Growth and Transformation of Vesicles Studied by Ferritin Labeling and Cryotransmission Electron Microscopy

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An investigation of the processes of growth and division of oleic acid/oleate and POPC/oleic acid/oleate vesicles (POPC stands for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) is presented. Preliminarily, kinetic turbidimetric studies show that the very slow process of spontaneous formation of vesicles from oleate micelles is dramatically accelerated by the presence of a small amount of preformed POPC vesicles ([POPC]/[oleic acid + oleate] = 1:25), suggesting in the latter case a quite different process of vesicle formation. The changes in the size distribution of preformed vesicles is then studied by cryotransmission electron microscopy (cryo-TEM) and by using an entrapped protein, ferritin, which, due to its dense iron core, can be detected by cryo-TEM. In this way, the size distributions of "filled" (ferritin-containing) and "empty" vesicles were determined. Addition of oleate to preformed POPC vesicles resulted in a shift of the size distribution of the ferritin-containing vesicles toward larger diameters, together with an increase in the number of vesicles. Most of these vesicles were empty, unilamellar, and often smaller than the preformed ones. This suggests the generation of new vesicles through a fission process of larger vesicles, which arise from the preformed vesicles. Oleic acid and oleate molecules produced in situ through the hydrolysis of oleic anhydride (added as a supernatant to an aqueous suspension containing preformed ferritin-containing oleic acid/oleate vesicles) led to a broadening of the vesicle size distribution and an increase of the percentage of empty vesicles. All of the data taken together give evidence of the growth of vesicles upon addition of fresh surfactant, as well as evidence of fission processes of larger vescicles that lead to a large number of small vesicles.

Introduction

The growth in size and number of vesicles is an interesting problem from the general point of view of the physicochemical behavior, and it has a particular meaning since lipid vesicles (also called liposomes or simply vesicles) are generally considered to be suitable models for protocells. Growth, budding, fission, and other vesicle transformations have been studied theoretically, and a few experimental investigations have been presented using mainly giant vesicles and light microscopy.[1−12] However, no systematic study on growth has been offered until now. In the case of submicrometer-sized vesicles, a lack in experimental data may be mostly due to a lack of a suitable methodology to follow such processes.

In this paper, we present a study about the formation and transformation of vesicles using a new methodology which involves the combination of two major tools, namely, the use of cryotransmission electron microscopy (cryo-TEM) and the use of an electron microscopically detectable marker entrapped in the internal aqueous volume of the vesicles.

Ferritin, a water-soluble protein, was chosen as the marker. Ferritin, which is an iron-storage protein in plants and mammals, consists of a hollow protein shell of ca. 12 nm containing an inorganic ferric "iron core" of ca. 7.8 nm in its center.[13,14] The very dense iron core gives rise to scattering contrast, which facilitates its detection in electron microscopy. The vesicle suspensions were frozen (vitrified) as thin aqueous layers and examined at low temperature in a transmission electron microscope. TEM of a frozen, hydrated sample provides a projection image of the object, thus permitting the determination of vesicle size and lamellarity as well as the visualization of macromolecules entrapped in the vesicle.[15]

Two types of vesicles were used in the present work: (a) oleic acid/oleate vesicles which were composed of oleic acid as well as oleate molecules (for the sake of simplicity, we will refer to these vesicles as oleic acid vesicles); (b) the classic POPC vesicles (POPC is the abbreviation for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine). Oleic acid vesicles are formed at intermediate pH (ca. pH 7.5−9.0), i.e., where both the deprotonated (the soap) and the protonated (the fatty acid) form coexist.[16−20] Oleic acid vesicles can be obtained "spontaneously"[21] (e.g., by adding a micellar solution of sodium oleate to an aqueous phase buffered at the required, intermediate pH). The formation of oleic acid vesicles has been studied to some extent in our group,[22−25] emphasizing the "self-reproduction" aspect of such fatty acid vesicle systems, namely, the autocatalytic process in the vesicle formation and growth as observed under certain experimental conditions.

The main procedure used here to induce size growth and transformations of vesicles is the addition of fresh oleate and oleic acid amphiphiles to preformed vesicles which had been first prepared with a defined mean diameter of about 100 nm. These preformed vesicles were either oleic acid or POPC

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vesicles. The choice of POPC was based mostly on the observation reported earlier, by other authors and by us, that phosphatidylcholine vesicles can incorporate oleic acid molecules in their bilayer, giving rise to mixed POPC/oleic acid/oleate vesicles,[21,26] and that, likewise, oleic acid vesicles may be able to efficiently bind and incorporate added oleate surfactant.[27]

The addition of oleate to preformed vesicles was carried out in two different ways: either as an unbuffered aqueous solution of oleate micelles to a buffered POPC vesicle suspension at pH 8.5 or generated in situ through the hydrolysis of the water-insoluble oleic anhydride which was added as a supernatant to a buffered solution containing the preformed oleic acid vesicles.

Materials and Methods

Chemicals. Sodium oleate (*cis*-9-octadecenoic acid sodium salt, >99%), *N*,*N*-bis(2-hydroxyethyl)glycine (bicine) (>99.5%), and boric acid (>99.5%) were obtained from Fluka, Buchs, Switzerland. Oleic anhydride (99%) and ferritin (from horse spleen) were purchased from Sigma, Buchs, Switzerland. The POPC used was from Avanti Polar Lipids, Alabaster, AL. The Sepharose 4B used was from Pharmacia, Uppsala, Sweden.

Turbidity Measurements. The vesicle formation was followed by recording time-dependent changes in the turbidity by using a UV/vis spectrophotometer as described before.[21]

Cryo-TEM. Copper grids (300 mesh) coated with a perforated carbon film according to a modified procedure described by Fukami and Adachi[28] were used for the preparation of the frozen hydrated specimen by the bare-grid technique.[29] The grids were rendered hydrophilic by glow-discharge before use. The thin aqueous films were prepared under controlled temperature (room temperature) and humidity conditions (97−99%)[30] within a custom-built environmental chamber.[31]

A total of 10 μl of the vesicle suspension was put on the grid. The excess liquid was blotted with filter papers 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 1K × 1K CCD camera (Proscan, Germany).

