Matrix Effect in the Size Distribution of Fatty Acid Vesicles

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Received: February 22, 1998; In Final Form: October 5, 1998

The cooperative mechanism of oleic acid/oleate vesicle formation is investigated under a variety of conditions. When a stock solution of sodium oleate is injected in a buffered aqueous solution of pH 8.8, the time progress of the vesicles formation follows a sigmoidal pattern, which is indicative of an autocatalytic process. Autocatalysis is demonstrated by running experiments in the presence of preadded vesicles, which shortens the lag phase and accelerates the process of vesicle formation as measured by turbidity and electron microscopy. The size distribution of the vesicle diameters obtained by injection of the surfactant stock solution in buffer solution covers a wide range, typically between 50 nm and 1.5 μm. However, in the presence of extruded vesicles having respectively 50 and 100 nm diameter, a quite different situation is observed: the size distribution is then much narrower and strongly biased toward the diameter of the preadded vesicles (i.e., 50 or 100 nm). It is as if the vesicles which are originally present would exercise a kind of template (or matrix) effect upon the new vesicles. This "matrix" effect is studied under a variety of concentration conditions of the preadded vesicles and the added surfactant stock solution. It is also investigated with a heterogeneous system, in which the water-insoluble and overlaid oleic anhydride is hydrolyzed in the presence and in the absence of preadded vesicles, and tendentiously the same matrix effect is observed. It is argued that this is most likely due to a prerequisite binding of the injected surfactant to the already existing vesicles, and it is this binding which determines the final size distribution.

Introduction

Over the past few years we have focused attention on the question of whether simple chemical reactions might be endowed with self-reproduction—where the term simple refers both to the chemical structure of the reagents (possibly prebiotic-like molecules) and to the nature of the driving forces (possibly a thermodynamically controlled process). In particular, we have described the self-reproduction of micelles and vesicles formed by long-chain carboxylic acids, such as caprylic (octanoic) acid,[1−3] oleic acid (*cis*-9-octadecenoic acid),[3,4] or 2-methyldodecanoic acid.[5] We have initially operated with a biphasic system constituted by an aqueous alkaline phase and a supernatant organic phase consisting of the water-insoluble precursor(s) of the surfactant itself, for example, the ester or the anhydride of the acid. Following the slow initial hydrolysis of the precursor at the macroscopic interface, the surfactant is released into water, and when the critical aggregate concentration is reached, the reaction acquires abruptly an exponential character: in fact, the so-formed initial hydrophobic micelles or vesicles are capable of rapidly solubilizing the water-insoluble precursor and efficiently hydrolyzing it.[6] This yields more aggregates, and the more aggregates are formed, the faster is the further solubilization of the remaining precursor, which in turn gives rise to more and more aggregates, and so on in a typical autocatalytic fashion. We define the process as self-reproduction,[7] since the aggregates catalytically induce their own multiplication.[1−5] This process has also been investigated theoretically.[6,8] In particular, Coveney and Wattis[8] have constructed and studied a nonlinear kinetic model that describes the formation and self-reproduction of vesicles.

In all these studies, however, little attention has been devoted to experimentally elucidate the molecular mechanism attending the self-reproduction processes. In the present paper, we have carried out a first analysis of such mechanisms in the case of the oleic acid/oleate vesicles. In particular, we will discuss the question of how the final size distribution of the vesicles is affected by the presence of preadded vesicles.

We will show that these studies permit one to shed light on the mechanism of autocatalytic self-reproduction of vesicles and at the same time disclose an unexpected perspective on the general mechanisms of vesicle formation.

Materials and Methods

Chemicals. Sodium oleate (>99%), oleic acid (puriss, standard for gas chromatography), and *N*,*N*-bis(2-hydroxyethyl)-glycine, bicine (>99.5%), were from Fluka, Buchs, Switzerland. Sodium cholate (analytical grade) was from Serva, Heidelberg, Germany, and POPC was from Avanti Polar Lipids, Inc., Pelham, AL.

Turbidity Measurements. Turbidity measurements were carried out at 25 °C with a Cary 1E UV/vis spectrophotometer from Varian International AG, Basel. The sample volume was usually 2.5 mL, and quartz cells with a path length of 1 cm were used.

Electron Microscopy. Samples containing oleic acid/oleate vesicles were analyzed by Michaela Wessicken in the laboratory for electron microscopy I at the ETH. Depending on the information required, either the freeze-fracturing method[9a] or cryoimmobilization[9b] was used.

Dynamic Light Scattering Measurements. Vesicle samples were analyzed with a fiber-optics-based spectrometer consisting of an argon laser, a digital autocorrelator, and a computer-

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10.1021/jp981234w CCC: $15.00
Published on Web 11/11/1998

controlled rotational stage.[10] Measurements were carried out at different scattering angels, and the values given are mean values and standard deviations from 10 measurements.

Oleic Acid/Oleate Vesicles. Vesicles composed of oleic acid and oleate[4,11−14] were prepared in basically four different ways.

Method i. A defined amount of oleic acid was dispersed in 0.2 M bicine buffer (pH 8.8) under magnetic stirring at room temperature overnight. The vesicles thus formed were rather polydisperse and often multilamellar.[4] For some experiments, the size and lamellarity of the vesicles were reduced by first applying a 10-times freeze−thaw cycle (freezing the vesicles in liquid nitrogen and thawing at 40 °C), followed by a 10-times passage through polycarbonate membranes[4,15] from Nucleopore, using "The Extruder" from Lipex Biomembranes, Vancouver, Canada.[15] The polycarbonate membranes were obtained from Sterico AG, Dietikon, Switzerland, and had cylindrical pores with diameters of either 100 or 50 nm, respectively. The resulting vesicle suspensions are abbreviated as "100 nm vesicles" or "50 nm vesicles", respectively.

