A Kinetic Study of the Growth of Fatty Acid Vesicles

Irene A. Chen and Jack W. Szostak
Howard Hughes Medical Institute and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts

ABSTRACT   Membrane vesicles composed of fatty acids can be made to grow and divide under laboratory conditions, and thus provide a model system relevant to the emergence of cellular life. Fatty acid vesicles grow spontaneously when alkaline micelles are added to buffered vesicles. To investigate the mechanism of this process, we used stopped-flow kinetics to analyze the dilution of non-exchanging FRET probes incorporated into preformed vesicles during growth. Oleate vesicle growth occurs in two phases (fast and slow), indicating two pathways for the incorporation of fatty acid into preformed vesicles. We propose that the fast phase, which is stoichiometrically limited by the preformed vesicles, results from the formation of a ''shell'' of fatty acid around a vesicle, followed by rapid transfer of this fatty acid into the preformed vesicle. The slower phase may result from incorporation of fatty acid which had been trapped in an intermediate state. We provide independent evidence for the rapid transformation of micelles into an aggregated intermediate form after transfer from high to low pH. Our results show that the most efficient incorporation of added oleate into oleic acid/oleate vesicles occurs under conditions that avoid a large transient increase in the micelle/vesicle ratio.

INTRODUCTION

Fatty acids and other simple amphiphilic molecules have been synthesized under simulated prebiotic conditions (Allen and Ponnamperuma, 1967; Yuen et al., 1981; McCollom et al., 1999; Rushdi and Simoneit, 2001; Dworkin et al., 2001) and have been isolated from carbonaceous chondrite meteorites (Yuen and Kvenvolden, 1973; Lawless and Yuen, 1979; Yuen et al., 1984; Deamer, 1985; Naraoka et al., 1999). Such molecules can form bilayer membrane vesicles with the ability to entrap macromolecules and highly charged small molecules, although remaining permeable to smaller polar solutes (Deamer and Pashley, 1989; Walde et al., 1994a; Apel et al., 2001). It has been suggested that such vesicles played an important role in the origin of life, either by providing a spatially limited microenvironment in which protometabolic reactions could have developed (Chakrabarti et al., 1994; Segre et al., 2001) or by providing a mechanism for the spatial confinement of genetic polymers, thus enabling Darwinian evolution (Szathmary and Demeter, 1987; Szostak et al., 2001; Cavalier-Smith, 2001).

Understanding the role of membranes in the origin of life requires a better understanding of the physical properties of membrane vesicles composed of simple single-chain amphiphiles. Although such vesicles were first examined 30 years ago (Gebicki and Hicks, 1973), their properties are much less well defined than those of phospholipid vesicles. In aqueous solution, fatty acids spontaneously form bilayer membranes when the pH is close to the pK_a of the membrane-incorporated acid; at higher pH only micelles form, whereas at lower pH oil droplets condense (Small, 1986; Cistola et al., 1988). The stability of the bilayer phase when the pH is close to the pK_a is thought to be due to hydrogen bonding between adjacent protonated and ionized carboxylates, decreasing the electrostatic repulsion of adjacent headgroups (Apel et al., 2001). This hypothesis is supported by the observation that vesicles composed of fatty acid-fatty alcohol mixtures are stable at higher pH values than vesicles composed only of fatty acids (Hargreaves and Deamer, 1978; Monnard and Deamer, 2003).

Fatty acid vesicles show a range of interesting properties and behaviors that are distinct from those of phospholipid vesicles. The major difference is that although fatty acid vesicles are long-lived supramolecular structures, their molecular components are in rapid dynamic equilibrium with the surrounding solution and with each other (Lange, 1986; Kamp and Hamilton, 1992; Kamp et al., 1995; Zhang et al., 1996; Kleinfeld et al., 1997; Hamilton, 1998). A fascinating observation that has brought fatty acid vesicles to the forefront of research on self-reproducing molecular assemblies is that the spontaneous assembly of fatty acid vesicles from alkaline micelles diluted into buffered solution is an autocatalytic process (Walde et al., 1994b). The self-assembly reaction has a long lag period, followed by an increasing rate of vesicle formation. The lag phase can be eliminated by the addition of preformed vesicles (Blochliger et al., 1998; Berclaz et al., 2001a). Vesicles formed in the spontaneous reaction are highly polydisperse; remarkably, vesicles formed after the addition of seed vesicles of defined size have a size distribution that is much narrower and closer to the size of the seed vesicles (Blochliger et al., 1998; Lonchin et al., 1999; Berclaz et al., 2001b). The mechanism of this so-called ''matrix effect'' remains unknown.

The first study to clearly demonstrate the growth of large unilamellar vesicles after the addition of fatty acid micelles at high pH to a strongly buffered solution of preformed vesicles (Berclaz et al., 2001a) used cryo-TEM of phospholipid vesicles containing encapsulated ferritin. This approach has the virtue of being able to distinguish between the growth of preformed vesicles and the appearance of larger vesicles formed de novo, and the resulting vesicles were found to be primarily unilamellar. Unfortunately, this method is tedious and cannot be used to follow growth in real time. Dynamic light scattering has also been used to follow the increase in vesicle size after micelle addition (Lonchin et al., 1999; Rasi et al., 2003). However, these experiments are complicated by the complex and changing size distribution of the growing vesicle population, and cannot distinguish new from preformed vesicles. Recently, our laboratory has used fluorescence methods to follow fatty acid vesicle growth, using the dilution of non-exchanging, membrane-localized FRET dyes to measure increasing vesicle surface area (Hanczyc et al., 2003). Fluorescence techniques have two important advantages: they allow quantitative measurement of the growth of preformed vesicles even when de novo vesicle formation is occurring simultaneously, and they allow such measurements to be made in real time during the process of membrane growth.

Previous work, although clearly establishing that fatty acid membrane vesicles can grow, has left the question of the mechanism of growth unanswered. In principle, the simplest mechanistic models of growth would be: 1), the direct fusion of micelles with vesicles in a single step or 2), the dissolution of micelles into molecular fatty acid followed by incorporation of these monomers into the preformed membrane. Our kinetic data show that neither of these simple models is sufficient to describe the rate-limiting steps, and we suggest a more complex pathway, involving a previously unsuspected intermediate. Our results provide guidance for the design of procedures for vesicle growth in the laboratory, and also constrain the range of conditions under which fatty acid vesicle growth in a prebiotic setting could have been efficient with respect to the competing formation of new vesicles.