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 obtained by plotting the relative number of vesicles (given in percentage of the total number of vesicles) against the vesicle diameter (nm). 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 (nm).

To check the validity of the cryo-TEM method, the size distribution of the vesicles was also deduced using the freeze−fracture technique (ffEM).[32] The size distributions determined by the two electron microscopic methods were in good agreement (data not shown).

Preparation of Ferritin-Containing POPC Vesicles. The POPC vesicles used in the present study were first prepared by the reverse phase method as described by Szoka and Papahadjopoulos.[33−34] POPC (23 mg) was dissolved in diethyl ether (5 mL) in a 50 mL round-bottom flask. A total of 1 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 (gel-like) suspension was obtained. The gel-like mixture was then vortexed until a low-viscosity suspension was obtained. An extra 1 mL of borate buffer was added, and the suspension was kept in a rotary evaporator at 100 mbar for 10 min and finally at 50 mbar for an additional 5 min. The obtained vesicles (a suspension of 2 mL) were sized down to approximately 100 nm using "The Extruder" from Lipex Biomembranes Inc., Vancouver, Canada.[35] The suspensions were 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.

Preparation of Ferritin-Containing Oleic Acid Vesicles. A desired amount of sodium oleate (typically 15 mg) was transferred into a 10 mL graduated reagent flask, and methanol (2 mL) was added. Methanol was removed with a rotary evaporator under reduced pressure ($T_{\text{water bath}}$ < 40 °C). The obtained oleate film was further dried under high vacuum overnight. A ferritin solution (25 mg/mL or 50 mg/mL) in bicine buffer (200 mM, pH 8.5) was then added to the flask so that the total oleic acid + oleate concentration was 80 mM, and the dispersion was vortexed until complete dissolution of the film. The suspension was then repetitively (10 times) frozen in liquid nitrogen (3 min) and subsequently thawed in lukewarm water (30 °C). Between each of these freezing−thawing cycles, the suspension was briefly vortexed. The vesicles were sized down using a "LiposoFast" device manufactured by Avestin Inc., Ottawa, Canada.[36] The suspensions (0.5−0.7 mL) were first forced 21 times through two stacked polycarbonate filters with pores of 400 nm diameter. The same procedure was then repeated with 200 nm and finally with 100 nm pore size filters.

Gel Permeation Chromatography. Nonentrapped ferritin molecules were removed by gel permeation chromatography using a Sepharose 4B column (length 50 cm, diameter 1.2 cm) equilibrated with the appropriate buffer prior to use. The vesicle suspension (0.3−1 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), the oleic acid concentration was determined by FTIR (Fourier transform infrared spectroscopy) (C═O stretch band at 1715 cm^{-1}) as described by Walde et al.,[22] and the POPC concentration was determined by using the Stewart assay.[37]

For each experiment, only the fraction containing the highest lipid concentration was examined by cryo-TEM and further used as preformed vesicles.

Addition of Oleate Micelles to Preformed POPC Vesicles.[21] An aqueous micellar solution of oleate (25 mM) was prepared by dissolving sodium oleate in H_2O. This oleate solution (0.2 mL) was then added to ferritin-containing POPC vesicles (0.8 mL, [POPC] = 0.25 mM). The vesicle suspensions were then examined before and after oleate addition by cryo-TEM. The molar ratio of POPC to oleic acid was 1:25.

Hydrolysis of Oleic Anhydride in the Presence of Oleic Acid Vesicles.[22,25] The ferritin-containing oleic acid vesicle suspension (1 mL, 5.5 mM oleic acid + oleate) was transferred

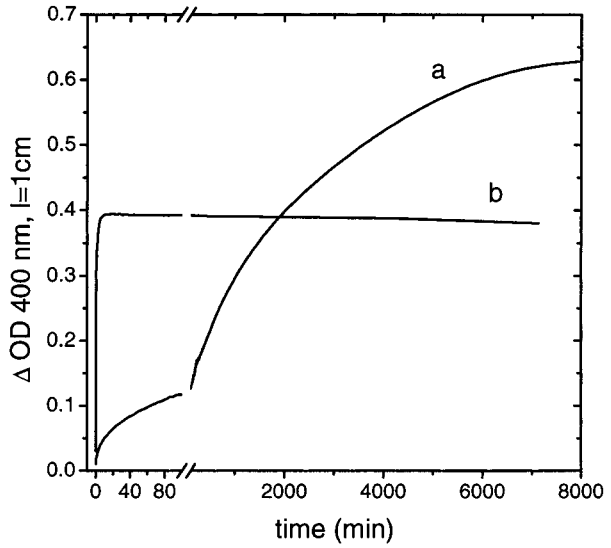


Figure 1. Effect of the presence of preformed extruded 100 nm POPC vesicles on the kinetics of mixed oleic acid/oleate/POPC vesicle formation at 25 °C, as followed turbidometrically at 400 nm. A solution of 0.2 mL of 25 mM sodium oleate in water was added either to 0.8 mL of 0.1 M borate buffer solution, pH 8.5 (a), or to 0.8 mL of 0.25 mM POPC vesicle suspension, pH 8.5 (b) in a 1 mL UV/vis quartz cell with a path length of 1 cm. [oleic acid + oleate] after mixing, 5 mM; (a) no preformed vesicles; (b) in the presence of preformed 100 nm POPC vesicles. [POPC] after mixing, 0.2 mM; [oleic acid + oleate]/[POPC] = 25:1.

to a flat-bottom flask (diameter 13 mm) containing a magnetic stirrer (10 mm × 5 mm). Oleic anhydride (12 mM with respect to the total volume) was laid on top of the vesicular suspension, and the reaction mixture was slowly stirred at room temperature for 4 days. The final oleic acid + oleate concentration was 28 mM. The total oleic acid + oleate concentration of the initial and of the final suspension was determined by FTIR.[22,25]

Results and Discussion

Preliminary Kinetic Analysis. One preliminary question is whether the kinetics of vesicle formation upon addition of oleate to a buffered solution of pH 8.5 is affected by the presence of preformed vesicles. In principle, both the rate process and the final size distribution may be influenced. In fact, previous work in our laboratory has suggested that this may be the case.[21,27]

To assess whether and to what extent the rate process of vesicle formation is affected by the presence of POPC vesicles under the conditions used in the present work, we have measured the time progress of optical density (turbidity) change upon the addition of oleate surfactant to an aqueous solution containing a small amount of extruded 100 nm POPC vesicles. A ratio of [POPC]/[oleic acid + oleate] = 1:25 was chosen.

