Coexistence and Mutual Competition of Vesicles with Different Size Distributions

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Vesicle populations having narrowly distributed size distribution are mixed with each other to investigate the extent and degree of mutual interaction. One basic question is whether these vesicular systems can be seen as a "kinetic trap" or whether they reequilibrate with each other to form a common energy minimum. To this aim, different size distributions of oleate and POPC vesicles (POPC stands for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) were prepared by extrusion methods. Accordingly, three different systems were investigated: POPC/POPC vesicles; oleate/oleate vesicles; POPC/oleate vesicles. Dynamic light scattering technique was used to follow the possible size changes in aggregation after the mixing of the two vesicles populations. In agreement with the "kinetic trap" notion, no interaction was observed for the first two systems; i.e., the two populations of vesicles coexisted for several days without change of their original size distribution. In the case of the oleate/oleate system, addition of Ca^{2+} ions brought about the turbidity increase, indicating an aggregation between the negatively charged oleate vesicles. These changes were however completely reversible, as shown by the addition of excess EDTA, which can be taken as evidence that the observed changes were not due to fusion but to a simple transient aggregation. For the system of POPC/oleate, the two peaks of the two different vesicles populations rapidly merged into only one peak, due to the disappearance of the oleate peak. This was explained by the uptake of monomeric oleate by POPC vesicles, mediated through the relatively large critical aggregate concentration (cac) of oleate vesicles. Finally, the oleate vesicles of different size were exploited to study the different rate of uptake of added surfactant monomer. It is shown that the larger vesicles are faster, even when care is taken of normalizing to the same overall surface.

Introduction

It is well-known that vesicles and in particular liposomes (vesicles made out of lipids) are generally nonequilibrium systems.[1−5] Thus, when vesicles are produced by the extrusion techniques in a given size, they generally remain as such without relaxing in a thermodynamically more stable distribution, for example in the case of POPC. This is generally true for all sizes that can be obtained by extrusion, and therefore, each size can be considered as a kinetic trap,[5] namely as a secondary energy minimum where the structure is confined because of the too high energy barriers that separate this state from the other energy minima. This situation prevents chemical equilibrium among the various vesicle sizes. If this is rigorously the case, then mixing two (or more) vesicles populations with different sizes should result in the stable coexistence of two different populations, as no thermodynamic reequilibration is possible. This paper is aimed at investigating this situation and in particular at investigating whether, and to what extent, the two families of vesicles interact with each other and, if not, to what extent these two different populations differ in physical properties and reactivity.

Particularly interesting is the case in which different vesicles from two different surfactants are used. In this sense, this research is related to our previous work,[6−9] in which mixed POPC/oleate vesicles were investigated by adding oleate micelles to POPC or oleate vesicles. And the present work is also related to the so-called "matrix effect", a term that refers to the observation that, under certain conditions, the preformed vesicles significantly affect the size distribution of the newly formed vesicles and their rate of formation.

In this paper, the mutual interaction of vesicles in three different systems is investigated: POPC vesicles with POPC vesicles; oleic acid vesicles with oleic acid vesicles; POPC vesicles with oleic acid vesicles. Also, oleate vesicles with different sizes are compared in terms of the rate of uptake of added oleate micelles—with the question namely of which one of the two families is more avid than the other in the uptake of added surfactant. This situation can be loosely considered as a model for the Darwinian competition for nutrients of different organisms coexisting in the same environment.

Results and Discussion

The three systems mentioned above are studied sequentially. Figure 1a shows the size distribution as determined by dynamic light scattering (DLS) after mixing two POPC families, previously extruded at 30 and 200 nm, respectively. As shown in Figure 1a, the sizes of the extruded vesicles differ somewhat from the size expected on the basis of the extrusion size. Using pore diameters of 30 and 200 nm, one should have obtained POPC vesicles with radius 15 and 100 nm, respectively. However, the radii for 30 and 200 nm extruded POPC vesicles are 30.0 and 70.2 nm (±4%), respectively. This discrepancy between the actual values of the radius and that expected from the polycarbonate extrusion filters can be ascribed to the interplay between the mechanical constraints of the extrusion and the energetic constraints of the vesicles.