MATERIALS AND METHODS

Materials

Oleic acid (C18:1) was from NuChek Prep (Elysian, MN); NBD-PE, Rh-DHPE, and pyrene were from Molecular Probes (Eugene, OR). Methanol and chloroform were from Fisher (Suwanee, GA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All vesicles were prepared and diluted in 0.2 M Na^+-bicine, pH 8.5 (adjusted with NaOH), unless otherwise noted.

Fatty acid micelles and vesicles

Oleate micelles were prepared as described (Hanczyc et al., 2003), at concentrations between 37.5 mM and 100 mM. Vesicles labeled with

fluorescent dyes were prepared by mixing stock solutions of dye dissolved in chloroform with oleic acid dissolved in a small volume of methanol in a round-bottom flask. Organic solvents were removed with a rotary evaporator, leaving a homogeneous oil. The oil was resuspended in buffer, pH 8.5, by hand mixing and vortexing, resulting in a turbid suspension, which was allowed to stand overnight before use. The final oleate concentration was 80 mM. Homogeneous 100 nm vesicles were obtained by extrusion for 11 passes through 100-nm pore filters using the Mini-Extruder system (Avanti Polar Lipids, Alabaster, AL). Extruded vesicles were used within 24 h. To assess whether residual oil droplets were a significant contaminant in these preparations, oleate vesicles were visualized by freeze-fracture electron microscopy after extrusion. No structures corresponding to oil droplets were seen (S.M. Fujikawa, Harvard University, personal communication, 2003). In addition, the results of the FRET assay for growth using vesicles prepared by adding oleate micelles to 0.2 M bicine, pH 8.5, were indistinguishable from vesicles prepared by resuspension from oleic acid oil (data not shown).

FRET measurement

FRET efficiencies (ε) were approximated as $1 - F_v/F_t$, where F_v is donor fluorescence in vesicles and F_t is donor fluorescence after addition of 1% Triton X-100. Donor fluorescence was measured at 530 nm with excitation at 430 nm. All fluorescence measurements were made on a Cary Eclipse fluorimeter (Varian, Mulgrave, Australia). Standard curves were generated using known dye concentrations in vesicles.

Stopped-flow kinetics

Oleate vesicles (100 nm) were diluted in 0.2M bicine, pH 8.5, to a concentration range of 1.5 mM to 6 mM and loaded into a 2.5 mL syringe of the RX-2000 rapid mix accessory (Applied Photophysics, Leatherhead, UK) to the fluorimeter. Micelles were loaded into a 100-μl syringe at 25 times the desired final concentration. Stopped-flow mixing was done according to manufacturer's instructions. Briefly, liquids from both syringes were pushed simultaneously into a mixing chamber/cuvette, from which FRET data were collected continuously. The stop volume for each reaction was 300 μL. The instrument dead time was $\sim$12 ms, as determined by a test reaction (Peterman, 1979). FRET data were converted to relative surface area using a standard curve of FRET efficiency versus dye concentration. No fluorescence changes were observed after rapid mixing of labeled and unlabeled vesicles, showing that the FRET dyes do not exchange on the timescale of these experiments (<1 min). F_t for the experiment was calculated from the equation $\varepsilon = 1 - F_v/F_t$, given the fluorescence of vesicles without added micelles and the FRET efficiency predicted by the standard curve. Final grown surface area relative to initial surface area was determined by averaging at least 10 data points after equilibrium was achieved, i.e., at least seven half-times after the start of the reaction. The difference between the final surface area and surface area at time t during the reaction (ΔSA) was fit to a double exponential decay ($\Delta SA = A \times \exp(-k_1 t) + B \times \exp(-k_2 t)$) using nonlinear regression.

For experiments designed to follow the micelle to intermediate transition, micelles were mixed with 0.2 M bicine, pH 8.5, at two times the desired micelle concentration, and loaded into a 2.5 ml syringe on the stopped-flow accessory. Preformed FRET dye-labeled vesicles were also loaded into a 2.5 ml syringe at two times the desired vesicle concentration. Stopped-flow mixing was initiated after the desired preincubation time, at a 1:1 volume ratio. These data were corrected for a slow decrease in FRET efficiency observed in the control mixture of unlabeled and labeled vesicles (apparent 10% change in surface area over 20 min, after which point the fluorescence stabilized). This background decrease was not the result of FRET dyes exchange among vesicles, since a mixture of 0.2 mol % NBD-PE-labeled vesicles and 0.2 mol % Rh-PE-labeled vesicles did not show a change in donor fluorescence after 20 h, but may be due to a perturbation of fatty acid membranes by the incorporated phospholipid dyes (Fujikawa, 2003).

Pyrene excimer/monomer ratio

Pyrene was incorporated into oleate micelles and vesicles at 0.5 mol % by addition from a 5 mM stock solution in methanol. This concentration of pyrene showed minimal excited-state dimer (excimer) fluorescence in micelles but significant excimer fluorescence in vesicles. Fluorescence emission spectra were measured at an excitation wavelength of 300 nm. Changes in pyrene monomer/excimer ratio were followed in stopped flow mixing experiments by recording fluorescence as a function of time, at emission wavelengths of 374 nm (monomer) and 470 nm (excimer).

Growth kinetics by light scattering intensity

Light scattering intensity from vesicle samples was measured by a PDDLS/Batch system (Precision Detectors, Bellingham, MA), equipped with a 100-mW, 800-nm diode laser, using a 90° detection angle. A small volume of micelles was added to oleate vesicles (0.75 mM oleate) in 0.2 M bicine, pH 8.5, and mixed by pipetting for 2–3 s. Vesicle growth was followed by measuring 90° light scattering intensity as a function of time. Two stages of increase were typically observed. The amplitude of the first stage was defined as the difference between the initial intensity and the intensity at the horizontal point of inflection. Raw data were smoothed (running median, sampling proportion = 0.1) and the first derivative was determined discretely. The position of the horizontal point of inflection was determined as the local minimum of the first derivative. The amplitude of the second stage was defined as the intensity difference between the horizontal point of inflection and the final intensity plateau. The half-time ($t_{1/2}$) of the each intensity increase was defined as the time from micelle addition to the point of half-maximal intensity change for that stage. All experiments were done with oleate concentrations between 0.75 mM and 2.5 mM.