The results are shown in Figure 1. Before analysis of the data, it is necessary to recall that turbidity measurements as those presented in Figure 1 are only a rough measure for the formation of vesicles. In fact, light scattering is a complex function of both the number of particles and of their respective volume, and actually the volume has an overwhelmingly statistical weight. In other words, a turbidity change is sensitive both to an increase in the of number of particles and (to a greater extent) to an increase in their size.

The rate process of vesicle formation shown in Figure 1 (curve a), which reflects the formation of pure oleic acid vesicles, is rather slow, and several days are needed in order to attain a kind of equilibrium. Note that in this experiment—and also in the next one—oleate is added as a micellar solution (see figure legend).

The kinetic pattern is completely different in the presence of a small amount of POPC vesicles (Figure 1, curve b): the turbidity change is extremely fast, and a plateau is immediately reached, which remains constant over several days. Clearly, the presence of a small amount of vesicles is sufficient to completely change the process of vesicle formation (in this case, mixed vesicles with ca. 4 mol % of POPC and 96 mol % oleic acid/oleate). Also notice that the final optical density is about 50% lower than that obtained with oleate alone—although the final surfactant concentration is about the same.

Clearly, a strong interaction between oleate and POPC must occur, which is in keeping with the effective oleic acid/oleate uptake by POPC liposomes as documented in the literature.[26] The first observation that stems from Figure 1 is that the "unperturbed" de novo formation of (POPC-free) oleic acid vesicles according to the slow process in Figure 1 (curve a) is no longer possible. Most of the vesicles which are now produced must reasonably be the outcome of the interaction between oleate and preformed POPC vesicles. It also appears possible to state, based on the final optical densities, that the size distributions in the two cases are different and that most likely the very large sizes, responsible for high scattering, are missing in the population of mixed vesicles. Thus, the presence of a small "catalytic" amount of POPC vesicles modifies the process of vesicle formation in three significant respects: (i) it increases the rate of vesicle production by at least an order of magnitude; (ii) it brings about a new mechanism of vesicle formation; and (iii) it induces a size distribution less biased toward larger sizes.

The necessary condition for all this is that the rate of formation of de novo vesicles is much smaller than the rate by which the POPC bilayer takes up the oleate molecules.

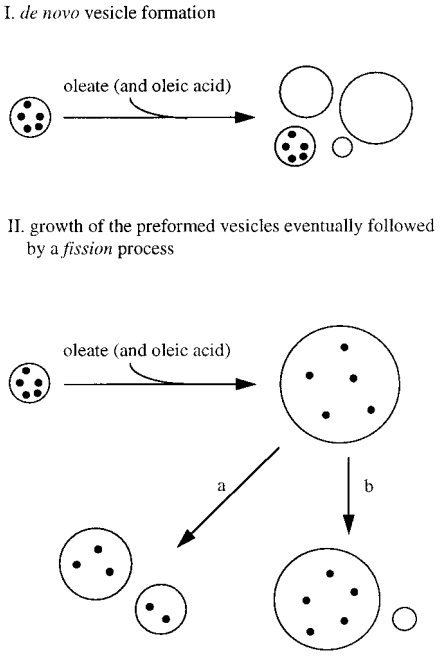
What then is the meaning of the mechanism outlined above? Once the formation of de novo vesicles is ruled out, one can envision the two following cases to account for the rapid increase of optical density in the case of curve b in Figure 1: vesicles grow in size by uptake of oleate and oleic acid in their bilayers, and/or the preformed vesicles incorporate oleic acid/oleate molecules in their bilayers and then divide by a fission process.

How can one experimentally distinguish between these different processes? One main approach is to label the preformed vesicles, so as to distinguish them from newly generated ones. This can be done, as already mentioned in the Introduction, by entrapping ferritin in the preformed vesicles and by examining the vesicle suspensions by cryo-TEM. In this way, by comparing the initial state (before addition) with the final state of the vesicular suspension (after addition), one can obtain information on the processes.

Scheme 1 illustrates the expected situations. The process I of Scheme 1 represents the de novo formation of vesicles, which should not be very significant in our case. In the case in which the vesicles are simply growing upon oleate addition (as illustrated by process II of Scheme 1), one would expect that the number of vesicles would remain constant while their average diameter would increase; also, one would expect that the ferritin distribution would not change. Conversely, if fission processes are present (as illustrated by the processes IIa and IIb in Scheme 1), one would expect that the number of vesicles would increase and that the mean number of ferritin molecules per vesicle would decrease.

Scheme 1 is the basis for the interpretation of the TEM data, as presented in the next sections.

SCHEME 1 . Schematic Representation of the Possible Vesicle Formation and Transformation Processes if Oleate (and Oleic Acid) Are Added to Preformed Vesicles Which Have Been Labelled with a Water-Soluble Marker[a]



[a] (I) shows the situation if only de novo vesicle formation occurs. (II) illustrates a growth in size of the preformed and labelled vesicles which may lead to a splitting (fission process), either yielding vesicles which all contain marker molecules (case a, a statistical redistribution of the ferritin molecules is thus obtained) or yielding vesicles which do not all contain markers (case b). See text for details.