Studies with population sizes closer to each other (e.g., extruded by 30 and 100 nm filter) are not easy to perform, due

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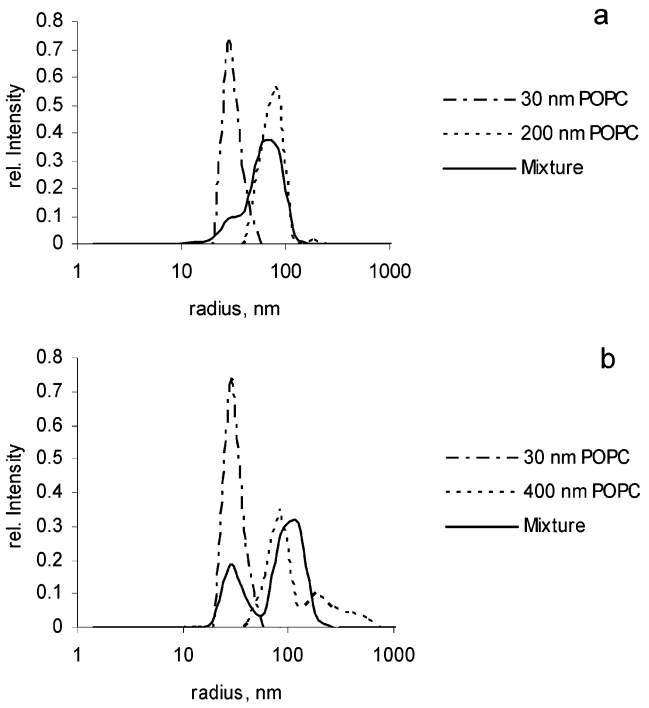

Figure 1. Intensity-weighted size distribution of POPC in 0.2 M bicine buffer solution, pH 8.5: (a) 0.5 mL solution of 1 mM 30 nm extruded POPC vesicles mixed with 0.5 mL solution of 1 mM 200 nm-extruded POPC vesicles; (b) 0.5 mL solution of 1 mM 30 nm extruded POPC vesicles mixed with 0.5 mL solution of 1 mM 400 nm extruded POPC vesicles. The measuring angle is 90°.

to the limitations of the DLS resolution.[10] To obtain more clear evidence, 400 nm extruded POPC vesicles (113.4 nm radius) were mixed with 30.0 nm POPC vesicles. Two peaks are seen clearly, as shown in Figure 1b. Each peak position is corresponding to the position of preadded POPC vesicles. These figures are given in terms of light scattering intensity, which is a complex function, depending on the sixth power of the radius and the first power of the particle number. The extreme dependence from the radius brings about a large overweight of the larger particles. Due to this, the first peak has a relatively low intensity in the normalized distribution after mixing.

As can be seen from Figure 1, the two main peaks coexist even after several days without significant changes. The two populations act as independent entities, and there is no tendency to fuse to search for a thermodynamic energy minimum. This is in agreement with the notion of kinetic trap, as mentioned earlier. Clearly, the activation energy for mixing is too high and more, in general, it is questionable whether there would be an energetic situation after mixing, which is more stable than the initial one.

The situation is not much different in the case of 30 nm extruded oleic acid vesicles mixed with 200 or 400 nm extruded oleic acid vesicles (data not shown). There may have been expected some difference with respect to the case of POPC because fatty acid vesicles appear to be more sensitive to thermodynamic factors.[5] Also, in this case, the cac is much higher (10^{-4} M of oleic acid against 10^{-10} M of POPC), which means that there is a larger amount of monomer in solution and in equilibrium with each vesicle species, so that one could have expected a monomer-mediated equilibration of the vesicles. But it is not so. In the work of Olsson, this phenomenon of monomer diffusion between vesicles of different sizes is also mentioned and suggested that the driving force for this process comes from the curvature energy.[11] Another emulsion system