Dynamic light scattering of de novo vesicle formation

Dynamic light scattering was measured by an ALV/DLS/SLS-5000 Compact Goniometer System (ALV-GmbH, Langen, Germany) with a CW Argon-ion laser and a detection angle of 90°. Data were analyzed by the method of cumulants (Koppel, 1972; Frisken, 2001). De novo vesicle reactions were prepared by mixing a small volume of micelles (1.5 mM oleate) with 0.2 M bicine, pH 8.5. To characterize the micelle-vesicle intermediate, the DLS measurement was begun ~30 s after mixing, and the measurement time was 20 s. Measurement times for de novo vesicle reactions incubated overnight and for oleate micelles (40 mM) were at least 1 min.

RESULTS

Fluorescence resonance energy transfer (FRET) between two fluorescently tagged phospholipid analogs has been used in the past to monitor membrane fusion between labeled and unlabeled vesicles, as the FRET efficiency is related to the surface density of FRET dyes (Fung and Stryer, 1978; Struck et al., 1981). In this study we labeled oleic acid/oleate vesicles with the FRET donor-acceptor pair NBD-PE and Rh-DHPE to monitor membrane growth after the addition of oleate micelles. As vesicles incorporate additional fatty acid, the surface density of the FRET dyes decreases, which decreases the FRET efficiency. We prepared vesicles containing 0.02 to 0.5 mol % donor and acceptor dyes, and measured FRET efficiency as a function of dye concentration. The resulting standard curve (Fig. 1 A) was used to
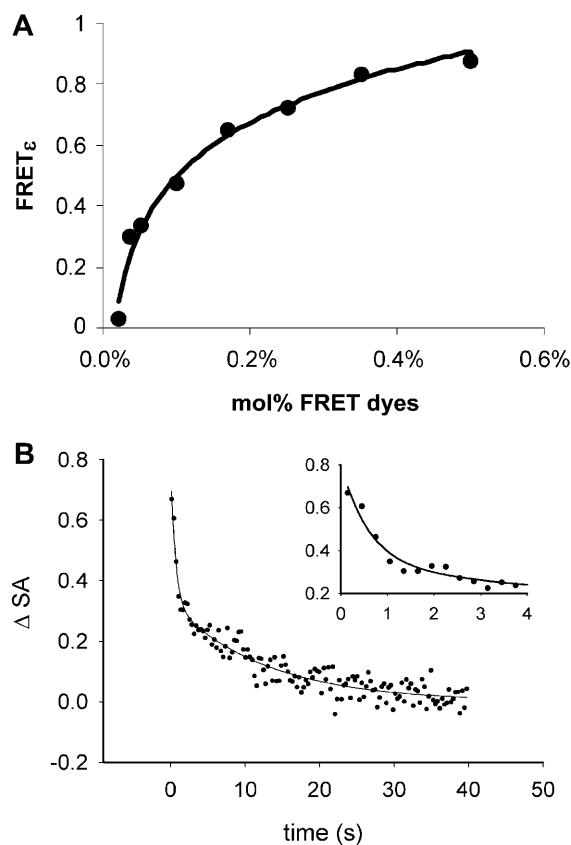

FIGURE 1 Membrane growth measured by a FRET assay. (A) Standard curve of FRET efficiency as a function of mol % FRET dyes in oleate vesicles. Each point represents a separate vesicle preparation. The solid line indicates the fitted curve, $y = 0.255 \ln(x) + 2.26$, $r^2 = 0.983$. (B) Typical growth experiment after stopped-flow mixing. Oleate vesicles (3 mM) were mixed with micelles (final 3 mM). Data were recorded every 0.3 s and converted into ΔSA using the FRET standard curve. The solid line indicates the fitted double exponential curve for this trial: $A = 0.48$, $B = 0.33$, $k_1 = 1.6/s$, $k_2 = 0.08/s$ (see text for definitions), and $r = 0.955$. Four replicates were done for each set of conditions. (Inset) Closer view of the first 4 s of the same experiment, showing the fast phase of growth.

interpret the results of subsequent vesicle growth experiments in terms of changes in total membrane surface area. Although de novo vesicle formation was likely to have been a concurrent process, only the growth of preformed vesicles was observed, since the fluorescent dyes did not exchange into newly forming vesicles on the experimental timescale. In contrast to the very slow mixing observed with dye-labeled vesicles, dye mixing was very rapid when the dyes were incorporated into oleate micelles. When micelles containing 2.5 mol % donor were mixed with an equal amount of 2.5 mol % acceptor-labeled micelles, the maximum change in FRET efficiency was reached in less than the 12-ms deadtime of our instrument.

The growth kinetics of preformed vesicles after micelle addition could thus be followed in real time using stopped-flow mixing in a fluorimeter cuvette. Our initial goal was simply to distinguish between growth by direct

micelle-vesicle fusion versus vesicle growth by the incorporation of free molecular fatty acid.

For a direct fusion model,

$$micelles(m) + preformed\,vesicles(p) \xrightarrow{k} grown\,vesicles(v)$$
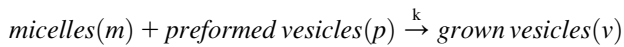
$$dv/dt = k*p*m.$$

If the rate of reaction depends on total vesicle surface area, p will increase during the reaction due to vesicle growth. In this case, growth should show autocatalytic kinetics, and the initial rate should be proportional to the concentration of micelles. In contrast, for the simplest model of vesicle growth by the incorporation of monomeric fatty acid:

$$micelle \rightleftarrows monomer$$

$$monomer + preformed\,vesicles(p) \xrightarrow{k} grown\,vesicles(v).$$
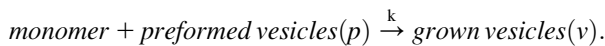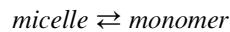
In this mechanism, micelles are in rapid equilibrium with monomers, which are maintained at the cmc. The rate equation is $dv/dt = k*cmc*p$. If the total vesicle surface area determines the rate of reaction, growth would show autocatalytic kinetics and the initial rate should be independent of the micelle concentration. However, if the monomer concentration were to increase beyond the cmc transiently, growth may show lagged kinetics.