Preparation of Ferritin-Containing Vesicles. The first step of the EM work consisted of entrapping ferritin molecules in vesicles. To this aim, the ferritin-containing vesicles were prepared by using the following established procedures (for details see Materials and Methods). In the case of ferritin-containing oleic acid vesicles, a dry thin oleate film was first prepared and then dispersed in aqueous buffer solution containing at least 25 mg/mL ferritin, which corresponded to a 7-fold excess of ferritin molecules over the calculated number of oleic acid vesicles (assuming for this calculation that the vesicles were all unilamellar and had a diameter of 100 nm). The suspension obtained was then treated by several freezing−thawing cycles.[38,39] This entrapment and vesicle formation procedure led to a rather heterogeneous vesicle suspension containing very large, multilamellar vesicles as well as small vesicles. To render the suspension more suitable for cryo-TEM investigations,[40] the vesicles were sized down by extrusion, the last extrusion step being performed by using 100 nm pore size polycarbonate membranes. In the case of ferritin-containing POPC vesicles, the so-called reverse phase evaporation method was first used,[33,34] followed again by a vesicle size reduction and homogenization with extrusion.

Nonentrapped ferritin molecules were removed from the suspension in both cases by gel permeation chromatography using a Sepharose 4B column, as shown in Figure 2 in the case of POPC vesicles. Free ferritin molecules (fractions 26−46, Figure 2) could be efficiently separated from the vesicles (fractions 18−23, Figure 2). These latter fractions contained ferritin-filled as well as empty vesicles.

Whereas the POPC vesicles present in the initial suspension formed a rather homogeneous vesicle population, and most of the vesicles contained entrapped ferritin (Figure 3A), the situation was considerably different after addition of oleate (see later on) (Figure 3B). Here the system was much more heterogeneous in size; furthermore, most of the large vesicles contained ferritin molecules, whereas the small ones were mainly empty.

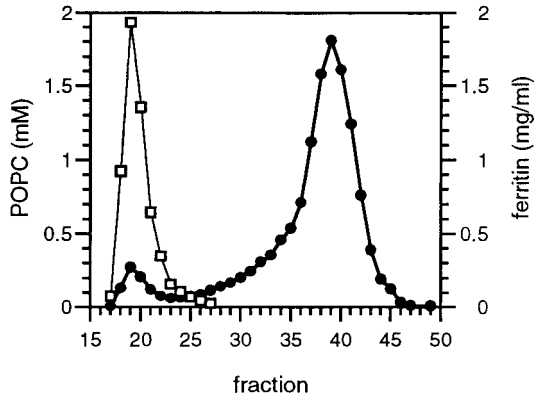


Figure 2. Separation of nonentrapped ferritin molecules from ferritin-containing POPC vesicles using a Sepharose 4B column (length 50 cm, diameter 1.2 cm). The POPC vesicles were prepared in borate buffer (0.1 M, pH 8.5) by the reverse phase evaporation method, followed by a sizing down to about 100 nm by extrusion. The vesicle suspension applied onto the column had a volume of 0.45 mL, and fractions of 1 mL were collected. The open squares represent the POPC, and the filled circles represent the ferritin concentrations.

On the basis of the cryo-TEM data, the mean number of ferritin molecules per vesicle, the mean vesicle diameter, and the mean number of lamellae per vesicle (see Table 1) were determined for each experiment. Table 1 shows the various mean values obtained for the POPC vesicles prepared and for the vesicles formed after oleate addition to these preformed POPC vesicles (see below). The POPC vesicles had an average diameter of ca. 100 nm, and 90% of the vesicles were unilamellar.

The distribution of the ferritin molecules among the vesicles is shown in Figure 4 (filled bars). Note that the ferritin molecules were not distributed homogeneously among all the vesicles, and although most of the vesicles were filled, empty vesicles were always also present. For example, 15% of the POPC vesicles did not contain any ferritin molecules while, interestingly, some others entrapped as many as 50 ferritin molecules (Figure 4, filled bars).

The POPC as well as the oleic acid vesicles prepared and used in the present study had a rather narrow size distribution, centered around the pore diameter used in the final extrusion step (100 nm); see Figure 5A for the POPC vesicles (filled bars) and later on Figure 9A for the oleic acid vesicles (filled bars). Under the experimental conditions used, ferritin could be successfully entrapped in both POPC and oleic acid vesicles.

In conclusion, it was possible to obtain rather homogeneous vesicle preparations in which 80−90% of the vesicles contained the water-soluble marker protein ferritin. The percentage of total ferritin entrapped and the ratio empty/filled vesicles was dependent on the ferritin concentration present during vesicle formation as well as on the vesicle preparation procedure used (data not shown).

It should be pointed out that the mean vesicle size and the vesicle size distribution reported in this work were rather reproducible. This observation is important in view of the fact that vesicles are generally not equilibrium systems,[41] and therefore, their average size and size distribution depend on the preparation method. In our work, the mean size and size