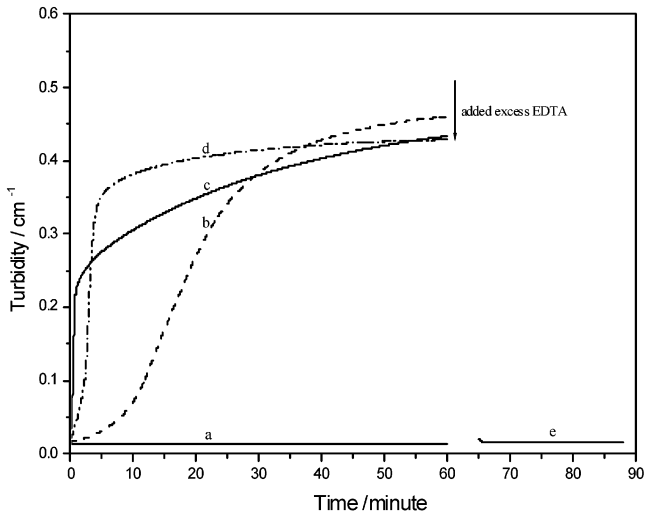

Figure 2. Effect of Ca^{2+} on the turbidity change upon mixing oleate vesicle solutions: 0.25 mL 1 mM 30 nm extruded oleic acid vesicles + 0.25 mL 1 mM 400 nm extruded oleic acid vesicles + 1.5 mL bicine buffer. Calcium ion concentration: (a) 0 mM; (b) 1 mM; (c) 2.5 mM; (d) 5 mM; (e) added excess EDTA to (d).

was investigated by Evilevitch, who reported that the mixing behavior of different sizes of oil droplets depend significantly on the concentration and sizes of large and small droplets.[12,13] In addition, Plant et al. found that the surface free energy of the supported alkanethiol monolayer had an effect for fusion of vesicles with this monolayer, and there is a kinetic barrier to the spontaneous fusion between them.[14,15] Although the above-mentioned systems are different from the one investigate here, it is clear that the fusion between vesicles is not at all an easy process, and actually a series of factors such as the size, composition, osmotic pressure, concentration, and curvature of the vesicles have an effect on vesicle fusion.

Oleic acid vesicles are charged, and we thought of possibly inducing their fusion by adding Ca^{2+} ions. It has been reported that these ions may induce vesicle fusion.[16−18] Figure 2 shows the effect of different concentration of Ca^{2+} added to oleic acid vesicles as studied by turbidity measurement. It can be seen that the time processes for these changes are influenced significantly by the concentrations of Ca^{2+}. In a low concentration range ($[Ca^{2+}] = 1$ mM), a lag process (curve b) was observed, whereas at higher concentration ($[Ca^{2+}] = 5$ mM) the turbidity reached rapidly a constant value (curve d).

To confirm the reversibility of this process, excess EDTA was introduced into the above system. The result is rather clear: the turbidity value is reduced sharply to the initial value (curve e). To confirm that real fusion does not take place during this process, fluorescence techniques were employed. Fusion measurements were carried out by the typical terbium−dipicolinate assay.[16] Tb^{3+} is encapsulated in one population of oleic acid vesicles, and DPA^{-}, in another. After the mixing of these two solutions, a fusion of the vesicles would then result in an increase of the fluorescence intensity, due to the formation of the Tb^{3+}/DPA^{-} complex. However, the fluorescence does not change in our case, with or without the presence of Ca^{2+}. Therefore, it appears that the turbidity increase observed in the presence of Ca^{2+} ions is not due to fusion but to an aggregation of oleic acid vesicles; namely, the oleate vesicles join together but do not fuse with each other.

Let us now consider the case of POPC vesicles in the presence of oleic acid vesicles. As a premise, one should recall that oleate monomer is rapidly uptaken by POPC or oleic acid vesicles, as shown previously in the literature[19] and by us.[6−9] Figure 3a