To test these models, we measured the rate of oleate vesicle growth as a function of added micelle concentration. Oleate vesicles were diluted to 3 mM whereas the concentration of added micelles was varied from 1.5 mM to 6 mM. We found that the kinetics of oleate vesicle growth were best described by a double exponential, indicating the presence of two pathways for vesicle growth (Fig. 1 B). The rate constants of the fast ($k_1 \sim 2.5/s$) and slow phases ($k_2 \sim 0.1/s$) differed by more than an order of magnitude, allowing good discrimination between the two phases. Neither rate constant depended significantly on the concentration of micelles (Fig. 2, A and B). These observations are inconsistent with the simple vesicle-micelle fusion model. The double-exponential kinetics also do not support the simplest model of micelle dissolution into monomers that was discussed above. However, monomer incorporation may still be an important step in a more complex pathway of vesicle growth. In addition, we examined vesicle growth kinetics at vesicle concentrations ranging from 1.5 mM to 6 mM, with a constant micelle concentration of 3 mM. Again, neither rate constant depended on the preformed vesicle concentration (Fig. 2, C and D). This observation is inconsistent with the autocatalytic mechanisms of growth mentioned above, in which vesicle surface area determined the rate of growth.

Although the biphasic kinetics indicate that vesicle growth is a composite reaction, we determined the concentration dependence of the initial rate of the fast phase of growth by
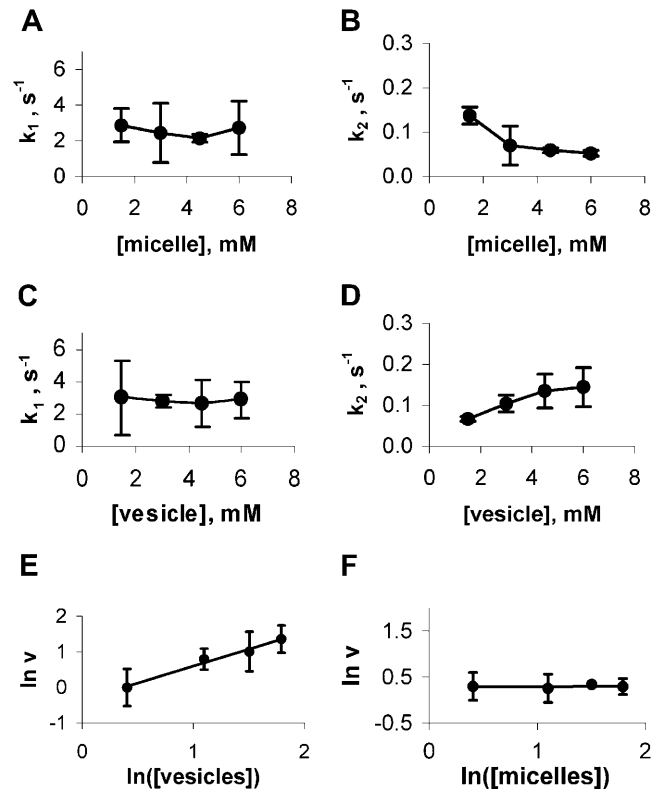

FIGURE 2 Observed rate constants k_1 and k_2 during vesicle growth by direct micelle addition, measured by the FRET assay: k_1 (A) and k_2 (B) versus micelle concentration (3 mM oleate vesicles), k_1 (C) and k_2 (D) versus vesicle concentration (3 mM oleate micelles). The van't Hoff plots of initial reaction rate (v) and vesicle (E) or micelle (F) concentration for high micelle/vesicle ratios (>0.4). Straight lines indicate linear fits to (E) $y = 0.95x - 0.36$ ($r^2 = 0.98$), and (F) $y = 0.01x + 0.28$ ($r^2 = 0.057$). Error bars indicate 95% confidence intervals calculated from replicates.

the van't Hoff method (Fig. 2, E and F), for micelle/vesicle ratios >0.4. In this regime, the fast phase of the reaction appeared to be first order with respect to vesicle concentration and 0th order with respect to micelle concentration. At low micelle/vesicle ratios (<0.4), the initial rate of reaction does depend on the micelle concentration. The initial rate of reaction at a micelle/vesicle ratio of 0.1 is ~sixfold slower than the initial rate at a micelle/vesicle ratio of 0.5. The order of the slow phase of the reaction could not determined by this method due to the uncertainty in determining the initial rate of the second step of the biphasic reaction from our data.

A closer examination of the vesicle growth data reveals that the fast growth phase corresponded to an ~40% increase in vesicle surface area, although the ratio of added oleate micelles to initial oleate vesicles varied from 0.5 to 2. The amount of oleate incorporated per vesicle during the fast phase was therefore stoichiometrically limited by the amount of preformed vesicle surface area. This phenomenon was best seen by comparison of the amplitudes A and B of the fast and slow phases of growth (Fig. 3, A and B). The fast phase amplitude A was

consistently ~40% at these micelle/vesicle ratios, whereas the slow phase amplitude B increased as the micelle/vesicle ratio increased, thus varying between 20% and 90%. Thus, at high ratios of micelle/vesicle, a preformed vesicle appeared to incorporate a fixed amount of oleate during the fast phase, whereas the amount of incorporation during the slow phase could vary with the amount of micelles added per vesicle. At low ratios of micelles to vesicles, i.e., <0.4, vesicle growth could be described by a single exponential function with a rate constant close to the previously determined rate constant of the fast phase of growth for higher micelle/vesicle ratios (Fig. 2, A and

FIGURE 3   Observed amplitudes A (*fast phase*) and B (*slow phase*) during vesicle growth by direct micelle addition, measured by the FRET assay. (*A*) Amplitudes versus micelle concentration (3 mM oleate vesicles). (*B*) Amplitudes versus vesicle concentration (3 mM oleate micelles). Error bars indicate 95% confidence intervals calculated from replicates. (*C*) Yield of vesicle growth by direct micelle addition as the amount of micelles added increases (3 mM oleate vesicles). The theoretical yield is the yield calculated by assuming that all micelles were incorporated into preformed vesicles.

C), and the total amplitude of growth was limited by the amount of micelles added (Table 1). If the amount of micelles added exceeded this ratio, vesicle growth proceeded rapidly up to a ~40% surface area increase, but then slowed considerably.

As more micelles were added per vesicle, the total amount of incorporation increased, but the yield expressed as the fraction of added fatty acid incorporated into the preformed vesicles decreased (Fig. 3 C). For example, if one-half equivalent of micelles were added, the yield of growth was close to 100%, but if two equivalents were added, the yield was ~50%. This decrease in yield reflects the presence of another reaction that consumes micelles, presumably de novo vesicle formation, which primarily affects the amplitude of the slow pathway of growth.