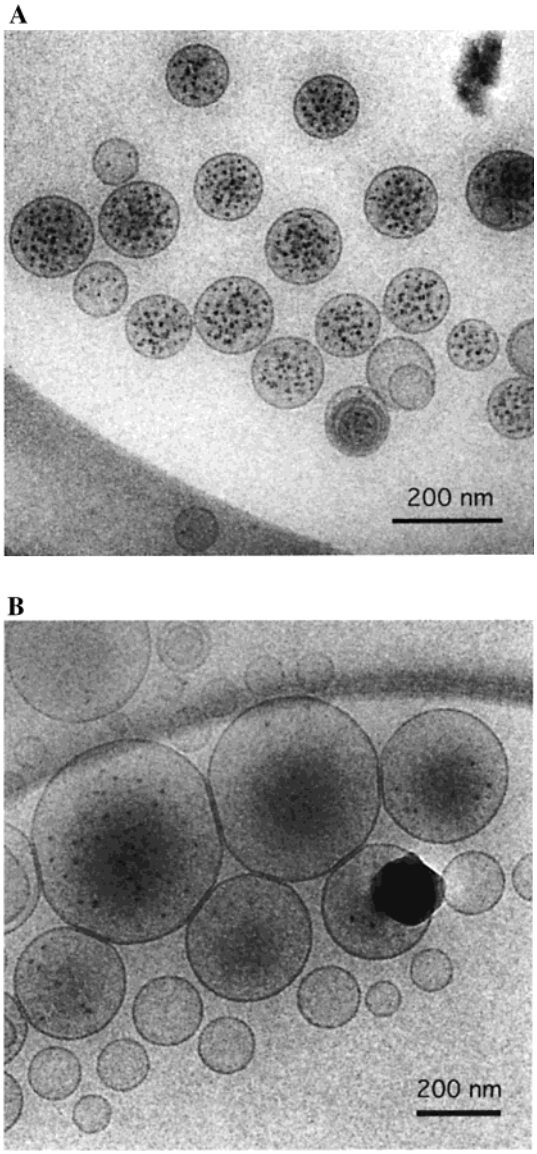


Figure 3. (A) Cryo-TEM micrographs of ferritin-containing POPC vesicles which have been obtained by the reverse phase evaporation method, followed by a sizing down by extrusion through polycarbonate membranes with 100 nm pore diameters. Some vesicles contained a large number of ferritin molecules. [POPC] = 1.9 mM. (B) Cryo-TEM micrographs of the vesicle suspension obtained after adding oleate to preformed ferritin-containing POPC vesicles. Most of the large vesicles detected contained ferritin molecules, while the small ones were mostly empty. [POPC] = 0.2 mM, [oleic acid + oleate] = 5 mM. Note: within the vitrified film, the vesicles are arranged according to their size, the larger vesicles being at the border of the hole, the smaller ones being more toward the center of the hole.

distribution of the preformed vesicles were determined by the extrusion procedure (see Materials and Methods).

Addition of Oleate to Preformed Ferritin-Containing POPC Vesicles. Let us now consider the results obtained by adding fresh oleate micelles to ferritin-containing 100 nm POPC vesicles. The molar ratio of POPC to oleic acid/oleate was 1:25 in the final suspension. The results are therefore relative to this particular set of concentration and should not be generalized too much. Consider in this regard that a systematic investigation of a wider concentration range would be extremely time-consuming; for the analysis presented in this section alone, ca. 250 micrographs had to be recorded and individually evaluated.

Figure 3A,B illustrates how the micrographs typically looked

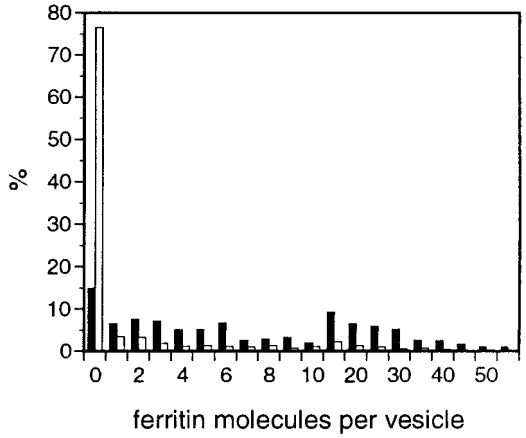


Figure 4. Ferritin distribution among all the vesicles as obtained by cryo-TEM for the preformed ferritin-containing POPC vesicles ([POPC] = 1.9 mM; filled bars) and for the vesicle suspension obtained after oleate addition to the preformed ferritin-containing vesicles ([POPC] = 0.2 mM, [oleic acid + oleate] = 5 mM; empty bars).

TABLE 1: Summary of the Main Observations Made in the Case of the POPC/Oleic Acid/Oleate System[a]

	POPC vesicles	mixed vesicles
mean diameter ± SD (nm)	97 ± 33 (811)	101 ± 63 (2167)
mean number of ferritin molecules per vesicle	11	2.4
mean number of lamellae per vesicle	1.1	1.1

[a] The mean vesicle diameter, the average number of ferritin molecules per vesicle, and the number of lamellae per vesicle were determined experimentally for the preformed ferritin-containing POPC vesicles and for the vesicle suspension obtained upon addition of oleate micelles to the preformed ferritin-containing POPC vesicles. All values reported in this table are based on the analysis of micrographs obtained by cryo-TEM. The numbers in parentheses indicate the number of vesicles counted. See text for discussion.

before (Figure 3A) and after (Figure 3B) oleate addition to the preformed ferritin-containing POPC vesicles. Figure 4—which compares the ferritin distribution among the vesicles before (filled bars) and after (empty bars) oleate addition—indicates that the addition of oleate resulted in a significant increase in the percentage of empty vesicles (0 ferritin molecule per vesicle). Looking at the change in the mean number of ferritin molecules per vesicle permits one to quantify more precisely the increase in the number of vesicles (see Table 1). The preformed POPC vesicles contained on average 11 ferritin molecules, whereas the vesicles obtained after oleate addition contained 2.4 ferritin molecules on average. This decrease indicates that the total vesicle population increased by a factor of ca. 4.5, assuming that the absolute number of ferritin molecules did not change upon oleate addition. In keeping with the kinetic observations of Figure 1 and with the discussion relative to Scheme 1, this significant increase in the number of vesicles is the result of fission processes.

Table 1 also shows that while the mean number of ferritin molecules per vesicle diminished after oleate addition, the mean vesicle diameter only slightly increased. The standard deviation, however, was larger after oleate addition, indicating that the polydispersity of the suspension increased. Furthermore, the number of lamellae per vesicle did not change on average.

It is of interest to compare the number- and mass-weighted size distribution of the vesicles present in the initial and in the final suspensions. This is shown in parts A and B of Figure 5, respectively. Following oleate addition, there was a shift of the