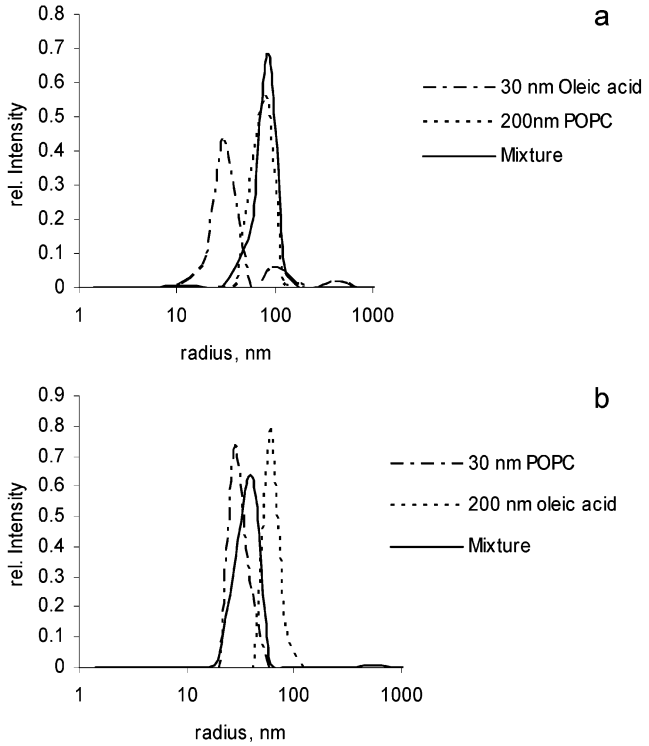

Figure 3. Size distribution of mixtures of oleic acid and POPC vesicles in 0.2 M bicine buffer solution, pH 8.5. (a) 0.5 mL solution of 1 mM 30 nm extruded oleate vesicles mixed with 0.5 mL solution of 1 mM 200 nm extruded POPC vesicles; (b) 0.5 mL solution of 1 mM 30 nm extruded POPC vesicles mixed with 0.5 mL solution of 1 mM 200 nm extruded oleate vesicles. The measuring angle is 90°.

shows the size distribution of 31.6 nm oleic acid vesicles mixed with the 70.2 nm POPC vesicles, represented by an intensity-weighted distribution. Only one peak is observed after the mixing. In addition, the position of this peak is very close to those of preformed 70.2 nm POPC vesicles.

Figure 3b shows the size distribution of 30.0 nm POPC vesicles mixed with 64.1 nm (200 nm extrusion filter) oleic acid vesicles. Again, only one peak is observed after mixing. However, the position of this peak is very close to that of preexisting 30.0 nm POPC vesicles. This experiment is particularly interesting. With recognition that the intensity of DLS is mainly controlled by large particles,[20] if nothing happens on mixing 30.0 nm POPC vesicles with large 64.1 nm oleic acid vesicles, the intensity should be dominated by the large premixed 64.1 nm oleic acid vesicles. The observed opposite experimental behavior suggests some change during this mixing process: in particular, data can be explained in terms of the disappearance of the oleate vesicles. In other words, it seems the oleate has been totally absorbed by the large number of the smaller POPC particles.

To visualize more clearly the interaction between vesicles, it is useful to convert the intensity distribution into a number-weighted distribution.[20] Notice preliminarily that, for the same surfactant concentration, the absolute number of 30 nm extruded vesicles is much larger than the absolute number of the 200 nm extruded vesicles. As shown in Figure 4a, the number size distribution after mixing 30.0 and 70.2 nm extruded POPC vesicles is very similar to that of the premixed 30.0 nm extruded POPC vesicles.

As shown in Figure 4b, a similar behavior is observed in the case of the 70.2 nm POPC vesicles: after mixing 31.6 nm oleic acid vesicles with 70.2 nm POPC vesicles, the number-weighted size distribution is very close to the number distribution of the