Method ii is based on the so-called "methanol injection method" originally described for the preparation of phospholipid vesicles:[16] A defined amount of oleic acid or sodium oleate was first dissolved in methanol, and a certain amount of this methanolic solution was added to a defined volume of concentrated bicine buffer solution (usually 0.2 M, pH 8.8) in a quartz cuvette used for UV/vis spectroscopy.

Method iii is similar to method ii with the exception that sodium oleate was first dissolved in water, and the vesicle formation was initiated by adding this aqueous oleate solution to a buffered bicine solution.

Method iv. Oleic acid/oleate vesicles were formed through the hydrolysis of oleic anhydride, starting with a heterogeneous two-phase system containing buffer solution (0.2 M borate, pH 8.5) and the water-insoluble anhydride.[4] This hydrolysis reaction was carried out under magnetic stirring at 30 °C, using flat-bottom test tubes with a diameter of 2.5 cm and using 10 mL of buffer solution and 0.12 mL (=0.20−0.25 mmol) of oleic anhydride. The reaction was run for 4.5 days to ascertain completion.

Determination of the Total Concentration of Oleic Acid and Oleate. The total concentration of protonated and deprotonated oleic acid in the vesicle suspension was determined by FTIR spectroscopy as described before,[4] using a Nicolet 5SXC or a Bruker IFS 66V FTIR instrument and a CaF_2 cell with a path length of 0.2 mm.

POPC Vesicles. POPC vesicles were prepared by the methanol injection method,[16] whereby POPC was first dissolved in methanol at a concentration of 12.7 mg/mL (16.7 mM). A 90 μL aliquot of this solution was then added to 3 mL of 20 mM Tris/HCl buffer, pH 7.4, under strong stirring (1000 rpm) with a Hamilton syringe. Since the injection speed considerably influenced vesicle size and polydispersity, the total solution volume was always added in the same way within a period of 30 s. Very fast additions (within 1 s) led to the formation of very small vesicles which did not show measurable turbidity.

Mixed POPC/Cholate Vesicles. Vesicle suspensions containing a defined fixed total amount of POPC and cholate were prepared by diluting a mixed micellar solution of 100 mM POPC (76 mg/mL) and 100 mM sodium cholate (43 mg/mL) in 135 mM NaCl.[17] Dilutions were made with 135 mM NaCl. With this procedure, the ratio of total POPC concentration to total cholate concentration was kept constant at 1.

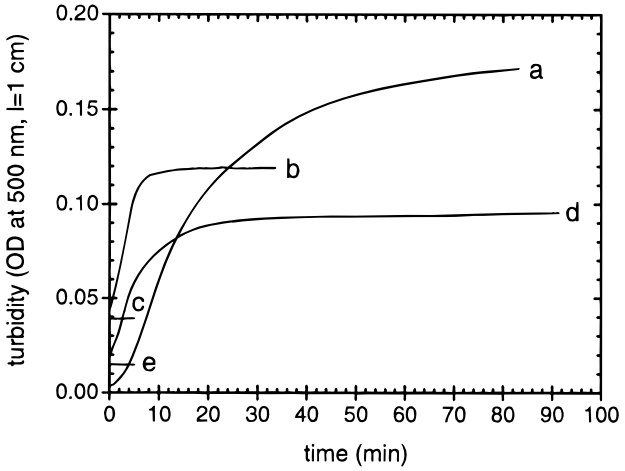

Figure 1. Effect of preadded vesicles on the formation of oleic acid/oleate vesicles. The turbidity measured at 500 nm (1 cm path length) is plotted as a function of time, $T = 25$ °C. Curve a: 62 μL of 80 mM aqueous sodium oleate was added to 2.438 mL of 0.2 M bicine buffer, pH 8.8 ([oleic acid/oleate] = 2 mM).[17] Curve b: 62 μL of 80 mM aqueous sodium oleate was added to 2.348 mL of a 2 mM oleic acid/oleate "100 nm vesicle" suspension (0.2 M bicine, pH 8.8, [oleic acid/oleate] = 4 mM). Curve c: turbidity of 2 mM oleic acid/oleate "100 nm vesicles". Curve d: the same as (b), but using a "50 nm vesicle" suspension. Curve e: turbidity of 2 mM oleic acid/oleate "50 nm vesicles".

Results and Discussion

In most of the micelle or vesicle reproduction experiments we have described in our earlier work,[1−5] we have been dealing with a biphasic heterogeneous reaction. In this case, a droplet of the neat water-insoluble precursor is added as a supernatant to the aqueous phase. The reproducibility can be considerably improved by working in a homogeneous phase. This can be achieved as illustrated in Figure 1, by injecting into a buffered solution of pH 8.8—a pH which induces vesicle formation[4]—a small amount (2.5%, v:v) of a concentrated aqueous solution of sodium oleate. Since in this case the starting stock solution is a micellar solution, oleate micelles are transformed into oleic acid/oleate vesicles. Alternatively, one can inject directly into a pH 8.8 buffer solution a concentrated methanolic stock solution of the water-insoluble oleic acid.

As shown in Figure 1 (curve a), under typical conditions the sigmoidal time process of the reaction reaches a plateau after 80 min.[18] This sigmoidal behavior is a first qualitative indication that we are dealing with an autocatalytic process.

Whether the process is indeed autocatalytic can be easily checked by running experiments in the presence of preadded vesicles: this should decrease the length of the initial slow phase (as in this case one does not have to wait for the first nucleation vesicle to be formed) and should accelerate the overall process. This is in fact what happens: as shown in Figure 1 (curves b and d), the presence of 2 mM oleic acid/oleate vesicles (extruded through polycarbonate membranes with pore diameters of 100 or 50 nm, respectively; see "Materials and Methods") gives a sizable initial optical density, from which the reaction starts upon the injection of fresh 2 mM sodium oleate. Now the reaction reaches a plateau in less than 10 min, and the lag phase has practically disappeared.