The increased partitioning of oleate into de novo vesicles with increasing initial micelle concentration was also evident from the changes in 90° static light scattering intensity. Although light scattering intensity is a function of particle size, shape, and concentration, it is a useful measure of overall vesicle dynamics (van Zanten, 1996), including growth and de novo vesicle formation. Micelle addition to preformed vesicles generated a biphasic profile (Fig. 4 A). The initial increase occurred over <30 s ($t_{1/2}$ ~10 s; Fig. 4 B), which encompassed both the fast and slow phases of preformed vesicle growth. The timescale of the initial increase was independent of the concentration of micelles, consistent with the FRET analysis of preformed vesicle growth. The initial increase is likely to reflect changes in the population of preformed vesicles. As greater concentrations of micelles were added, the amplitude of the initial increase reached a plateau above a 1:1 ratio of micelles to vesicles (Fig. 4 D), confirming that the additional oleate was not incorporated into the preformed vesicles. The second stage of increase, which occurred after >1 min, most likely corresponds to de novo vesicle formation, since no changes in FRET efficiency for labeled preformed vesicles were seen after 1 min. This second stage was sigmoidal, indicative of an autocatalytic process. Furthermore, the timescale of the second stage became faster as the concentration of micelles added increased (Fig. 4 C). This was also consistent with an autocatalytic process, assuming micelles aggregate to form the ''seeds'' for vesicle formation. The amplitude of the second stage continued to increase as more micelles were added, beyond the point at which the growth of preformed vesicles reached a plateau (Fig. 4 D), indicating that de novo vesicle formation became the primary pathway for the consumption of micelles in this concentration regime.
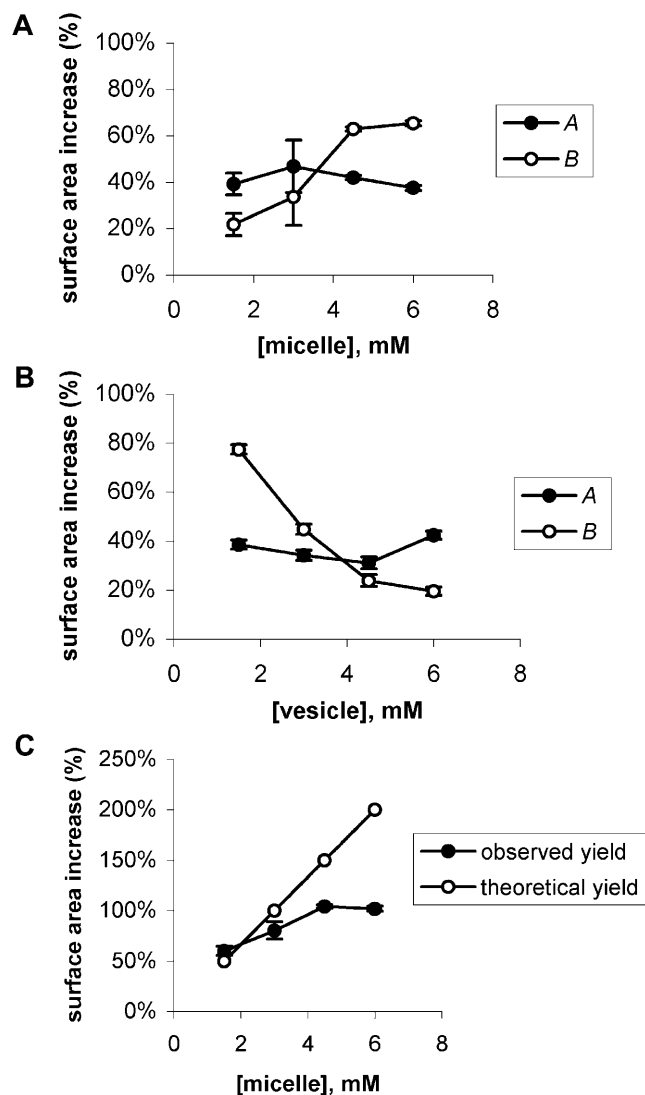
TABLE 1   Vesicle growth at low micelle/vesicle ratio

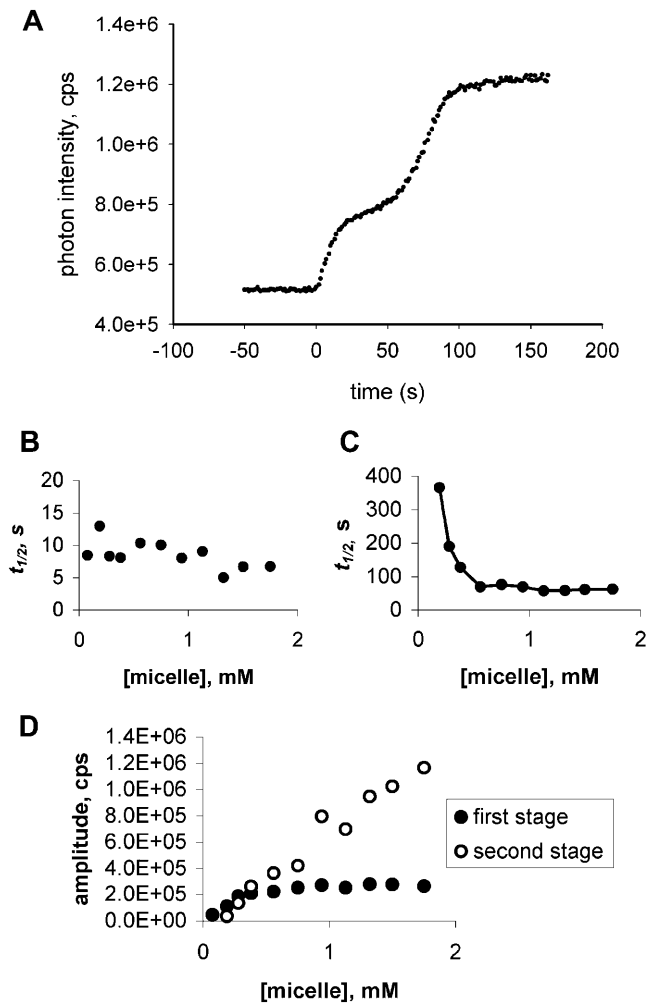| [Vesicles] (mM)	[Micelles] (mM)	Surface area increase	k (s^{-1})
6	0.6	9 ± 1%	1.4 ± 0.4
6	1.2	17 ± 1%	1.9 ± 0.5

FIGURE 4 Light scattering intensity increases during vesicle growth by direct micelle addition. (*A*) A typical example of light scattering intensity during the growth of preformed vesicles. At time zero, one equivalent of micelles were added to a solution of preformed vesicles (0.75 mM oleate in 0.2 M bicine, pH 8.5). (*B* and *C*) The timescales of the first and second stages, respectively, as the micelle concentration is varied. (*D*) The amplitudes of the first and second stages.

