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Shrink-wrap Vesicles

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Abstract

We describe a simple approach to the controlled removal of molecules from the membrane of large unilamellar vesicles made of fatty acids. Such vesicles shrink dramatically upon mixing with micelles composed of a mixture of fatty acid and phospholipid (POPC), as fatty acid molecules leave the vesicle membrane and accumulate within the mixed micelles. Vesicle shrinkage was confirmed by dynamic light scattering, fluorescence recovery after photobleaching of labeled vesicles, and fluorescence resonance energy transfer between lipid dyes incorporated into the vesicle membrane. Most of the encapsulated impermeable solute is retained during shrinkage, becoming concentrated by a factor of at least 50-fold in the final small vesicles. This unprecedented combination of vesicle shrinkage with retention of contents allows for the preparation of small vesicles containing high solute concentrations, and may find applications in liposomal drug delivery.

Introduction

Vesicles are of interest as agents of drug delivery because they reduce the toxicity and improve the pharmacokinetics of certain drugs, while providing a protective barrier for entrapped macromolecules¹⁻³. Nanosized vesicles are of special interest because the efficiency of delivery generally increases as particle size decreases^{1,4-6}. Small vesicles may be able to penetrate small pores (e.g., endothelial defects) and also exhibit increased circulation times, and these features can lead to greater accumulation in the hypervascularized tissue of solid tumors^{4,6-8}. The smallest vesicles currently used are still relatively large (~100 nm), so vesicles of even smaller size may have additional utility. Small particles could also be useful for inhaled drugs, having an increased probability of deposition as well as an enhanced ability to cross the pulmonary epithelium^{9,10}.

The amount of drug encapsulated by small vesicles is usually quite low, since the encapsulated volume decreases with the third power of the radius. Here we describe a method for preparing small vesicles (outer diameter 20-25 nm) with highly concentrated internal contents, by first preparing large unilamellar vesicles (outer diameter ~70 nm) from myristoleic acid (MA) and then shrinking these membranes in a process that retains the majority of encapsulated dye molecules.

Vesicle shrinking is a surprising phenomenon that is a consequence of the dynamic behavior of single-chain amphiphiles in bilayer membranes. Fatty acid vesicles can incorporate

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Supporting Information Available: Standard curve of donor fluorescence vs. probe density for the FRET assay (Fig. S1). Standard curve of self-quenching of calcein vs. concentration (Fig. S2). Release of calcein during and after shrinking of MA vesicles (Fig. S3). Final vesicle diameter after shrinking using micelles composed of varying mole percentages of POPC and MA (Fig. S4). Fluorescence of encapsulated calcein after the addition of hypertonic solution of bicine (Fig. S5). Shrinking of oleate vesicles after adding MA/POPC (89% MA, 11% POPC) micelles (Fig. S6). Supplementary text and references. This material is available free of charge via the Internet at <http://pubs.acs.org>.

additional fatty acid (supplied as micelles) into their membranes and thereby grow larger¹¹⁻¹³. We have previously observed both the growth of osmotically swollen vesicles and a small degree of shrinkage of osmotically relaxed vesicles following the mixing of these vesicles, as fatty acid molecules are transferred from the relaxed to the swollen vesicles¹⁴. Vesicle growth proceeds through incorporation of fatty acid into the outer leaflet of the membrane, followed by flip-flop of approximately half of these molecules into the inner leaflet¹⁵. The flip-flop of fatty acid molecules is much faster than that of most phospholipids, such as POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine)¹⁶⁻¹⁹. We attempted to take advantage of the slow flip-flop of phospholipids in an effort to generate asymmetric membranes with an excess of phospholipid in the outer leaflet, by mixing MA vesicles with MA micelles doped with POPC (11 mol% POPC; 89 mol% MA).

However, instead of incorporating into the vesicles, the mixed myristoleic acid/phospholipid micelles extracted fatty acid from the vesicles. Most of the initially encapsulated dye remained entrapped in the vesicles after shrinkage was complete. This yielded very small vesicles with highly concentrated contents (50-100-fold increase in concentration).