But there is another, and quite surprising, observation arising from curves b and d in Figure 1: one would have in fact expected that the turbidity plateau reached in this experiment would be about twice that obtained without preadded vesicles—as the final lipid concentration is twice as large. Instead, the

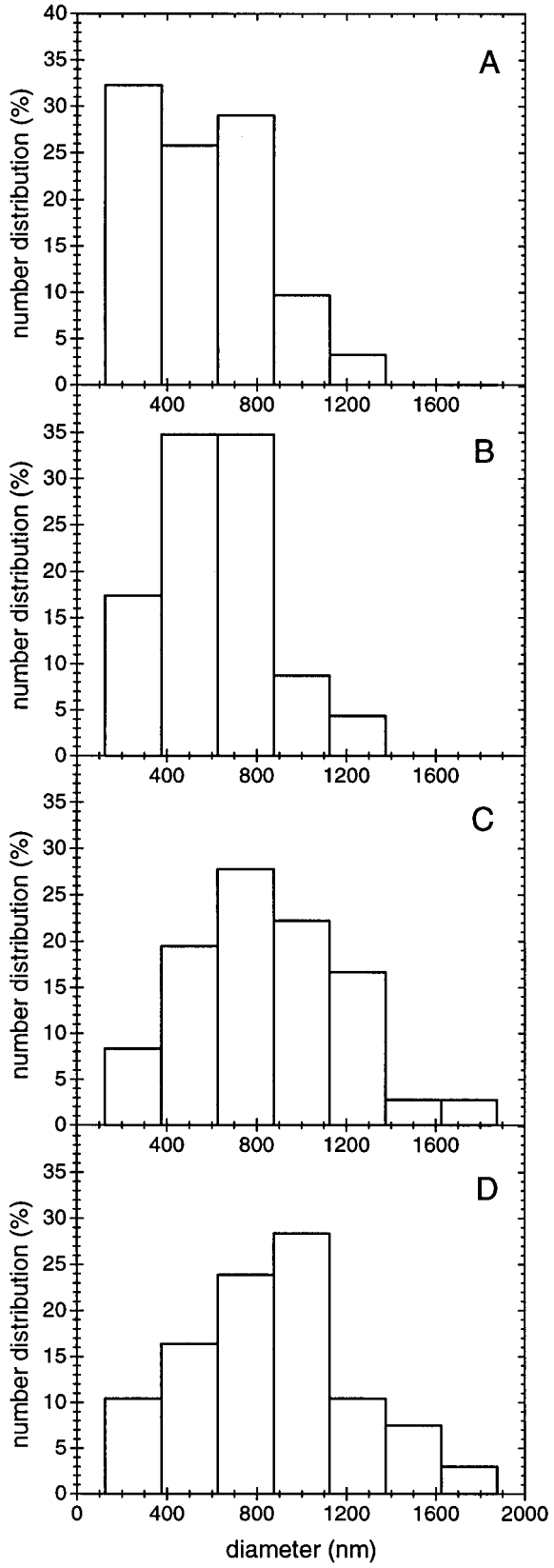

Figure 2. Freeze-fracture electron microscopic analysis of the vesicle size distribution after adding 62 μL of 80 mM aqueous sodium oleate into 2.438 mL of 0.2 M bicine buffer, pH 8.8 ([oleic acid/oleate] = 2 mM). Electron micrographs were taken 5 (A), 10 (B), 20 (C), and 40 min (D) after oleate addition. The relative number of vesicles (given in percentage of the total number of vesicles) is plotted against vesicle size (total number of vesicles counted: 157). The corresponding turbidity change is shown in Figure 1, curve a.