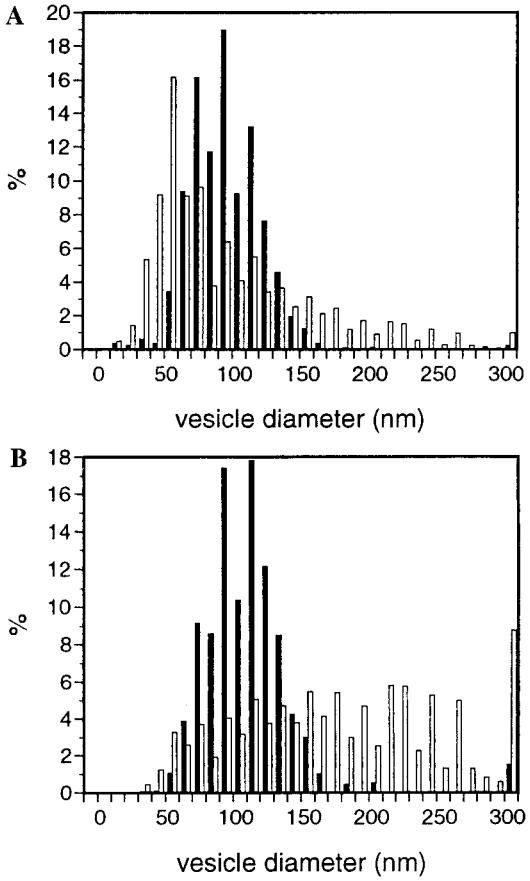


Figure 5. (A) Number-weighted and (B) mass-weighted vesicle size distributions as obtained by cryo-TEM for the preformed ferritin-containing POPC vesicles ([POPC] = 1.9 mM; filled bars) and for the vesicle suspension obtained after addition of oleate to preformed ferritin-containing POPC vesicles ([POPC] = 0.2 mM, [oleic acid + oleate] = 5 mM; empty bars). For the calculation of the mass-weighted size distribution, the vesicles were assumed to be spherical and unilamellar. The last bar of the histogram in both figures corresponds to all the vesicles larger than 300 nm.

main peak of the number-weighted size distribution (Figure 5A) toward smaller diameters whereas the overall number-weighted size distribution got broader, indicating again that the final suspension was more polydisperse than the initial one. Note in particular the presence of many more vesicles larger than 150 nm, which contribute significantly to the mass-weigthed size distribution (Figure 5B). For example, recognizing that a 200 nm vesicle is composed of about four times more amphiphiles than a 100 nm vesicle, it is interesting to consider the corresponding mass-weighted size distributions (Figure 5B). Whereas the number- and mass-weighted distribution of the preformed POPC vesicles were very similar, significant differences were observed after oleate addition; in this case, 19% of the vesicles larger than 150 nm represented 62% of the total lipid mass (compare parts A and B of Figure).

To visualize the ferritin distribution in the system, the entire vesicle population has been divided into two classes: empty and filled vesicles. Figure 6A shows that the preformed POPC vesicles smaller than 40 nm were generally empty, whereas the majority of the larger vesicles entrapped at least one ferritin molecule. After oleate addition, the ratio of empty/filled vesicles depended on the path followed by the newly added amphiphilic molecules. As shown in Figure 6B, after oleate addition, the vesicles smaller than 160 nm were mainly empty while the ones larger than 160 nm were mostly filled. Figure 6B suggests that

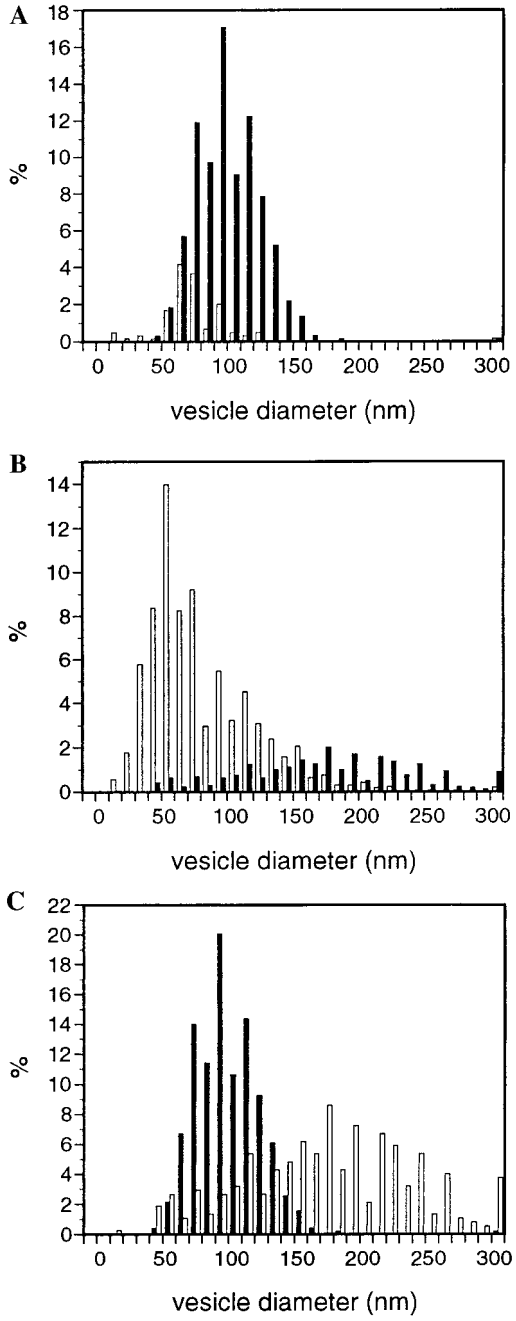


Figure 6. Number-weighted size distributions as obtained by cryo-TEM (A) for the preformed POPC vesicles ([POPC] = 1.9 mM) and (B) for the vesicle suspension obtained upon addition of oleate to preformed ferritin-containing POPC vesicles ([POPC] = 0.2 mM, [oleic acid + oleate] = 5 mM). Empty (empty bars) and ferritin-containing (filled bars) vesicles are represented individually in the histogram. (C) Direct comparison of the number-weighted size distribution of the preformed POPC vesicles which contained at least one ferritin molecule (filled bars) with the number-weighted size distribution of the ferritin-containing vesicles obtained after oleate addition to preformed POPC vesicles (empty bars). Note that the total of all ferritin-containing vesicles was set to 100%.

oleate addition gave rise to many small vesicles (main peak between 50−60 nm), smaller than the preformed ones (main peak between 90 and 100 nm, Figure 5 A, filled bars). The direct comparison of the size distribution of the filled vesicles present in the suspension before and after addition of oleate yielded information about the transformations undergone by the vesicles, since the ferritin-containing vesicles found after addition of oleate are assumed to be related to the vesicles