Methods and Materials

Vesicle preparation

Vesicles were prepared as described¹³. Briefly, myristoleic acid (Nu-Chek Prep, Inc., Elysian, MN) was mixed with one equivalent of NaOH in water, followed by addition of 1 M bicine to obtain a visibly turbid solution of 100 mM MA in 0.2 M bicine, pH 8.5. After extrusion through 0.1 μ m polycarbonate filters using a Mini-extruder (Avanti Polar Lipids, Alabaster, AL), vesicles were incubated overnight for equilibration. All subsequent vesicle dilutions were done in 4 mM MA, 0.2 M bicine, pH 8.5. 4 mM is approximately equal to the critical aggregate concentration (cac) of MA and was necessary to preserve membrane integrity after dilution.

For the FRET experiments, vesicles were labeled with 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rh-DHPE) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) (Molecular Probes, Eugene, OR). Vesicles contained 0.025 mol% of each dye and were prepared as described (Hanczyc et al., 2003). The standard curve was prepared using 0.025-0.4 mol% of each dye.

To prepare vesicles encapsulating calcein or HPTS (Molecular Probes, Eugene, OR), 0.2-20 mM calcein or 0.5 mM HPTS was mixed with bicine before addition to MA micelles. Extruded vesicles were purified from unencapsulated dye by size-exclusion chromatography on Sepharose 4B (Sigma-Aldrich, St. Louis, MO).

For FRAP experiments, vesicles were labeled with 0.5% NBD-PE. At this concentration of NBD-PE, vesicle formation from micelles resulted in relatively small vesicles, as determined by DLS. Instead, these vesicles were prepared by resuspending the oil in a solution of 0.2 M bicine for a final pH of 8.5¹⁵.

The concentration of MA vesicles in each preparation was determined using the ADIFAB fatty acid assay (Molecular Probes)²⁰.

MA/POPC micelle preparation

MA/POPC micelles were prepared by combining solutions of MA in methanol and POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Avanti Polar Lipids, Alabaster, AL) in chloroform at an 89:11 mol/mol ratio, unless otherwise specified. Volatile solvents were removed by rotary evaporation. The resulting oil was dissolved in water with one equivalent of NaOH to yield a clear solution with a final total lipid concentration of 50 mM and pH >10.

For FRAP experiments with labeled micelles, 0.5 mol% NBD-PE was added to the mixture prior to rotary evaporation.

Micelle addition to vesicles

Shrinking reactions were initiated by simultaneously adding MA/POPC micelles and 1 M bicine, pH 8.5, to MA vesicles in 0.2 M bicine, pH 8.5. The added volume of 1 M bicine, pH 8.5, ensured that the final solution contained 0.2 M bicine, pH 8.5. Reactions were mixed by pipetting and vortexed for ~3 seconds. Control mixtures contained MA vesicles diluted into buffer. In general, shrinking reactions contained 3.2 mM vesicles and 4 mM MA/POPC micelles unless otherwise specified. To determine the dependence of rate on micelle concentration, 3.2 mM vesicles were mixed with 4–30 mM micelles. To determine the dependence of rate on vesicle concentration, 4 mM micelles were mixed with 0.42–3.2 mM vesicles. To attain acceptable signal strength, FRAP experiments contained 32 mM vesicles and 20 mM micelles. Reaction kinetics were fit to a first-order equation ($y = y_0 + a \cdot \exp(-kx)$) to determine the rate constant (k) and the final diameter (y_0).

Dynamic light scattering (DLS) and turbidity measurements

DLS measurements of vesicles and shrinking reactions were performed using a PDDLS Coolbatch/PD2000DLS instrument at 25°C (Precision Detectors, Inc., Franklin, MA). This instrument is insensitive to very small particles (<5 nm) due to the lower limit on the correlation times that it can measure. Also, because of the strong dependence of scattering intensity on particle size, DLS measurement of shrinking reactions effectively measured the progress of changes in vesicle size. For the measurement of small micelles in the absence of vesicles, DLS was performed using a similar instrument capable of detecting shorter correlation times, the PDDLS Coolbatch 90T/PD2000DLS^{Plus} (Precision Detectors, Inc. Franklin, MA). Turbidity was measured by absorbance at 500 nm using a Cary 1E UV-Visible spectrophotometer (Varian, Inc., Walnut Creek, CA).