The biphasic kinetics of preformed vesicle growth observed by FRET led us to investigate the nature of the intermediates generated after oleate micelle addition. Micelles diluted into a solution buffered at pH 8.5 are thermodynamically unstable at concentrations above the critical aggregate concentration (cac), since they ultimately transform into vesicles. We therefore hypothesized that micelles exposed to pH 8.5 might aggregate into metastable structures before being assimilated into vesicles. Indeed we were able to detect a very rapid aggregation event after a pH drop by observing a shift in the spectrum of pyrene, a fluorescent dye which is solubilized in hydrophobic environments such as membranes and micelle cores (Almgren et al., 1979). The emission spectrum of pyrene shifts depending on whether the dye is present as a monomer

or excited-state dimer (excimer) (Vanderkooi and Callis, 1974; Schenkman et al., 1981). At a low concentration (0.5 mol %), pyrene is statistically distributed among micelles primarily as monomers due to the small volume of a single micelle (Lichtenberg, 1993). However, aggregates with a larger contiguous hydrophobic volume allow the formation of pyrene excimers. When the pH of micelles was quickly dropped to 8.5 by stopped-flow mixing, we observed an immediate (<12 ms) spectral shift corresponding to the appearance of pyrene excimers (Fig. 5 *A*). This immediate shift was not due to vesicle formation, which takes several minutes to progress beyond the lag phase. The excimer/monomer ratio remained unchanged throughout vesicle formation, which was complete within an hour under these conditions (data not shown).

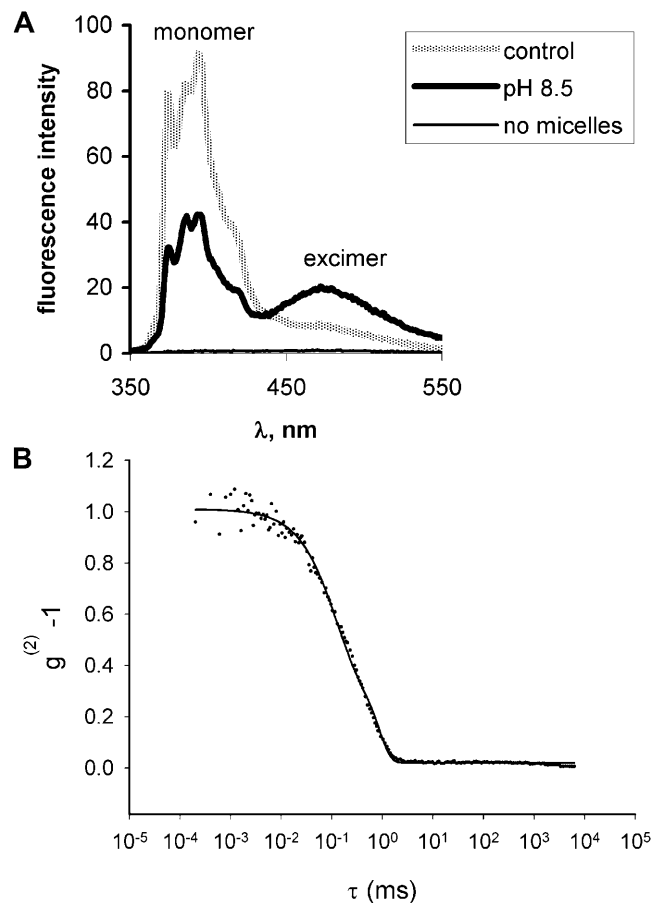We then determined the hydrodynamic radius of the aggregated intermediates by dynamic light scattering. Using

FIGURE 5 Characterization of micelles mixed with buffer at pH 8.5. (*A*) Pyrene emission spectrum of micelles mixed by stopped-flow. The monomer peaks can be seen at ~374 nm, and the broader excimer peak can be seen at 470 nm. Micelles were mixed with 0.2 M bicine, pH 8.5. In the absence of oleate, pyrene fluorescence is much weaker. Control: micelles in 1 mM NaOH. (*B*) Autocorrelation data from DLS of micelles mixed with 0.2 M bicine, pH 8.5. The solid line is a cumulant fit up to the second moment ($r^2 = 0.997$).

this method, the radius of oleate micelles was found to be 1.3 nm, consistent with the expected size of a spherical oleate micelle. The radius of unextruded vesicles prepared from micelles after overnight equilibration was 130 nm (polydispersity index = 0.7). Because a significant lag period exists before de novo vesicle formation (Blochliger et al., 1998), the intermediates could be characterized during this time. The intermediates, observed ~30 s after micelles were diluted to pH 8.5, were found to have an average hydrodynamic radius of 45 nm, with a broad distribution (polydispersity index = 1; Fig. 5 *B*). This indicated that micelles were quickly transformed into quite large, heterogeneous aggregates after a pH drop.

Because the aggregated structures observed above form so quickly, they may be intermediates on the pathway that leads from micelles to fatty acid incorporation into preformed vesicles. However, the limited extent of incorporation into preformed vesicles, together with the subsequent formation of de novo vesicles, suggested that the initial aggregate structures might be transient intermediates that undergo further rearrangements at pH 8.5. We measured the amplitude and rate of growth of preformed vesicles using the FRET assay, after the addition of micelles which had been premixed with buffer at pH 8.5 for varying incubation times. Significant growth was observed using preincubated micelles, but the amplitude of vesicle growth decreased quickly as the preincubation time increased (Fig. 6 *A*). Preincubation times >30 min did not result in significant growth. This timescale closely matched the timescale of de novo vesicle formation, confirming that the process of de novo vesicle formation competes with the growth of preformed vesicles for available fatty acid.