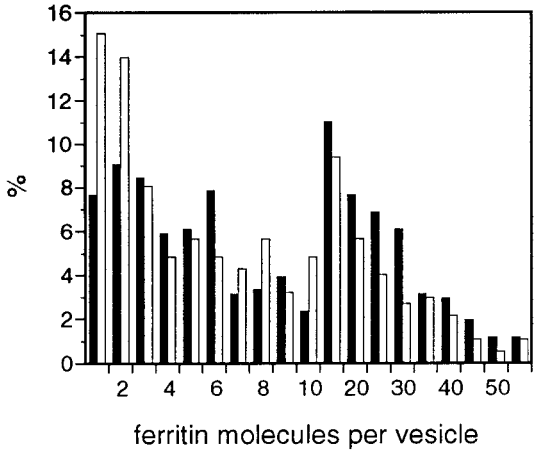


Figure 7. Ferritin distribution among the filled vesicles as obtained by cryo-TEM for the preformed ferritin-containing POPC vesicles ([POPC] = 1.9 mM; filled bars) and for the vesicle suspension obtained after oleate addition to preformed ferritin-containing vesicles ([POPC] = 0.2 mM, [oleic acid + oleate] = 5 mM; empty bars). In this graph, only vesicles that contained at least one ferritin molecule have been considered, and the sum of all filled vesicles was set to 100%. Note that the scale of the *x*-axis changes after 10.

present in the initial suspension. Figure 6C clearly shows that the size distribution of the filled vesicles is shifted toward larger diameters upon oleate addition, providing a clear evidence for a growth in size of the original ferritin-containing POPC vesicles.

Let us consider now the significant increase of small empty vesicles obtained after addition of oleate, as apparent from the comparison of Figure 6A (empty bars) with Figure 6B (empty bars). The most likely interpretation, as already mentioned, is that the small vesicles are produced by fission of larger growing ones. To demonstrate that this is indeed the case, one can analyze the ferritin distribution among the filled vesicles before and after oleate addition. This final ferritin distribution is reported in Figure 7 and shows that there is a significant increase of vesicles containing one or two ferritin molecules (up to 70−100%). These "new" vesicles that contained so little ferritin must have reasonably originated from the splitting processes of larger ferritin-containing vesicles.

Hydrolysis of Oleic Anhydride in the Presence of Preformed Ferritin-Containing Oleic Acid Vesicles. Let us now consider the second experimental configuration used in this work, the one in which oleic acid and oleate were produced in situ through the hydrolysis of the water-insoluble oleic anhydride, which was added as a supernatant to a buffered aqueous suspension of ferritin-containing oleic acid vesicles. As already mentioned, this (or a similar) experimental setup has been used before in our group to investigate the autocatalytic self-reproduction of vesicles[22−25] (and of micelles).[42−44] Furthermore, the hydrolysis of oleic anhydride has also been investigated within the frame of the so-called matrix effect in the formation of vesicles.[27,45] The autocatalytic vesicle self-reproduction and the matrix effect were not, however, the focus of this investigation. The emphasis of the present work was set on the in situ delivery of oleate and oleic acid molecules to preformed oleic acid vesicles to possibly induce transformations of the preformed vesicles. In the experiment which is reported here, the total oleic acid concentration was 5-fold higher after oleic anhydride hydrolysis than before hydrolysis. The entire experimental setup can be seen—with respect to what has been described above—as an alternative chemical way of oleate (and oleic acid) delivery to preformed vesicles. It was therefore interesting to study this system and to compare the data with the vesicle system described above.

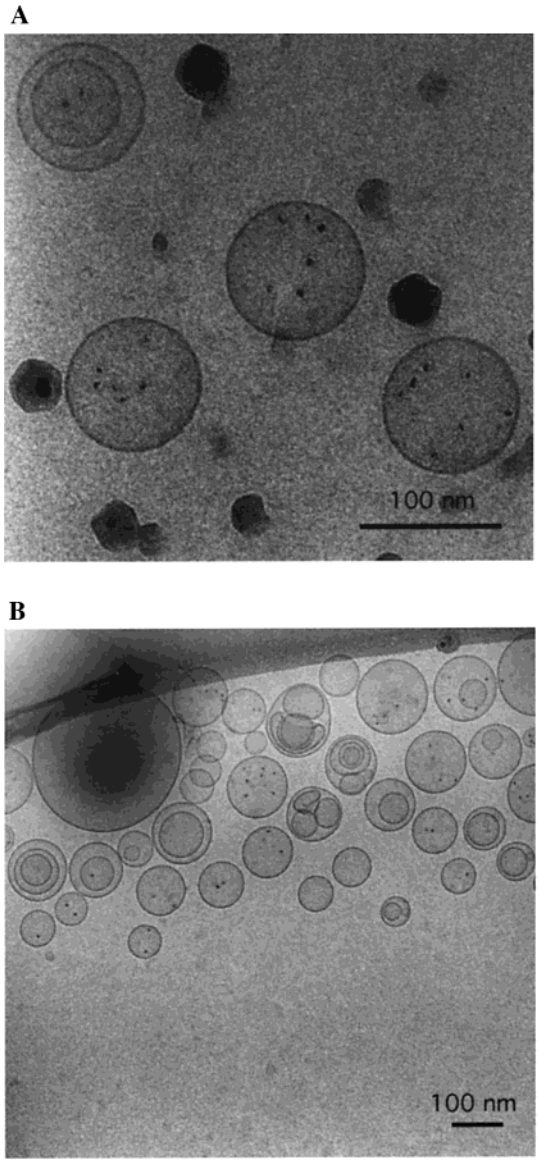


Figure 8. Cryo-TEM micrographs of (A) oleic acid vesicles prepared in the presence of 25 mg/mL ferritin in bicine buffer (200 mM, pH 8.5) (see Materials and Methods for details on the vesicle preparation) and of (B) the vesicular suspension obtained after complete hydrolysis of oleic anhydride in the presence of preformed oleic acid vesicles. [Oleic acid + oleate] before oleic anhydride hydolysis was 5.5 mM, while after hydrolysis it was 28 mM.