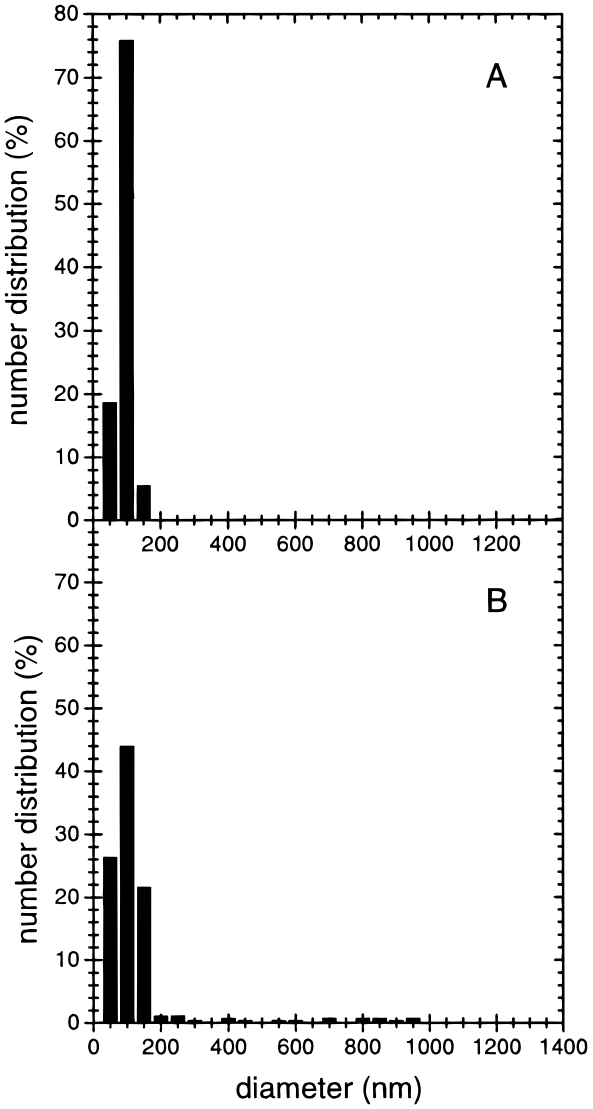

Figure 3. Freeze-fracture electron microscopic analysis of the vesicle size distribution of 2 mM oleic acid/oleate "100 nm vesicles" (A) and of the vesicles that were obtained after adding 2 mM sodium oleate to these "100 nm vesicles", 0.2 M bicine buffer, pH 8.8 ([oleic acid/oleate] = 4 mM). (B) The relative number of vesicles is plotted as a function of vesicle size (total number of vesicles counted: 364). The corresponding turbidity change and the experimental conditions are given in Figure 1 (curve b) and in the legend of Figure 1.

turbidity intensity reached values that are *lower* than the plateau obtained without preadded vesicles. This observation holds also for other combinations of oleic acid/oleate concentrations and sizes of the preadded vesicles.

We have therefore decided to study this unexpected phenomenon. Keeping in mind that the turbidity reflects not only the absolute concentration but also—and primarily—the mean size and size distribution, one possible rationalization which comes to mind is the following: that the size distribution of the vesicles in the case of curves b and d in Figure 1 is much smaller than the one obtained for curve a in Figure 1. In other words, it appears that in the distribution corresponding to curve b the larger sized vesicles are absent.

We have then measured by freeze fracture electron microscopy the mean size and size distribution of the vesicles, and results are illustrated in Figure 2 and Figure 3. Figure 2 shows the relatively broad size distribution obtained after injecting an aqueous solution of sodium oleate into 0.2 M bicine buffer at pH 8.8. The total oleic acid/oleate concentration was 2 mM,

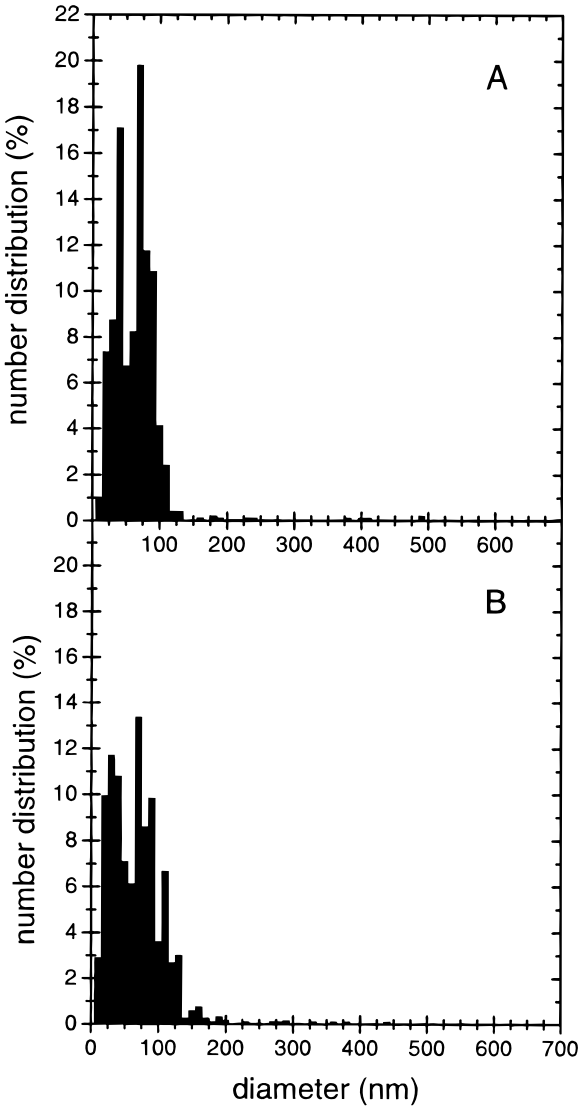

Figure 4. Freeze-fracture electron microscopic analysis of the vesicle size distribution of 10 mM oleic acid/oleate "100 nm vesicles" (A) and of the vesicles that were obtained after adding 15 mM sodium oleate to these "100 nm vesicles", 0.2 M bicine buffer, pH 8.8 ([oleic acid/oleate] = 25 mM). (B) A 465 μL aliquot of a 80 mM aqueous oleate solution was added to 2.035 mL of buffer solution. The relative number of vesicles is plotted as a function of vesicle size (total number of vesicles counted: 2857).