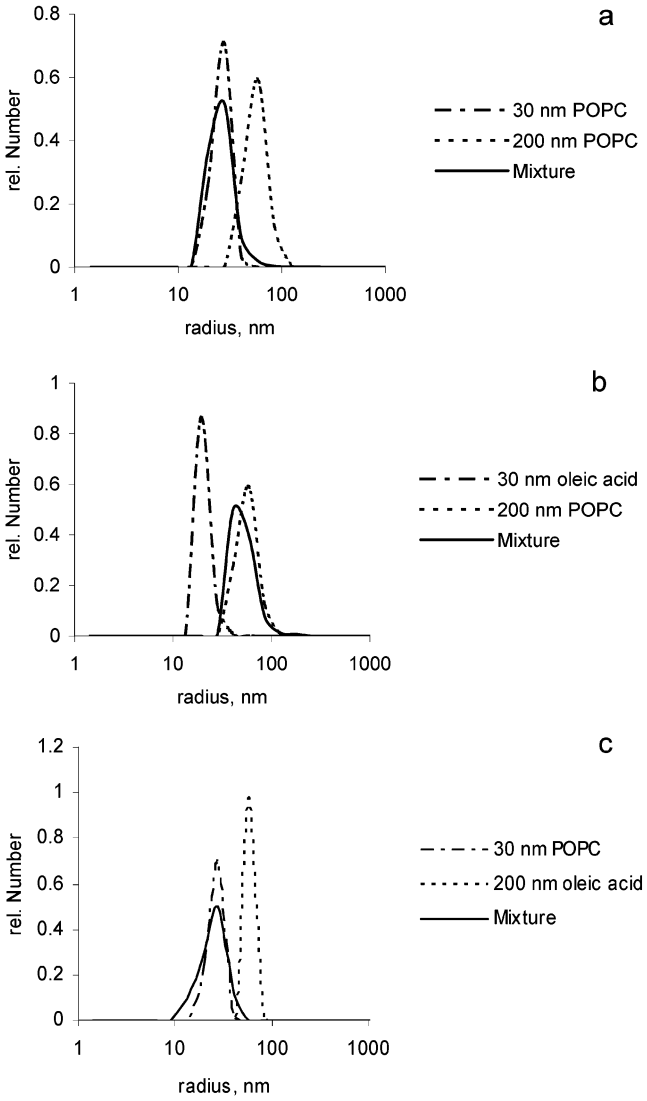

Figure 4. Relative number distribution, obtained from corresponding relative intensity distribution. (a)−(c) are corresponding to Figures 1a, 3a, and 3b, respectively.

premixed 70.2 nm extruded POPC vesicles. Notice that this is a normalized distribution and not a representation of the absolute number of particles: the latter would be much larger after the mixing.

Let us consider in more detail the mechanism of this process. As mentioned before, it seems that the oleate vesicles disappear. As reported in the previous work, the uptake of oleate by POPC vesicles is rapid.[6−9] Due to the relative large cac oleate acid, there will be a good amount of surfactant monomer in equilibrium with the oleate vesicles.[21] If these findings are combined, a reasonable mechanism can be proposed, and this is shown in Figure 5. The oleate monomer in equilibrium with the oleate vesicles will be uptaken by the POPC vesicles, and this would displace the equilibrium and eventually destroy all oleate vesicles. As a consequence, mixed oleic/POPC vesicles are formed. This mechanism is actually in keeping with our previous studies on the so-called "matrix effect".[6−9]

Finally, let us consider the rate of uptake when oleate micelles are added to oleic acid vesicles of different sizes. The interesting question in this regard is: is there a difference in the rate of uptake between the two populations with different size? In other words, which one of the two sizes will grow faster? We will consider first, for the sake of simplicity, the two systems separately from one another.

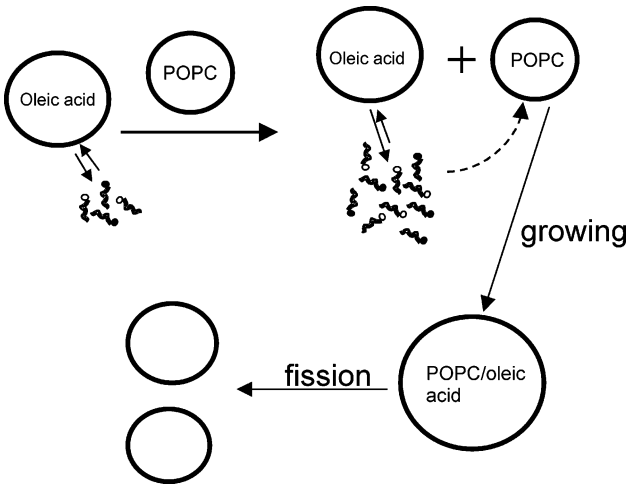

Figure 5. Schematic representation of the time process attending the mixing of oleic acid and POPC vesicles.