Fluorescence resonance energy transfer (FRET) assay

Shrinking reactions of labeled vesicles and MA/POPC micelles were measured over time in a quartz cuvette using a Cary Eclipse fluorimeter (Varian Inc., Walnut Creek, CA). The FRET signal was determined from the extent of quenching of donor fluorescence (NBD-PE; ex. 430 nm, em. 530 nm), as described^{15,21,22}. Vesicles were disrupted using Triton X-100 (1%) to measure the unquenched fluorescence of the donor. A standard curve for FRET signal vs. probe density was used to translate measured fluorescence into probe density (Fig. S1) and therefore relative surface area (1/relative probe density). The corresponding relative diameter was calculated as the square root of the relative surface area. The relative diameter was converted to nm units by multiplying by the initial diameter of the vesicles, determined by DLS. The presence of the FRET dyes did not affect the rate of shrinking as determined by DLS.

Calcein self-quenching and percent encapsulation

Aliquots from a shrinking reaction were run on a Sephadex G-25 size-exclusion column to separate vesicles with encapsulated calcein from free calcein. To determine the internal concentration of calcein in the vesicles, the fluorescence (ex. 415 nm, em. 600 nm) of the vesicle fraction was measured before (F_{ves}) and after (F_{ves}^0) addition of 1% Triton X-100.¹³ The extent of quenching (F_{ves}/F_{ves}^0) was related to calcein concentration by a standard curve (Fig. S2). The corresponding relative diameter was calculated as the (concentration factor)^{-1/3}, after adjusting for dye leakage. The relative diameter was converted to nm units by multiplying by the initial diameter of the vesicles, determined by DLS.

The percent dye encapsulated was determined as $F_{ves}^0/(F_{ves}^0 + F_{free})$, adjusted for the volumes of the fractions. For calcein, because dye release occurred while the vesicle size was decreasing as well as after the vesicle size had equilibrated, the percent dye encapsulated at the end of shrinking was determined by fitting a single exponential decay curve to time points collected in <1000 minutes (i.e. <5 half-lives) (for an example, see Fig. S3). For HPTS, the end of the shrinking reaction was defined to be ~4 half-lives, as determined by the decrease in vesicle size measured by DLS.

Fluorescence recovery after photobleaching (FRAP) assay

Vesicles and micelles were labeled with 0.5 mol% NBD-PE in separate experiments. Shrinking reactions were incubated at room temperature, transferred to a glass slide, covered with a cover slip and sealed with clear nail polish. The diffusion constant of the labeled species was determined by FRAP,²³ using a Leica TCS SP confocal laser scanning microscope (20x objective, NA = 0.7). A selected point was photobleached using the 488 nm argon laser line at 100% intensity for one second. The initial width and extent of bleaching were determined from a sample of labeled micelles that were dried onto a slide. Recovery data were collected at 11% intensity and successive frames were 600 ms apart (4x zoom, pinhole = 222 μ m). Data were normalized by total image intensity. The characteristic diffusion time was determined by fitting to the series solution for 2-D diffusion with a Gaussian beam profile, truncated after 40 terms, to calculate the diffusion constant (D).²⁴ The diameter was calculated according to Stokes' law, assuming spherical particles ($D = kT/(6\pi\eta r)$). Results were averaged from at least five replicates. The presence of 0.5% NBD-PE did not affect the rate of shrinking determined by DLS.

Results and Discussion

We expected that the incorporation of molecules derived from the MA/POPC micelles into the vesicle membrane would cause an increase in average particle size, as seen for vesicle growth following the addition of MA micelles¹³. However, in initial experiments we observed a decrease, instead of the expected increase, in turbidity (Fig. 1). Because changes in turbidity may reflect changes in particle size, shape, or number, we also measured the diffusion constant of the particles by dynamic light scattering (DLS). DLS indicated that the mean hydrodynamic diameter (d_H) of the vesicles decreased from 73 nm to 20 nm, in an apparent first-order process ($k_{DLS} \sim 0.004 \text{ min}^{-1}$) (Table 1; Fig. 2A). This decrease was also observed using micelles of different POPC content (5.5-16.5 mol%) (Fig. S4).