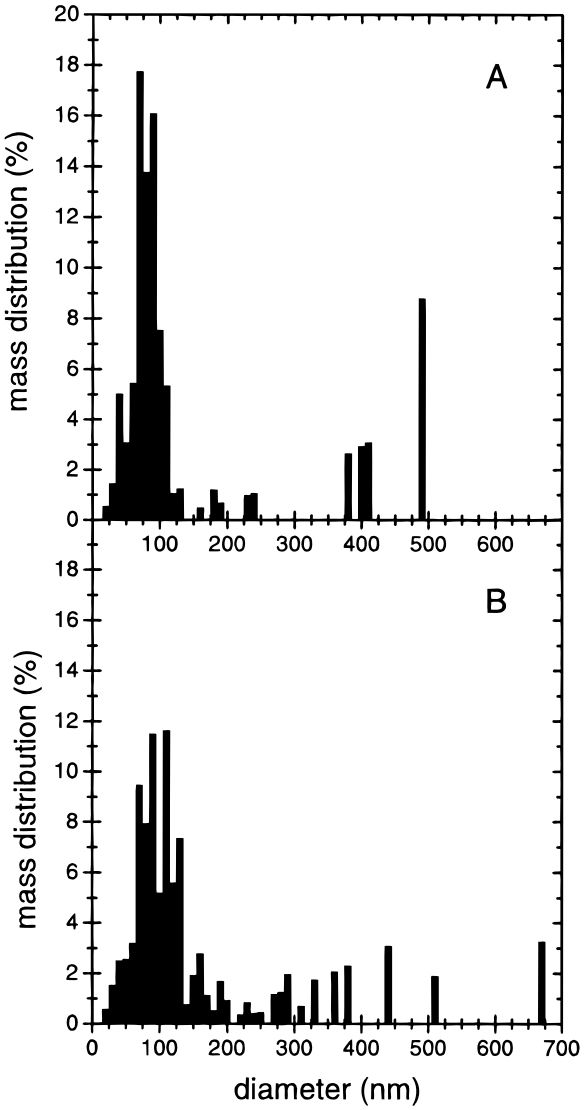

Figure 5. The same data as plotted in Figure 4 but showing the mass distribution as a function of vesicle diameter (see text for details).

and the time progress of the size distribution during the first 40 min is shown (see curve a in Figure 1 as a reference). Although less than a total of 200 vesicles were counted, it is evident that the number-weighted size distribution after 40 min extends in a range from 100 nm to 1.6 μm, with a higher size density centered around 0.5−1.0 μm. Notice also how the initially high population around 200 nm decreases with time in favor of larger aggregates.

Let us consider now Figure 3 which shows the effect of preadded vesicles. Notice the relatively narrow distribution for the "100 nm vesicles", obtained after extrusion through polycarbonate membranes with pore diameters of 100 nm (Figure 3A). Most of the "100 nm vesicles" have diameters between 50 and 150 nm, with a main peak at 100 nm. The size distribution obtained after adding the same amount of oleate to these "100 nm vesicles" is shown in Figure 3B. If the vesicle formation process would be unaffected by the presence of preadded vesicles, one would expect a distribution that corresponds to the weighted sum of the distributions shown in Figures 3A and 2D.

Quite surprisingly, about 90% of the vesicles are distributed now around the 100 nm peak. Although the small number of larger vesicles takes away a proportionally higher amount of surfactant, the distribution is now clearly very different from that originally expected. It is actually as if the presence of "100 nm vesicles" would have favored the formation of vesicles with tendentiously a similar size.

The same kind of results are also obtained when the amount of injected material is larger—actually in the range 1−5 mM added to 2 mM preformed vesicles about the same results as in Figure 3 are observed (data not shown). And qualitatively the same results are obtained when a much larger amount of lipid is involved: Figure 4 shows the number distribution obtained when 10 mM oleic acid/oleate vesicles are extruded through 100 nm filters and the corresponding distribution after addition of 15 mM oleate to these "100 nm vesicles".

It is important to consider in these processes not only the number-weighted distribution but also the mass-weighted distribution. A rough quantitation can be attempted on the basis of the freeze-fracturing electron microscopy data by calculating the frequency of each size, weighted by the corresponding surface area,[19] thus obtaining for the data of Figure 4 the

TABLE 1: Dynamic Light Scattering Measurements of 2 mM Oleic Acid/Oleate "100 nm Vesicles" and of the Vesicles Obtained upon Addition of 2 mM Sodium Oleate to These "100 nm Vesicles", 0.2 M Bicine, pH 8.8[a]

vesicle system	(apparent) hydrodynamic vesicle radius (nm) for scattering angle of 60°	90°	120°
2 mM oleic acid/oleate "100 nm vesicles"	55.6 ± 0.8	55.1 ± 0.3	54.5 ± 0.3
after addition of 2 mM sodium oleate to 2 mM oleic acid/oleate "100 nm vesicles"	76.6 ± 3.1	67.4 ± 0.8	64.2 ± 0.1
after addition of 2 mM sodium oleate to buffer, without preadded vesicles	164 ± 4	119 ± 1	97 ± 4

[a] For comparison, scattering data are also given for a vesicle suspension obtained by adding sodium oleate into buffer solution in the absence of preadded vesicles.

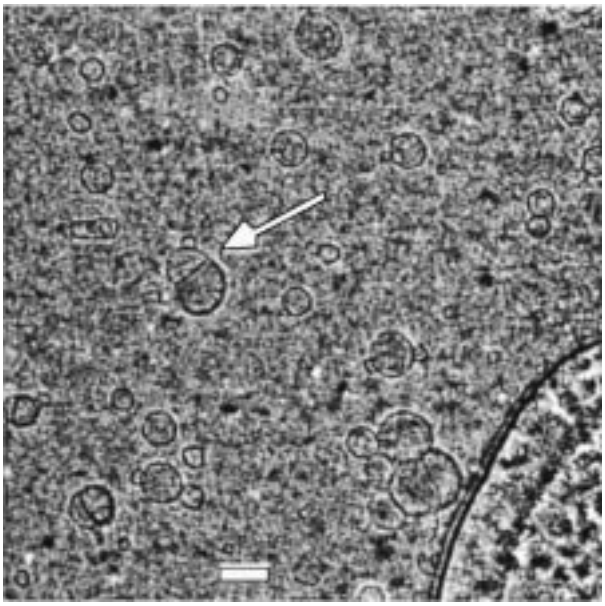

Figure 6. Cryo-electron micrograph of a "100 nm vesicle" sample (2 mM oleic acid/oleate, 0.2 M bicine, pH 8.8) to which 2 mM sodium oleate has been added (compare with curve b in Figure 1). The arrow indicates the presence of "paired vesicles". Length of the bar: 100 nm.

distribution shown in Figure 5. The results indicate that most of the mass of the oleic acid/oleate molecules remains in vesicles of about 100 nm size, but the distribution tends slightly to shift toward larger vesicle sizes. Dynamic light scattering measurements confirm these tendencies, as reported in Table 1. The slight size increase is paralleled by an increase in polydispersity. Again, without preadded vesicles, the addition of sodium oleate to 0.2 M bicine buffer, pH 8.8, leads to the formation of a very heterogeneous population of vesicles which cannot be analyzed quantitatively by light scattering as the (apparent) hydrodynamic radius strongly varies with the scattering angle (Table 1). Generally, all the observations are in good agreement with the turbidity and freeze-fracturing electron microscopy data.