This kinetic process is readily followed by the increase in turbidity of the sample with time,[22] whereby the simplest kinetic treatment would be based on the half-life of the reaction in terms of a first-order exponential ($t_{1/2}$ is the time when the turbidity reaches half of the maximum value). For the sake of simplicity, it is assumed that all oleic acid vesicles are unilamellar and have initially a uniform spherical size. In this case, the two systems are considered to have a radii of 31.6 and 64.1 nm (in the case of the "30 nm extruded oleate vesicles" and "200 nm extruded oleate vesicles"), as obtained from dynamic light scattering measurements at 90°. For the same total surfactant concentration, the total surface areas are the same for the two types of vesicle, but the absolute vesicles number of the 31.6 nm vesicles is 4.4 times larger than that of the 64.1 nm vesicles. On the other hand, if one wishes to compare the rate of uptake by keeping a constant number of particles of the two systems, then the concentration of oleate is accordingly decreased when preparing the 31.6 nm vesicles (these calculations neglect the difference in aggregation number between the inner and outer leaflet of the vesicles).

Some typical data are shown in Figure 6, and half-time ($t_{1/2}$) values are listed in Table 1. The vesicle number concentrations of 31.6 and 64.1 nm vesicles are roughly the same for curves c−f. Under those conditions, it is clear that the larger oleate vesicles grow faster (curve e: $t_{1/2} = 2.25$ min) than the smaller ones (curve c: $t_{1/2} = 6.49$ min). This is reasonable due to the fact that the larger vesicles have now a larger total surface and the probability of uptake is correspondingly larger.

It might have been expected that the final turbidity value of curve e should be higher than that of curve c because of the higher final lipid concentration. This is not so. In fact, turbidity reflects not only the absolute lipid concentration but also the mean size and size distribution of vesicles. In our experiments, the ratio of preadded oleic acid vesicles and added oleate micelle (1: 2.27) in curve e is much higher than that of curve c (1:10). Thus, for the condition in curve e, a stronger matrix effect is expected, which leads to a narrower size distribution of newly formed vesicles. Comparatively, for curve c, a weaker matrix effect leads to a relatively larger size distribution of newly formed vesicles in the solution.

Note that the half-time of the curve e ($t_{1/2} = 2.25$ min) is less than that of curve c ($t_{1/2} = 6.49$ min) and curve f ($t_{1/2} = 3.68$ min). It is also interesting to compare the rate of vesicle formation without preadded oleate vesicles. From curve a ($t_{1/2} = 19.92$ min) and curve b ($t_{1/2} = 28.88$ min), it is clear that the process without preadded vesicles is much slower. This is in agreement with the matrix effect.