Two processes could lead to a decrease in average vesicle size: division or shrinking. To distinguish between these possibilities, we prepared MA vesicles containing a pair of fluorescent phospholipid dyes incorporated into the membrane. These dyes form a fluorescence resonance energy transfer (FRET) pair and do not exchange detectably among vesicles for many hours^{13,14,22}. The efficiency of FRET depends strongly on the distance between dye molecules and thus measures the surface density of the dyes (Fig. S1)²¹. If the vesicles were dividing, the surface density of the dyes would not change. But if vesicles were shrinking by extraction of MA out of the membranes, the surface density of the dyes would increase. We found that the surface density of the dyes increased upon mixing labeled vesicles and MA/POPC micelles. After translating these data into relative diameter (assuming the vesicles were spherically shaped), the decrease of vesicle size over time measured by the FRET assay was similar to that measured by DLS ($36 \pm 1\%$ of the initial diameter; Table 1; Fig. 2B). This result indicated that the vesicles were shrinking, not dividing.

An important question was whether the internal contents of the vesicles were concentrated during shrinking, rather than being released into bulk solution. We encapsulated a water-soluble, self-quenching fluorescent dye (calcein) in MA vesicles. As shrinking progressed,

aliquots of the reaction were purified by size-exclusion chromatography to separate the vesicles, containing entrapped calcein, from calcein that had leaked into free solution. Although there was some leakage, most of the initially encapsulated calcein copurified with the vesicle fraction (Fig. S3), and this percentage was similar for different concentrations of initially encapsulated calcein. For vesicles initially encapsulating 20 mM calcein, $58 \pm 6\%$ of the dye was retained after shrinking, and $52 \pm 13\%$ of the dye was retained by vesicles initially encapsulating 2 mM calcein. Another fluorescent dye, HPTS, showed greater retention at the end of the vesicle shrinking reaction ($\sim 80\%$) (Fig. 3). The shrinking reaction also increased the permeability of the membrane, leading to the slow release of dye after the vesicle size had equilibrated, a potentially interesting property for a delivery vehicle.

The self-quenching efficiency of calcein fluorescence in the vesicle fraction is a measurement of the internal concentration of calcein (Fig. S2)^{13,25}. Although the dynamic range of self-quenching is limited (~ 7 -30 mM calcein), the effective dynamic range of the assay could be made quite large by preparing vesicles with different initial concentrations of calcein ($c_0 = 0.2$ - 20 mM). The change in the inner diameter (d_i) of the vesicles as a function of time was determined by compiling self-quenching data from several experiments, such that later time points were taken from experiments containing lower c_0 (e.g., 0.2 mM).

Using this assay, we found that encapsulated calcein was concentrated by a factor of 50-100, from 0.2 mM to 10-20 mM. The resulting shrinking time course closely resembled that determined by DLS and the FRET assay (Table 1; Fig. 2C). The relative decrease in d_i is expected to be greater than the relative decrease in hydrodynamic diameter, because of the non-zero thickness of the membrane. For an estimated membrane thickness of 4 nm^{26,27}, the decrease of d_H from 73 nm to 20 nm, measured by DLS, would correspond to a decrease of d_i from 65 nm to 12 nm, or 18% of the initial d_i . From the calcein quenching experiments, we observed a final relative encapsulated diameter of 19%, which agrees well with the value expected based on the DLS data.

While these data confirmed that the encapsulated volume was shrinking, we wondered whether this degree of dye concentration was reasonable given the consequent rise in osmotic pressure. The maximum tolerable osmotic pressure gradient is given by Laplace's law for a sphere: $\tau^* = (\Delta\Pi^*)r/2$, where τ^* is the rupture tension of the membrane, $\Delta\Pi^*$ is the maximum sustainable osmotic pressure gradient, and r is the radius of the sphere.^{28,29} For $\tau^* \sim 10$ dyn/cm¹⁴, the maximum gradient for a sphere of inner diameter = 12 nm would be ~ 0.7 Osm. Given the observed 50-100-fold increase in concentration of calcein, for $c_0 = 2$ mM (~ 10 mOsm due to the negative charges on calcein), the final internal osmolarity would be ~ 0.5 -1 Osm after shrinking, close to the calculated maximum gradient. The osmotic contribution of the buffer (bicine) could be ignored because it equilibrates across the membrane within the timescale of the experiments (Fig. S5). However, the same calculation for $c_0 = 20$ mM would imply an osmotic pressure gradient greater than $\Delta\Pi^*$. Since the final degree of dye concentration was determined from measurements at low c_0 , we tested whether vesicles with $c_0 = 20$ mM shrank less than vesicles with $c_0 \leq 2$ mM calcein. Indeed, DLS measurements showed that vesicles with $c_0 = 20$ mM shrank less (Table 1), suggesting that the increased internal osmotic pressure inhibited further shrinking.