Cryo-electron microscopy shows that the "100 nm vesicles" are mainly unilamellar and that this unilamellarity remains after oleate addition. The addition of sodium oleate to preadded "100 nm vesicles" seems to lead in many cases to the formation of "paired vesicles"—one vesicle adhering to another one—as shown in Figure 6.

The inescapable conclusion we can draw from all these data (Figures 1−5) is that the presence of preformed oleic acid/oleate vesicles influences the distribution of the vesicles that are formed in their presence and in particular that the size distribution of the "new" vesicles does not follow the broad distribution statistics obtained in the absence of preadded vesicles. Finally, the size of the "new" vesicles is biased toward the size of the preadded vesicles. It is as if there were a kind of template effect. Since the term template is used in the literature mostly for linear molecular structures, we will use here the more general term "matrix effect".

We have also investigated whether the same effect is present in the heterogeneous system. As mentioned before, in these experiments we let the water-insoluble anhydride of oleic acid hydrolyze at the surface of a slightly alkaline aqueous solution, whereby oleic acid/oleat vesicles form spontaneously as the hydrolysis reaction proceeds.[4] The vesicle size distribution when 25 mM overall anhydride (affording 50 mM oleate/oleic acid) is hydrolyzed is shown in Figure 7A. Figure 7B shows the size distribution of 10 mM oleic acid/oleate vesicles extruded through 50 nm polycarbonate filters ("50 nm vesicles"): notice that most of the vesicles are in fact distributed within the 50−100 nm region; however, a small number of vesicles are to be found at higher diameters, which accounts for a relatively large amount of lipid material. Finally, Figure 7C shows the size distribution of the vesicles after complete hydrolysis of 20 mM overall oleic anhydride in the presence of 10 mM oleic/oleate "50 nm vesicles". One might in this case expect—in the simplest possible approximation—a combination of the two previous distributions, Figure 7, A and B. It is not so, and actually the final size distribution is strongly biased toward the 50 nm size of the preformed vesicles. Again, it is clear that the presence of the preformed vesicles strongly affects the final size distribution, in particular strongly decreasing the probability of vesicles with dimension larger than 100 nm.

A comparison of parts B and C of Figure 7 in terms of the number of vesicles is interesting. One way to illustrate pictorially this difference is to make a difference distribution, namely the algebraic difference of the two number distributions (e.g., Figure 7, C and B). This has been also studied with experiments in which the preformed vesicles were "100 nm vesicles" or "200 nm vesicles", respectively. Results are shown in Figure 8: the decrease in the 20−50 nm region of Figure 8A indicates that these small vesicles have disappeared after the addition of oleic anhydride; by contrast, a good amount of "new" vesicles in the 50−100 nm region has been built, together with a significant amount of larger aggregates. In the case of preadded "100 nm vesicles" (Figure 8B) notice, in addition to the decrease in the number of small vesicles, the large increase in the 70−150 nm region, indicating the formation of such new vesicles in the size of the preadded ones—accompanied again by the formation of a significant amount of larger aggregates. Finally, with the 200 nm extruded vesicles, the increase of the number of larger vesicles is very significant (Figure 8C). On going from part A to part B to part C, notice that the size distribution of the "new" vesicles becomes larger and larger again. However, the most interesting result is that in the experiments with the "50 nm vesicles" and with the "100 nm vesicles" there is no maximum at 200 nm, namely such large vesicles are maximally formed only when the 200 nm extruded vesicles are preadded (Figure 8C).