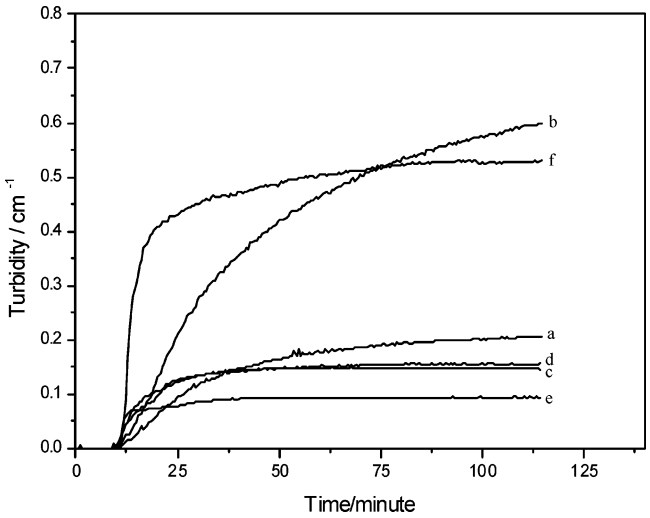

Figure 6. Effect of preadded vesicles on the formation of oleic acid/oleate vesices. Curve a: 20 μL of 50 mM aqueous sodium oleate was added to 1 mL of 0.2 M bicine buffer, pH 8.5. Curve b: the same as (a), but using 88 μL of 50 mM aqueous sodium oleate. Curve c: 20 μL of 50 mM aqueous sodium oleate was added to 1 mL of a 0.1 mM oleic acid/oleate "30 nm extruded vesicle" suspension (0.2 M bicine, pH 8.5, $X = 1{:}10$). Curve d: the same as (c), but using a "200 extruded nm vesicle" suspension ($X = 1{:}10$). Curve e: 20 μL of 50 mM aqueous sodium oleate was added to 1 mL of a 0.44 mM oleic acid/oleate "200 nm extruded vesicle" suspension (0.2 M bicine, pH 8.5, $X = 1{:}2.27$). Curve f: the same as (e), but using 88 μL of 50 mM aqueous sodium oleate ($X = 1{:}10$). X represents the ratio of [preadded oleic acid + oleate]/[added sodium oleate].

TABLE 1: Effect of the Preadded Oleate Vesicles on the Half-Time of Oleate Micelles Uptake at Different Conditions

	condition[a]					
	a	b	c	d	e	f
half-time (min)[b]	19.92	28.88	6.49	5.83	2.25	3.68

[a] Conditions a−f corresponding to those of curves a−f in Figure 6, respectively. [b] Relative standard deviation (RSD) is less than 5% in each condition.

As mentioned before, oleate micelles can also be uptaken by POPC vesicles.[6] However, this process is very fast and cannot be monitored by simple spectrophotometry. Stopped flow techniques[22] are necessary in this case, which is however out of the framework of this work.

In conclusion, the rate of uptake of larger vesicles is "faster" than that of the smaller one. This greater reactivity of the larger vesicles may be ascribed to a higher energy, probably resulting from the smaller curvature. It is however not easy to understand why this should be so as actually one could have expected the opposite; the greater curvature might result in a lower tendency to relax into even larger dimensions. Probably, this kind of generalization is not possible, in the sense that the chemical structure of the surfactant may determine which size is actually more stable and therefore less prone to change.

Concluding Remarks

This work shows that different size populations of vesicles can coexist in the same solution without tendency to fuse with each other to search for an energy minimum. As mentioned in the literature, this phenomena is in agreement with the notion of "kinetic trap", which notes that vesicle systems are not chemical equilibrium systems, as micelles generally are.[5] The

different vesicle sizes correspond to energy minimum that are probably very close to each other and separated by rather high activation energy barriers, so that there is no tendency to an equilibration. The existence of kinetic traps does not prevent however that the monomer is in local equilibrium with the aggregate, as dictated by the corresponding cac. In the case of oleate, the presence of monomer surfactant in equilibrium with the aggregate is the cause of the disappearance of oleate vesicles in the presence of POPC vesicles. In this way, a complementary confirmation of the matrix effect is observed: whereas in the previously published experiments[6] the oleate was added in a micellar form, here it is furnished directly in a vesicular form.

The interaction of oleate vesicles with each other in the presence of Ca^{2+} is also of some general interest. An increase of size is observed by turbidity measurement, which is reversible, and fluorescence experiments confirmed that there was no fusion. This point is important, because in the literature the term of "fusion" is used in a very vague context. Very often no discrimination is made between simple vesicle aggregation and real fusion. We believe this last term should be limited to the case in which demonstrably the two aqueous compartments of the vesicles fuse with each other, thus mixing their content in an irreversible way.

A new question addressed in this work is whether vesicle populations differing only in the average size distribution may differ in reactivity and possibly in other physicochemical properties. Here only the rate of uptake of fresh surfactant is examined, and it is discovered that larger vesicles are more reactive—in the sense that they show a larger uptake rate. This is observed also when the two systems have the same total surface, so that the different uptake rate must reflect an intrinsic tendency of the larger vesicles to relax into an even larger state. Why this should be so is at the present not clear; more systems should be studied to reach some more general conclusions. At present, we can only say that, in the limited case of oleate vesicles, the smaller ones (31.6 nm radius) appear to be more stable, in the sense that they have less tendency to react away from their size.