We also investigated the fate of the MA/POPC micelles after mixing with MA vesicles. Because spherical micelles are quite small, they scatter little light compared to vesicles, such that DLS primarily monitors the vesicles in the reaction. However, micelles can be labeled with a non-exchanging fluorescent phospholipid (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; NBD-PE), and the size of these particles can be monitored by fluorescence recovery after photobleaching (FRAP)³⁰. In FRAP, a sample is placed on a microscope slide and a small region is photobleached by a high intensity

laser pulse. The recovery of fluorescence into this region measures the diffusion constant, and thus the size, of the fluorescent particles^{23,31}. If labeled MA/POPC micelles are mixed with buffer at pH 8.5, they show only a slight increase in size (Fig. 2D), whereas labeled MA micelles show a dramatic increase in size as they aggregate into large vesicular structures visible under a light microscope (up to several microns)¹³. This indicates that adding POPC to MA micelles inhibits the transition from micelles to a vesicular phase, consistent with the observed lack of incorporation of these micelles into preformed vesicles.

When labeled MA/POPC micelles are mixed with vesicles, the micelles show a slight increase in size (Fig. 2D), as expected. If all of the fatty acid extracted from the vesicles had been incorporated into the micelles, the diameter of the micelles would have increased by only 36%. For particles of this small size, a change in micelle diameter of this magnitude is difficult to measure accurately by FRAP, but our data are consistent with this possibility. These results do indicate that MA/POPC micelles remain as small particles throughout the vesicle shrinking reaction.

In a separate FRAP experiment, the vesicles were labeled and monitored after the addition of MA/POPC micelles, confirming that the vesicles decreased in size (Fig. 2E; Table 1). Because FRAP required a relatively high amount of fluorescence to attain an acceptable signal to noise ratio, a higher initial concentration of MA vesicles was used for these experiments. Nevertheless, the results were similar to those obtained from the other techniques. Also, the sizes of the micelles and vesicles measured by FRAP were distinct from each other, indicating that NBD-PE did not exchange substantially between the two populations, consistent with the absence of exchange of the FRET dyes among vesicles.

By the DLS, FRET, calcein quenching, and FRAP assays, the shrinking of vesicles appeared to follow a first-order integrated rate law. For a true first order reaction, the initial rate of shrinking would be directly proportional to the initial vesicle concentration, resulting in a van't Hoff plot with slope = 1. However, the initial rate of shrinking decreased with increasing vesicle concentration, indicating that vesicles inhibited the reaction (Fig. 4A). In contrast, increasing the concentration of micelles increased the reaction rate dramatically; in some cases, a 2-fold increase in the concentration of micelles resulted in a ~20-fold increase in initial rate (Fig. 4B).

A possible explanation for these results is that MA/POPC micelles associate with MA vesicles soon after mixing, forming reactive complexes. Similar complexes have been implicated as intermediates in the growth of fatty acid vesicles¹⁵ (supplementary text). The initial rate of transfer would depend on the structure of the complexes, such that more micelles per vesicle would lead to a greater rate of transfer. This accounts for the inhibition of the reaction by vesicles and the increase in reaction rate at higher micelle concentrations. Furthermore, the formation of complexes containing more micelles may lead to an apparent increase in the reaction order, consistent with the observed van't Hoff plot for micelles (Fig. 4B). The driving force for the decrease in vesicle size may be that the geometry of POPC, which usually prefers to form bilayer membranes, destabilizes spherical micelles. The uptake of MA into these micelles could result in the formation of bicelle-like structures with the exposed edges stabilized by fatty acid molecules (Fig. 4C)^{32,33}. If the driving force is inherent to the structure of MA/POPC micelles, the shrinking reaction might be applied to other vesicle compositions. Indeed, oleic acid vesicles also shrink when mixed with MA/POPC micelles (Fig. S6). Vesicles made from a polymerizable fatty acid (tricosadiynoic acid) also shrink when mixed with MA/POPC micelles, suggesting a possible way to stabilize the final small vesicles^{34,35}.