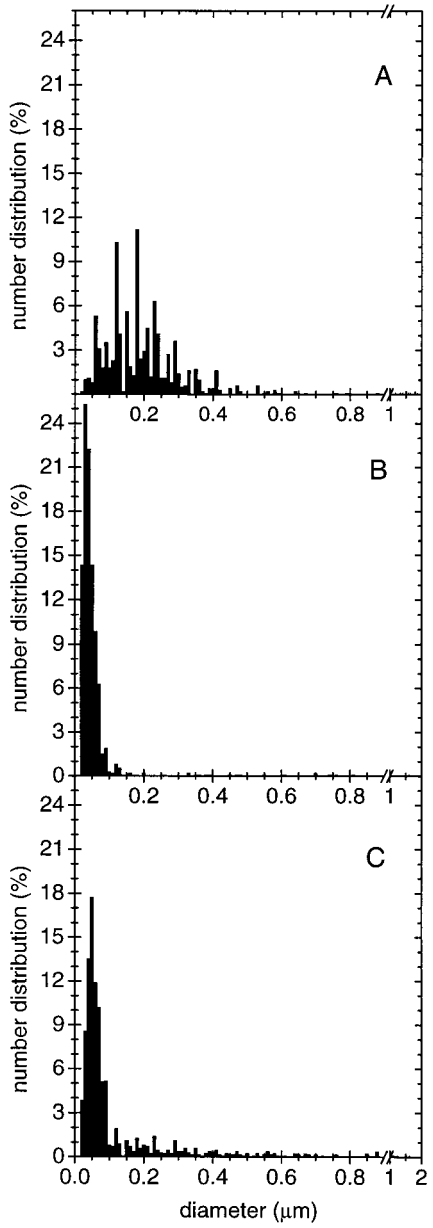

Figure 7. Freeze-fracture electron microscopic analysis of the size distribution of oleic acid/oleate vesicles. Buffer used: 0.2 M borate, pH 8.5. (A) Vesicles formed upon hydrolyzing 25 mM oleic anhydride (overall) at 30 °C, yielding 50 mM oleic acid/oleate. (B) Extruded "50 nm vesicles" (10 mM oleic acid/oleate). (C) Vesicles formed upon hydrolyzing 20 mM oleic anhydride (overall) at 30 °C in the presence of preadded extruded "50 nm vesicles" of a concentration of 10 mM oleic acid/oleate. The final oleic acid/oleate concentration was always 50 mM.

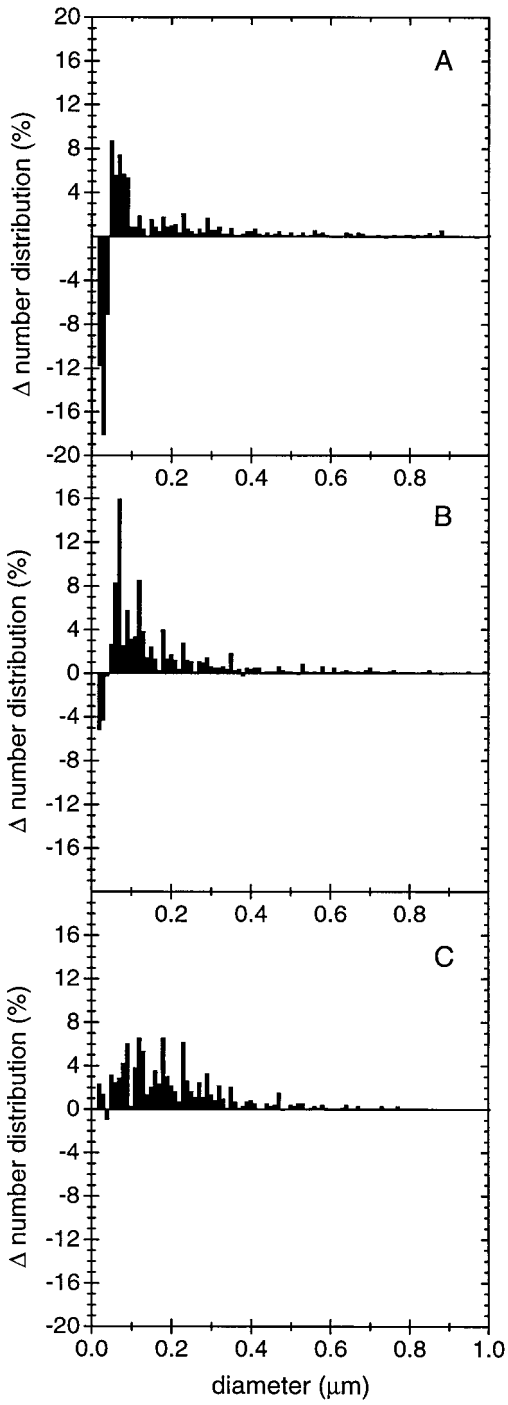

Figure 8. Freeze-fracture electron microscopic analysis of the size distribution of oleic acid/oleate vesicles obtained from the hydrolysis of oleic anhydride carried out in the presence of preadded extruded vesicles. Representation of the differences in the number distribution as obtained by subtracting the number distribution of the preadded vesicles from the number distribution obtained after hydrolysis (0.2 M borate buffer, pH 8.5, 30 °C) from evaluating equal real size areas. Concentration of preadded vesicles: 10 mM oleic acid/oleate. Concentration of anhydride at the beginning of the hydrolysis: 20 mM (overall). Final concentration of oleic acid/oleate, after hydrolysis: 50 mM. The preadded vesicles were extruded "50 nm vesicles" (A), "100 nm vesicles" (B), and "200 nm vesicles". (C) Total number of vesicles counted before hydrolysis: 1115 (for A), 875 (for B), and 528 (for C). Total number of vesicles counted after hydrolysis: 1354 (for A), 1542 (for B), and 1245 (for C).

An interesting question is whether this matrix effect is also observed in other, more classic vesicle systems, such as those composed of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine). We have carried out experiments with POPC by adding to an aqueous system a methanolic solution of POPC.[20] The first observation is that in this case there is a linear relationship between concentration and optical density, namely the anomaly such as that represented in Figure 1 for oleic acid/oleate is not present. If a methanolic POPC solution is added to an aqueous solution under the conditions used (see "Materials and Methods"), vesicles rapidly form, leading to an instant appearance of a slight turbidity (0.5 mM POPC). The vesicle formation process is so rapid that it cannot be followed by a conventional spectrophotometer. This is in clear contrast to what is observed in the case of oleic acid/oleate (see Figure 1). Further addition of a methanolic POPC solution to yield 1 mM POPC leads to a further increase in turbidity. As already