The growth of preformed vesicles after the addition of preincubated micelles showed only one phase of growth, corresponding to the slow phase of growth from direct micelle addition. This is consistent with the hypothesis that slow phase growth proceeds through an aggregated in-

termediate formed subsequent to the exposure of micelles to a low pH (pH 8.5) environment. Interestingly, the rate constant for vesicle growth decreased as the preincubation time increased (Fig. 6 *B*). This trend did not simply reflect the depletion of available fatty acid, since the rate constant of slow phase growth did not depend on the concentration of micelles. Instead, this trend implies that the aggregated ''intermediate'' is actually a series of intermediates that become progressively less reactive over time.

DISCUSSION

We have studied the process by which preformed oleate vesicles grow by incorporating additional oleate, supplied in the form of micelles. We initially sought to determine whether micelles fused directly with vesicles, or whether the micelles simply dissociated into oleate monomers which were incorporated into the vesicles. However, for the range of concentrations tested, neither simple pathway was sufficient to explain all of our data. A FRET-based assay for growth of preformed vesicles revealed that growth occurred in a single exponential phase for low micelle/vesicle ratios, but two phases were observed when the micelle/vesicle ratio exceeded 0.4. The rates of both fast and slow phases were independent of initial oleate micelle or vesicle concentration, thus excluding a single-step direct micelle-vesicle fusion model. In addition, linear models with one or more intermediates between micelles and growing vesicles were also ruled out, since such models can predict lagged but not double-exponential kinetics.

The overall growth reaction is a composite process, as indicated by the presence of two distinct timescales. The double-exponential character of the reaction suggests the presence of a stable intermediate. Apparently, some micelles are initially quickly consumed by a fast reaction (up to 40% growth), whereas the remaining micelles are ''trapped'' in a kinetically stable intermediate state which contributes to growth via a slower pathway.

The fast phase of growth appeared to be first-order with respect to the concentration of vesicles, indicating that the rate-limiting step involves the bilayer. The fact that the fast phase of growth had a constant amplitude *A,* corresponding to a 40% increase in vesicle surface area (when the micelle/vesicle ratio exceeded 0.4), indicated that the amount of micelle incorporation during the fast phase was stoichiometrically limited by the amount of preformed vesicles. More vesicles resulted in proportionally more fast phase incorporation in total, but the amount incorporated per vesicle was the same. This stoichiometric limitation cannot be explained as a branching ratio between two pathways, which would give proportional changes in the amplitudes of both phases of growth. Instead, we propose that the stoichiometric limitation on fast phase growth reflects a physical interaction between the added oleate micelles and the preformed vesicles. One possible interpretation is
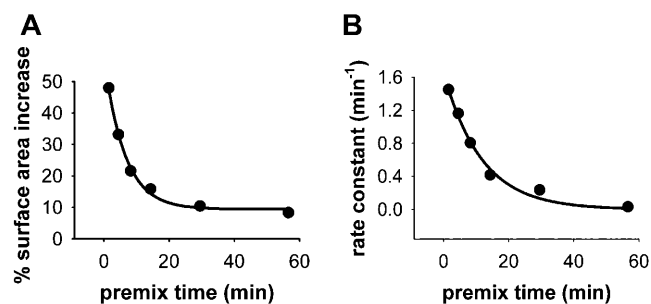

FIGURE 6 Growth of preformed vesicles using one equivalent of micelles preincubated for varying times in 0.2 M bicine, pH 8.5, as measured by the FRET assay. (*A*) Surface area increase versus micelle preincubation time; solid line indicates the fitted curve, $y = 9.44 + 48.7\ e^{-0.159(x)}$, $r^2 = 0.996$. (*B*) The rate constant of growth versus micelle preincubation time; solid line indicates the fitted curve, $y = 1.66\ e^{-0.085(x)}$, $r^2 = 0.989$.

that incoming negatively charged micelles may displace the bicine anions of the outer Helmholtz plane at the double layer of the preformed vesicles (An et al., 1996; Grahame, 1947; Hunter, 2001). Recent work indicates that correlations among counterions may decrease the magnitude of electrostatic repulsion between negatively charged surfaces, an effect which can lead to attractive interactions (for experimental studies, see Angelini et al., 2003, and Butler et al., 2003; for theoretical studies, see Allahyarov et al, 1998; Levin, 1999; Linse and Lobaskin, 1999; Ha, 2001). The displacement at the outer Helmholtz plane may also be a ''depletion'' effect, driven by an increase in entropy due to the net release of anions into bulk solution (Dinsmore et al., 1998; Kaplan et al., 1994). Such effects are expected to be quite sensitive to ionic conditions, and indeed, the growth of oleate vesicles can be abolished by the use of borate buffer (S. M. Fujikawa and J. W. Szostak, unpublished data). Thus a micelle-vesicle interaction may plausibly arise from a combination of nontrivial entropic and electrostatic sources. We suggest that the resulting micelle ''coat'' of the preformed vesicle may then be incorporated into the vesicle, resulting in the observed fast phase of growth. Because no lag period was detected before vesicle growth, the formation of a micelle-vesicle interaction is presumed to be faster than the rate-limiting step. Although the rate-limiting step of the fast phase of growth cannot be determined from our data, the possibilities include the translocation of incorporated monomers across the bilayer (Kleinfeld et al., 1997) or desorption of monomers from the micelles (Zhang et al, 1996; Thomas et al., 2002).

Such a structural picture is consistent with the apparent first-order kinetics observed for the initial fast phase of growth, since the rate-limiting step might correspond to a rearrangement of the bilayer upon fatty acid incorporation. This model also explains the observation that the fast phase amplitude of growth cannot exceed a certain amount, since the stoichiometry would be limited by the surface area of the preformed vesicles. At low micelle/vesicle ratios, only the fast phase of growth was observed, and growth was limited by the amount of micelles, as would be expected if the available micelles are assembled around the vesicles. Also, the initial rate of growth was slower for low micelle/vesicle ratios, but was approximately constant for micelle/vesicle ratios >0.4, consistent with fast formation of a limited micelle ''coat'' that is incorporated into the bilayer. The observed value of the amplitude of the fast phase (~40%) agrees well with the theoretical value calculated by assuming that spherical micelles assemble in a closely packed arrangement on the vesicle surface (42%). The lack of additional fast growth in the presence of more micelles does not appear to result from a limitation on the amount of possible growth per se, because the amplitude of slow phase growth did increase as more micelles were added. An alternative hypothesis is that, after rapid growth to a surface

area increase of ~40%, a relaxation process must occur before further growth is possible.

