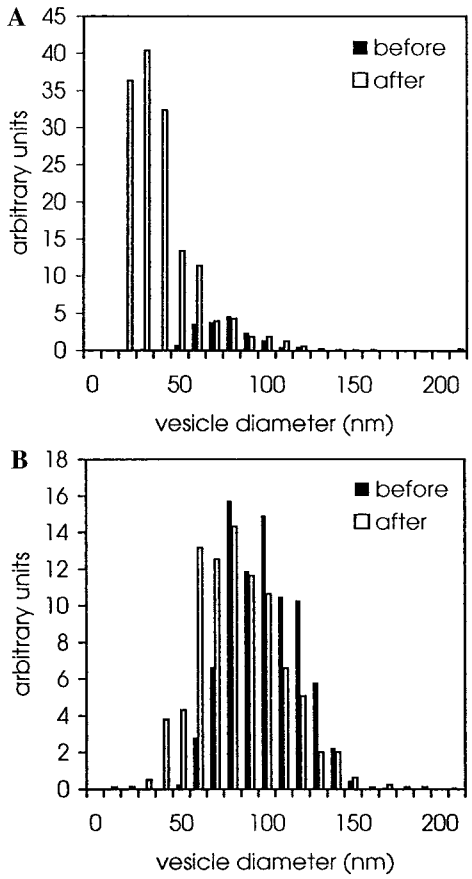

Figure 7. Comparison of the "absolute"[25] number-weighted size distribution (A) of the empty and (B) of the filled preformed POPC liposomes ([POPC] = 6.1 mM; black bars) with the vesicles obtained after addition of oleate ([POPC] = 3 mM, [oleic acid + oleate] = 3 mM; empty bars).

this second mechanism is unlikely in view of the fast kinetics underlying the formation of nascent mixed vesicles.

To clarify this point, let us consider Figure 7A,B, which compare the "absolute" number-weighted size distributions of the empty and of the filled vesicles, respectively, before and after addition of oleate. Figure 7A evidences that the newly formed vesicles, which do not contain any ferritin molecules, are smaller than 60 nm. On the other hand the distributions of the filled vesicles are very similar (Figure 7B): the size range is confined between 40 and 150 nm, with a main peak in the 70−100 nm region. However, beyond this first qualitative analogy, a significant difference is also apparent after oleate addition: the relatively higher number of small filled vesicles after oleate addition. This observation is mechanistically very important: in fact, these new small filled vesicles (diameter 30−50 nm) can only derive from division processes of the initial filled POPC liposomes. Notice from Figure 7B that the number of filled vesicles having a diameter between 50 and 70 nm also increased after oleate addition. On the other hand, the number of large filled vesicles (diameter between 100 and 130 nm) decreased. These two observations are consistent with each other and supportive of a fission mechanism.

Concluding Remarks

Aside from the question of the matrix effect and fission processes of vesicles, the present study could shed light on some properties of oleic acid vesicles obtained by spontaneous vesiculation upon addition of oleate micelles to an aqueous solution. This process gives rise to the formation of oleic acid vesicles distributed around a main peak at 50 nm. This might indicate that this is a very stable size for such vesicles under the studied conditions. The fact that the "nascent" oleic acid vesicles obtained from fission processes are also centered around 30−40 nm may also be taken as an indication of a particular stability of these small sizes.

The very low lamellarity degree of these oleic acid vesicles is another surprising feature of this spontaneous vesiculation. Furthermore, the observation that a higher concentration of oleic acid surfactant gives rise to a sharper size distribution is per sé very interesting and might have some bearing with the matrix effect (a possible contribution from the excluded volume to the size distribution of vesicles).

The main purpose of this work was to study by cryo-TEM the matrix effect and to shed light on the vesicle transformations underlying this mechanism. Two main features have appeared from this investigation: the substantiation of the matrix effect, at least under the particular conditions used here, and the evidence of fission processes that yield small vesicles. Concerning the matrix effect, cryo-TEM analyses have shown that the freshly added surfactant mostly concentrates in vesicles with a size that does not exceed the one of the pre-added, extruded ones. This is particularly evident when we consider the mass-weighted distribution shown in Figure 6A.

In relation to the matrix effect, it must be emphasized again that in this work we limited ourselves to a restricted set of conditions, so that the findings outlined here do not have the character of generality. Also, cryo-TEM gives information only on the final state of the suspension, and not on the intermediate steps that remain unknown.

One would also like to understand by which mechanism the very many small vesicles, empty or filled, originate. It seems clear that this must be the result of a prerequisite oleate uptake by the POPC liposomes. Could the formation of the small vesicles simply be the result of excluded volume effect caused by the presence of preformed POPC liposomes? It does not seem possible, in view of the kinetic evidence presented in Figure 1, in view of literature data that advocate an extensive binding of fatty acids to POPC liposomes,[20−24] and in view of the fact that several small vesicles also contain ferritin molecules.

If fission is preceded by an extensive uptake, then most likely there is an initial growth of the original vesicles. Why and at which point in time there is eventually fission is not clear.

Our previous studies, mostly based on DLS,[6,7] failed to provide evidence on the formation of these small vesicles because DLS is not sensitive enough to detect small particles in a polydisperse suspension. In fact, this work can be taken as an illustration of the power of cryo-TEM analysis in the study of vesicle transformations and vesicle size distributions.

Fission processes of vesicles are important as part of their general physicochemical behavior. In addition, vesicle fission can be seen as a first very simple model for cellular fission. In particular, notice that in this case simple physical factors govern the division of self-organized bilayer structures and that such a fission may yield products with controlled size dimensions.

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(25) As the number of ferritin molecules does not change upon oleate addition, if the mean number of ferritin molecules per vesicle decreases, it means that the number of vesicles increases. 2.4 is defined as the"increase in number" factor and will be used to calculate "absolute" number-weighted size distributions. In these representations, the number of vesicles is normalized to 100 before oleate addition and to 240 after oleate addition. It permits to observe more directly what kind of vesicles (e.g., small/large ones) was newly formed.