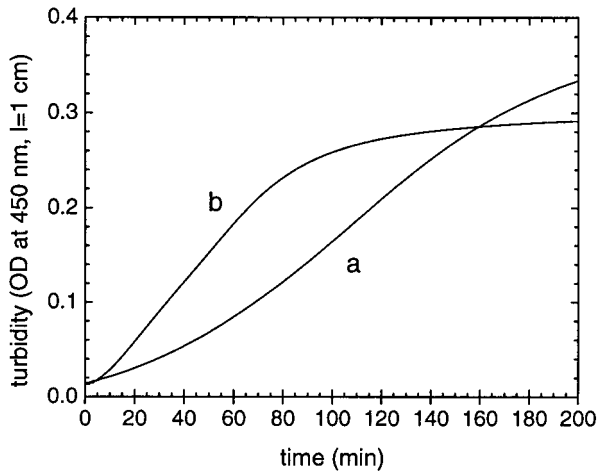

Figure 9. Formation of mixed POPC/cholate vesicles from mixed POPC/cholate micelles. The turbidity measured at 450 nm (1 cm path length) is plotted as a function of time, upon addition to 3 mL of 135 mM NaCl 65 (a) or 80 μL (b) of a mixed micellar solution containing 100 mM sodium cholate and 100 mM POPC in 135 mM NaCl, T = 25 °C. The total final concentrations of POPC and of cholate were 6.5 (a) and 8.0 mM (b).

mentioned, the turbidity increases linearly with the concentration of added POPC. The same is observed if extruded "100 nm vesicles" are used. Again, this observation is in clear contrast to the case of oleic acid/oleate (see Figure 1). In other words, in the case of POPC, none of the matrix effects observed for oleic acid/oleate seem to be present.

In the homogeneous experiments of Figures 1−6, the starting oleate solution was a micellar solution. Recognizing this, we have performed a series of experiments with POPC starting also from micellar solutions. As is well-known, the presence of bile salts, such as cholate, in a given concentration range induces the formation of mixed micelles.[21] When a concentrated mixed micellar solution of POPC/cholate is injected into a water solution, the typical sigmoidal behavior of vesicle formation is again observed, as shown in Figure 9 and reported earlier using egg phosphatidylcholine instead of POPC.[22] Under those conditions, there is then an analogy with the case of oleic acid/oleate. The system is, however, much more complex due to the presence of cholate and due to the fact that systematic studies as a function of concentration are made difficult by the peculiar concentration relation of POPC/cholate in the phase diagram.[21,22] For these reasons, we have not carried out at this time a deeper investigation into the cooperativity of vesicles POPC formation in the presence of cholate.

Concluding Remarks

There are several interesting questions arising from the experiments described in this paper. The most interesting is certainly the one related to the matrix effect: why, and by which mechanism, can such a strong effect take place?

There are in principle two arguments one can think of: one based on the interaction between preadded vesicles and the added surfactant and one in which the presence of preadded vesicles alters the initial thermodynamic conditions, so that, for example, because of volume-excluded effects, vesicles of larger size are improbable, and only those of one restricted size range are formed. It is difficult to see how this argument might account for the data in which vesicles with different sizes are preadded.

The most likely general mechanism is one that is based on the binding between preadded vesicles and the newly added surfactant molecules.[23] Having said that, details of the mechanism are still not clear. If surfactant molecules bind to the vesicles, they should just grow in size, and the radius should increase proportionally with the root-mean-square of the vesicle surface (spherical vesicle surface area = $4\pi r^2$, with vesicle radius r). Therefore, it is still not clear why the binding should eventually produce vesicles about in the same size range than the preadded ones. What are the intermediates of the process of vesicles formation? One possibility is that a second shell is built around the preadded small vesicles—with the formation of intermediate double lamellar vesicles, which then lose the outer shell giving unilamellar vesicles. Processes of this kind have been observed for micromanipulated giant vesicles, using cationic and anionic lipids.[24]

Another aspect to discuss is the question about the role of the micelles. In the homogeneous case, for both oleate and POPC/cholate, the autocatalytic behavior is observed when vesicle formation starts from micellar solutions. In the case of POPC alone, there is a rapid formation of vesicles without a preliminary lag phase. Thus, the lag phase observed in the autocatalytic processes may correspond to the transition from micelles to vesicles.

In turn, this means that we are dealing with a system in which monomer surfactants and aggregates exist in the presence of each other and interact with each other reversibly. This property can be considered as being a prerequisite for the observed autocatalysis.

It is apparent that we are touching on new and partly unexpected aspects of the kinetics and thermodynamics of vesicle formation and that new avenues of study must be conceived to shed light on them. Clearly, more investigations of kinetic nature are needed in order to solve this puzzle, as well as theoretical studies (now in progress in our group).

Acknowledgment. The authors thank Michaela Wessicken for performing all the electron microscopy measurements and Beat Flachsmann for very relevant experimentation during his diploma thesis at the ETH. We also thank Fabio Mavelli for his comments and suggestions.

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(7) Notice the use of the term self-reproduction instead of self-replication (Luisi, P. L. In *Self-Production of Supramolecular Structures*; Fleischhaker, G. R., Colonna, S., Luisi, P. L., Eds.; Kluwer Academic: Dordrecht, 1994; pp 196−197): in the latter case, we are dealing with mechanisms that determine a perfect replica of the original (usually a template); a self-reproduction process is instead dominated by statistical laws and the copying process is generally not perfect; for example, although the same kind of micelles or vesicles, respectively, are formed, the size and size distribution can be different. More generally, the difference between these two processes may be relevant from the point of view of origin of life: as noted by Dyson (Dyson, F. *Origins of Life*, Cambridge University Press: Cambridge, 1985), most likely the less sophisticated self-reproduction processes have preceded the exact molecular mechanisms of self-replication.

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(17) Please note that the preparation of the mixed micellar solution needs 2−3 days to form and equilibrate at room temperature.
(18) Long time measurements indicate that a plateau is reached after about 400 min with a measured optical density at 500 nm of 0.22 (compare with curve a in Figure 1).
(19) In these calculations unilamellarity and sphericity of the vesicles are assumed.
(20) Adding an alcoholic solution of phospholipids to an aqueous solution is one of the well-known methods for preparing phospholipid vesicles.[14]
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