Conclusion

We have described an unprecedented phenomenon in which a gradual removal of molecules from a bilayer membrane vesicle leads to a decrease in vesicle size and a concomitant ‘shrink-wrapping’ of encapsulated impermeable contents. The vesicle contents become highly concentrated, by a factor of 50-100, reaching the maximum osmotic concentration allowed by the tensile strength of the membrane. Small vesicles can therefore be made to encapsulate large amounts of solutes despite their low volumes. For drug delivery applications, more work is needed to stabilize these vesicles to physiological conditions; one possibility is to polymerize the vesicles, perhaps by using a cross-linkable fatty acid or by encasing them in polymerizable shells³⁶⁻³⁹. This strategy for shrinking vesicles may then be useful for encapsulating substantial amounts of drugs in very small vesicles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Turbidity of MA vesicles after addition of MA/POPC (89:11) micelles (solid line) compared to addition of pure MA micelles (dotted line). The mixture contained 20 mM vesicles and 25 mM micelles. Micelles were added at $t = 4$ min.

Figure 2.

Decrease in size of MA vesicles (3.2 mM, unless otherwise indicated) after mixing with MA/POPC micelles (89% MA, 11% POPC; 4 mM lipid, unless otherwise indicated). Solid lines show curve fits with parameters given in the text. (A) Diameter measured by dynamic light scattering. Empty circles: vesicles alone; filled circles: mixture. (B) Diameter relative to initial diameter, measured by FRET assay. Empty circles: vesicles alone; filled circles: mixture. (C) Diameter relative to initial diameter, measured by the calcein self-quenching assay. Data were compiled from seven separate shrinking experiments with initial [calcein] = 0.2, 2, or 20 mM. Vesicles alone showed <8% dye leakage and change in quenching efficiency after 22 hours. (D) Diameter of labeled MA/POPC micelles determined by FRAP. Empty circles: 40 mM

micelles in buffer only; filled circles: mixture (20 mM micelles and 32 mM vesicles). Micelles in water have an apparent diameter of 8 nm by this assay. (E) Diameter of vesicles determined by FRAP (final diameter = 28 nm; $k_{\text{FRAP}} = 0.004 \text{ min}^{-1}$). Empty circles: 32 mM vesicles alone; filled circles: mixture (20 mM micelles and 32 mM vesicles).

Figure 3.

Shrinking of MA vesicles initially containing 0.5 mM HPTS. Filled circles: MA vesicles mixed with MA/POPC micelles; empty circles: vesicles alone. The average vesicle diameter decreased over time, as measured by DLS (A; solid line shows a single exponential decay curve fit, with $k \sim 0.0044 \text{ min}^{-1}$ and final diameter $\sim 23 \text{ nm}$). Encapsulated HPTS was released slowly (B), with a timescale much slower than the decrease of vesicle size.

Figure 4.

Dependence of initial rate of vesicle shrinking on the concentration of reactants. (A) Van't Hoff plot indicating inhibition of the reaction rate by vesicles (slope ~ -1). (B) Van't Hoff plot showing the effect of micelles on initial rate. The dotted lines are meant to guide the eye (slope ~ 0.6 and 4), suggesting the presence of more than one reaction order. (C) Diagram of the transfer of MA (black) from vesicles encapsulating calcein (green) to micelles containing POPC (red). Transfer of MA to micelles might create a local membrane-like geometry, relieving strain in the micelles caused by the geometry of POPC.

Table 1

Rate and extent of vesicle shrinking.*

technique		initial [calcein] (mM)	final outer diameter (nm)	$k(\text{min}^{-1})$
DLS	control	-	73 ± 2	-
	mix	-	20 ± 3	0.0037 ± 0.0002
	mix	0.2, 2	20 ± 2	0.006 ± 0.001
	mix	20	26 ± 3	0.007 ± 0.001
FRET		-	26 ± 3	0.0073 ± 0.0004
calcein self- quenching		0.2 - 20	20**	0.008
FRAP		-	28	0.004

* Mixture: 3.2 mM MA vesicles + 4 mM MA/POPC micelles, except for FRAP experiment (32 mM MA vesicles + 20 mM MA/POPC micelles). Errors given are standard deviations.

** assuming a membrane thickness of 4 nm