The observed double-exponential kinetics indicate the presence of an intermediate responsible for slow phase vesicle growth. We suggest that any micelles that are not immediately sequestered by interaction with the preformed vesicles are quickly shunted into a metastable intermediate state from which slow phase growth occurs. Although the rate-limiting step of the slow phase cannot be determined from our data, a significant possibility is the desorption of oleate monomers from the intermediates (Zhang et al, 1996; Thomas et al., 2002). Such intermediates have been observed by time-resolved static and dynamic light scattering and cryo-TEM studies of micelle to vesicle transitions in other systems, including phospholipid-surfactant mixtures (Egelhaaf and Schurtenberger, 1999; Konikoff et al., 2000) and anionic/cationic surfactant mixtures (O'Connor and Hatton, 1997; Schmolzer et al., 2002). These intermediates were observed during micelle-vesicle transformations of the entire amphiphile system, as sustainable growth of preformed vesicles has not been observed in these systems. In our system, the amplitude of slow phase growth increased as the input micelle concentration increased, as expected. Since some micelles are consumed by de novo vesicle formation, it is likely that the metastable intermediates also lead to de novo vesicle formation. This model is summarized in the following kinetic scheme, and pictorially in Fig. 7.

SCHEME
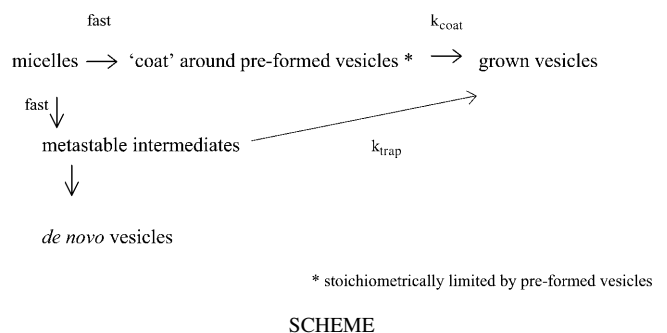
In this model, the rate constants k_{coat} and k_{trap} would be observed as the apparent rate constants k_1 and k_2. The back reaction from the metastable intermediate to micelles is presumably slower than k_{trap} and k_{coat}, since the observed rate constants did not vary with micelle concentration. Rapid dynamics of micelle transformations relative to vesicle formation have also been observed in other surfactant systems (Egelhaaf and Schurtenberger, 1999; Schmolzer et al., 2002). The nature of the metastable intermediate is unknown, but candidate structures are bilayer patches and cup-like membrane structures, such as those observed during solubilization of phospholipids by surfactants and anionic vesicle formation upon addition of salt (Vinson et al., 1989; de la Maza and Parra,
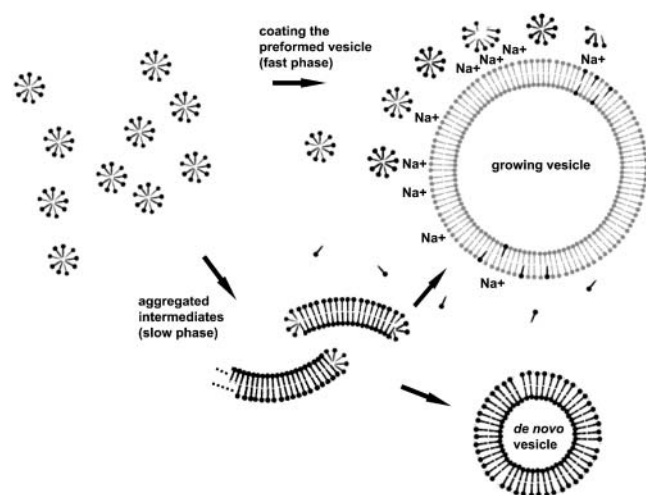
FIGURE 7 Proposed scheme of dynamic processes occurring during micelle addition to preformed fatty acid vesicles.

1994; Egelhaaf and Schurtenberger, 1999; Konikoff et al., 2000; Lasic et al., 2001; Grillo et al., 2003). Additional possible intermediate structures are long cylindrical micelles, observed during the solubilization of phospholipid vesicles by surfactants (Vinson et al., 1989), as well as during a reverse process, the aggregation of mixed phospholipid-surfactant micelles into vesicles (Long et al., 1994). Our experiments suggest that the metastable intermediate is not one well-defined structure, but may be better thought of as a mixture of polydisperse structures that change with time. The rapid appearance of pyrene excimers suggested that when micelles were mixed with buffer at pH 8.5, they quickly aggregated (<12 ms). The aggregates were observed to have a hydrodynamic radius of ~45 nm by dynamic light scattering, much larger than spherical micelles. Although the aggregates were still capable of being incorporated into preformed vesicles, their reactivity declined over time, suggesting that they were gradually transformed into more stable structures, until they eventually became vesicles.

The aggregation of micelles appears to be an immediate consequence of the pH drop. Extrapolating the rate constant of growth to zero premixing time gives a rate constant ($k = 2$/min) close to the value of k_{trap} estimated by the kinetics of direct micelle addition. During the direct addition of one equivalent of micelles to preformed vesicles, ~60% of the added micelles were shunted into metastable intermediates. Approximately two-thirds of these intermediates were then incorporated into preformed vesicles during the slow phase of growth. This proportion agrees with the yield of growth from intermediates formed when micelles were premixed with buffer (58%), extrapolated to zero premixing time.

Our interpretation of the data indicates that if preformed vesicles are present when micelles are added to a buffered solution, some micelles may be adsorbed and rapidly incorporated into the vesicles. The remainder, however,

may be converted into large metastable structures. Some of these structures may eventually form new vesicles, rather than contribute to the growth of preformed vesicles. Although our results did not support the simplest models of growth, monomer dynamics (e.g., desorption and incorporation) are nevertheless likely to be important within the framework of the proposed mechanism (Kamp and Hamilton, 1992; Thomas et al, 2002). Because this mechanism involves the physical assembly of micelles onto preformed vesicles through a combination of electrostatic and entropic effects, the temperature and ionic strength of the reaction may significantly affect the reaction. Our observations suggest that de novo vesicle formation can be minimized by avoiding the rapid introduction of more micelles than can be accommodated by adsorption to the surface of the preformed vesicles. Thus slow addition of new fatty acid should lead to optimal growth of preformed vesicles.

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