This different reactivity, together with the fact that two different size populations can coexist in the same solution, permits one to have a system in which two populations of very similar species may compete with each other, for example for the uptake of reagents. This may simulate the competition between two different species, or organisms, for the uptake of nutrients from the environment. Although in this paper we have not carried out such experiments, the evidence presented here offers the basis for future work on models for Darwinian evolution systems. This is now in progress in our group.

Materials and Methods

Chemicals. Sodium oleate (99%), oleic acid (puriss, standard for gas chromatography), *N*,*N*-bis(2-hydroxyethyl)glycine (bicine) (99.5%), dipicolinic acid (DPA, pyridine-2,6-dicarboxylic acid), terbium chloride, trisodium citrate hydrate, and sodium cholate were obtained from Fluck. The POPC was purchased from Avanti Polar Lipids, Alabster, AL. The Sepharose 4B was from Pharmacia, Uppsala, Sweden. All other chemicals were of the highest purity available from Fluka. The 0.2 M bicine buffer solution was prepared by dissolving bicine in distilled water, adjusted to pH 8.5 with 1 M NaOH. Ultrapure water from Millipore (Volketswil, Switzerland) was used in all experiments.

Turbidity Measurement. Turbidity measurements were carried out at $\lambda = 450$ nm with a Cary 1E UV/vis spectrophotometer from Varian International AG, Basel. The quartz cells with a path length of 1 cm were used unless otherwise stated. All of the experiments were thermostatically controlled at 25 °C.

Dynamic Light Scattering Measurements. DLS measurements were done with a fiber-optics-based spectrometer consisting of an Innova 70 coherent argon laser ($\lambda = 488$ nm), a digital autocorrelator ALV 5000, and a computer-controlled rotational stage. Hydrodynamic radius of the vesicle was determined from the cumulant analysis of the intensity autocorrelation function. The vesicle size was taken as a mean value of 10 measurements. All of the experiments were thermostatically controlled at 25 °C.

Fluorescence Spectroscopy. Fluorescence spectroscopy was measured with a Spex spectrofluorometer. Excitation wavelength was 270 nm, and emission spectra were recorded between 350 and 600 nm.

Preparation of Vesicles.

(1) Oleic Acid/Oleate Vesicles. A defined amount of oleic acid was dispersed in 0.2 M bicine buffer (pH 8.5) under magnetic stirring at room temperature overnight. The oleic acid concentration was typically 20 mM. Then, vesicles were treated by first applying a 10× freeze−thaw cycle (freezing the vesicles in liquid nitrogen and thawing at 35° C aqueous solution), followed by a 10× passage through polycarbonate membranes from Nucleopore, using "The Extruder" from Lipex Biomembranes (Vancouver, Canada). The polycarbonate membranes have cylindrical pores with diameters of 400, 200, 100, or 30 nm, respectively. The obtained vesicles suspensions are abbreviated as "400 nm vesicles", "200 nm vesicles", "100 nm vesicles", or "30 nm vesicles", respectively.

(2) Oleic Acid/Oleate Vesicles Containing Tb^{3+} and DPA. A desired amount of sodium oleate was transferred into a round-bottom flask, and methanol was added. Methanol was removed by a rotary evaporator under reduced pressure. The obtained oleate film was further dried under high vacuum overnight. Oleic acid/oleate vesicles (80 mM lipid) containing Tb^{3+} were obtained by suspending the dried oleate film in 0.2 M bicine (pH 8.5) containing 2.5 mM $TbCl_3$ and 50 mM sodium citrate ($TbCl_3$ was encapsulated in the presence of citrate to prevent the interaction of Tb^{3+} with oleate). The dispersion was vortexed until complete dissolution of the film. Then, freeze−thaw and extrude were performed in the same way as described before. For the separation of nonentrapped $TbCl_3$ from vesicles containing entrapped $TbCl_3$, a sepharose 4B column (length 50 cm, diameter 1.6 cm) was used. The concentration of oleic acid/oleate in the fractions was determined by FTIR spectroscopy. The same procedure was operated for DPA containing oleic acid vesicles, while 50 mM DPA is used instead of 2.5 mM $TbCl_3$ and 50 mM sodium citrate described above.

(3) POPC Vesicles. The POPC vesicles were prepared in the same way as described in our previous paper.[9]

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