Parts A and B of Figure 8 show typical micrographs of the oleic acid/oleate suspensions obtained before and after hydrolysis , respectively. The oleic acid vesicles were more heterogeneous in size (and shape) after hydrolysis; note in particular the presence of larger vesicles entrapping other ones. Similar structures have been observed before in the case of giant oleic acid vesicles.[25] As in the case of POPC vesicles—compare Figure 8A with Figure 5A—the relatively homogeneous oleic acid vesicle size distribution before hydrolysis turns into a much broader one after oleic anhydride hydrolysis (Figure 9A).

Figure 9B shows the number-weighted size distribution of empty and filled vesicles after hydrolysis. Notice the formation of a significant number of empty vesicles both on the small and on the large side of the scale. In particular, after hydrolysis, most of the filled vesicles (85%) had diameters between 100

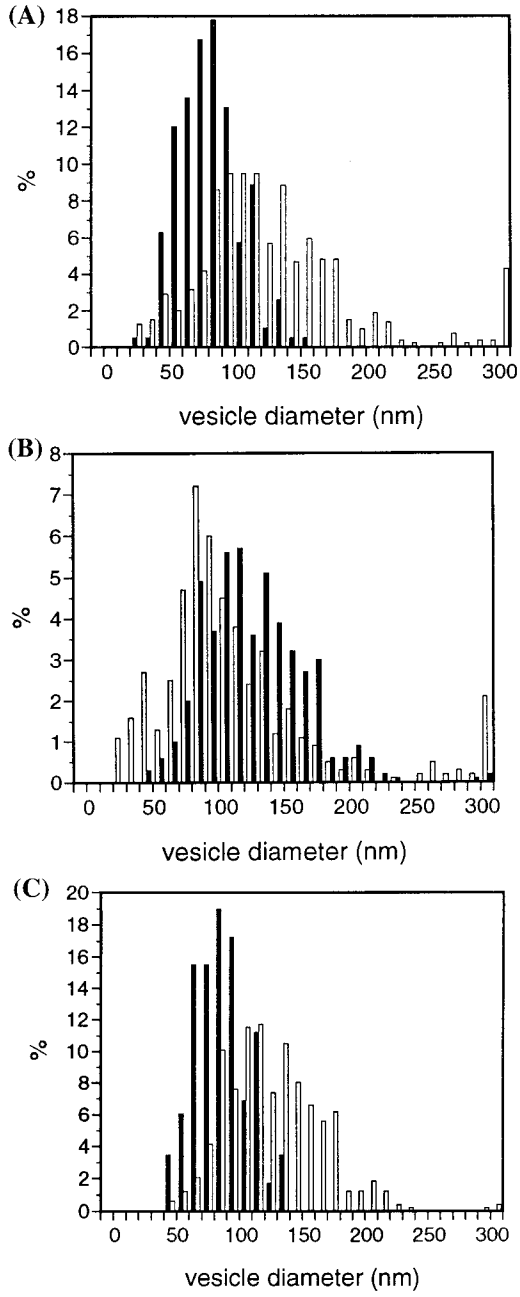


Figure 9. (A) Number-weighted size distribution as obtained by cryo-TEM for the oleic acid vesicle suspension analyzed before hydrolysis (filled bars) and after oleic anhydride hydrolysis (empty bars). (B) Number-weighted size distribution as obtained by cryo-TEM for the oleic acid vesicle suspension examined after oleic anhydride hydrolysis. Empty (empty bars) and ferritin-containing (filled bars) vesicles are represented individually. (C) Comparison of the number-weighted size distribution of the filled oleic acid vesicles obtained before (filled bars) and after (empty bars) oleic anhydride hydrolysis. The total of all ferritin-containing vesicles was set to 100%. The last bar of the histogram in the three figures corresponds to all the vesicles larger than 300 nm.

and 250 nm, whereas the majority of the other vesicles (diameters below 100 nm or above 250 nm) were empty. In other words, the hydrolysis of oleic anhydride resulted in the formation of both relatively small and relatively large vesicles. This is in contrast to the situation obtained in the case of oleate addition to preformed POPC vesicles.

Figure 9C shows another way of presenting the data and compares directly only the filled oleic acid vesicles before and after oleic anhydride hydrolysis. The significant shift of the size distribution of the filled vesicles toward larger diameters upon hydrolysis clearly indicates a size growth of the preformed oleic acid vesicles.

Conclusions and Outlook

There are two kinds of considerations which arise from this work. One concerns the general use of this novel technique in the field of vesicles in order to obtain information at least about their size and transformations; the other one concerns the particular case of oleic acid vesicles and vesicle suspensions obtained after addition of oleate to preformed POPC vesicles (a mixed POPC/oleic acid/oleate vesicle system).

Concerning the generality of the method, it appears that the combination of ferritin labeling with cryo-TEM enables one to obtain first-hand qualitative information on two major aspects of vesicle formation/transformation processes: growth in size and fission processes. The necessary condition for observing a growth in the size of vesicles is that the preformed vesicles are able to bind and uptake the added surfactant molecules and that this rate process is faster than the de novo formation of vesicles. We therefore believe that this method—also in view of the relative easy entrapment of ferritin in vesicles—can be of general applicability in the field of vesicles, despite the fact that the final size distributions are not, generally, equilibrium distributions.

It is also important to mention that the information gathered by this technique cannot be obtained by other physical methods which yield average values, such as turbidity or light scattering. Light scattering, for example, can provide valuable qualitative information about a shift of the vesicle size. However, it cannot provide any detailed picture of the relative distribution of empty and filled vesicles.

Concerning the particular case of the vesicular systems investigated in this work, one general observation is the increase of polydispersity upon addition of oleate surfactant. A growth in size of oleic acid and POPC vesicles upon oleic anhydride hydrolysis and oleate addition, respectively, could be clearly evidenced by the observed shift of the vesicle size distribution of the ferritin-containing vesicles toward larger diameters. The formation of small vesicles from the preformed vesicles through fission is important also in view of the phenomenon of the matrix effect described before.[21,27] A subsequent investigation will be devoted to this topic.

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