Supporting Information

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SI Text

Statistics of the Membrane Tube Formation in Deflated Vesicles. In our experiments, membrane tube formation is a common phenomenon observed during the deflation of vesicles containing polyethylene glycol (PEG)/dextran homogeneous aqueous solution. Vesicles with spherical shape are typically under some tension. Upon significant deflation, the vesicles gain excess area, which can undertake several different paths. First, the excess area may participate in membrane fluctuations, which are optically visible under the microscope. Second, the vesicle may adopt nonspherical morphology (e.g., prolate, oblate or sessile-shape) when deformed by gravity. In the third path, which is characteristic for the case of vesicles with phase separation, the excess area gets involved in the formation of tubes. The latter is also associated with elevation in the membrane tension, as indicated by the overall geometry of the vesicles, which is either a sphere or an assembly of spherical caps. One example is illustrated in Fig. S1, showing the deflation of a vesicle made of PEG-membrane in S_d (the deflation of S_p vesicles is illustrated in Fig. 1B in the main text); for composition of the polymer solutions S_d and S_p , see section Deflation Trajectories in the Phase Diagram in the main text. Upon significant deflation, the spherical vesicle, Fig. S1A, adopts sessile-shape (Fig. S1B). The latter indicates the decrease of the membrane tension, which cannot overcome gravity. As soon as the new phase appears (last snapshot in Fig. S1B), the vesicle becomes spherical again. The morphological change from sessile-shape to sphere indicates the elevation of the membrane tension: The tension can overcome gravity now. The excess area forms membrane tubes; see arrows in Fig. S1C.

Observations on vesicles using single-step deflation indicated that membrane tubes formed in approximately 95% of all deflated vesicles in each of the four systems studied. This statistic was obtained from 188 G_{M1}-membrane vesicles (as in the main text) prepared in S_p (see also Fig. S3), 163 G_{M1} -membrane vesicles prepared in S_d (as in Fig. S2), 312 PEG-membrane vesicles prepared in S_p (see also Fig. S4), and 435 PEG-membrane vesicles prepared in S_d (as in Fig. S1). The specific membrane compositions are given in Table S1. When tubes are not observed after the initial deflation step, they are observed upon further deflation when phase separation within the droplets occurs. The external solution does not affect the tube formation. Tubes form in vesicles diluted and deflated by sucrose aqueous solution. We also explored the behavior of vesicles encapsulating solutions without phase separation, such as aqueous sucrose solution and PEG solution. No tubes are observed in these cases.

Calculation of Membrane Tension and Vesicle Apparent Area During Micropipette Aspiration. The tension induced by the micropipette in the *pe* membrane (i.e., the membrane segment separating the PEG-rich droplet from the external phase) can be calculated from the following equation (1):

$$\hat{\Sigma}_{pe} = PR_p/2(1 - R_p/R_v), \qquad [S1]$$

where *P* is the pressure difference between inside and outside the pipette, R_v is the radius of the vesicle (or the curvature radius of the PEG-rich phase when the vesicle is nonspherical), and R_p is the radius of the spherical part of the vesicle inside the micropipette.

The effect of gravity is neglected because of the insignificant density difference between the interior and exterior of the vesicle. For the predeflated vesicle shown in Fig. 4 in the main text (r = 1.4), the density of the external medium is 1.0110 g/cm³, and the density of the polymer solution inside the vesicle is 1.0165 g/cm³ (see also Fig. S6).

In all measured cases, the vesicles adopt an axisymmetric shape. Under low suction pressure, the part in the pipette is very small and the vesicle geometry is an ensemble of spherical caps (Fig. S5*A*). Under high suction pressure, the vesicle contour is a combination of spherical caps and a cylindrical part inside the pipette (Fig. S5*B*). To measure the apparent area of the vesicle under different pressures, we fit the vesicle shape in the digitized images with circles and straight lines (Fig. S5).

Note that the vesicle protrusion inside the pipette was observed to oscillate slightly under low suction pressure. This oscillation can still be observed half an hour after applying a constant suction pressure. It may be caused by thermal fluctuations of the membrane or perturbation of the surrounding. In general, the membrane stops fluctuating when the suction pressure exceeds a few pascals, corresponding to tension of about 0.03 mN/m.

Membrane Tensions and Area Dilation. The *pd* interface pulls the membrane along the contact line with the interfacial tension Σ_{pd} , which represents a force per unit length of the contact line. In mechanical equilibrium, this force must be balanced by two membrane tensions $\hat{\Sigma}_{pe}$ and $\hat{\Sigma}_{de}$ acting tangential to the *pe* and *de* membranes, respectively. Because both the *pe* and the *de* membrane segments are fluid, they are both characterized by uniform tensions.

The interfacial tension Σ_{pd} was experimentally determined for pd coexistence of the bulk phases. This tension has a magnitude of the order of 10^{-5} N/m and increases monotonically with increasing osmolarity, from about 0.4×10^{-5} N/m to about 1.8×10^{-5} N/m over the osmolarity range studied here. The membrane tensions can be expressed in terms of the interfacial tension Σ_{pd} and the (effective) contact angles θ_p , θ_d , and θ_e , under which the three surface segments meet at the contact line (2). These three angles are defined in such a way that their sum is equal to 360°, whereas each individual angle must be smaller than 180° because of the force balance along the contact line. The latter balance also implies the relations

$$\hat{\Sigma}_{pe} = \Sigma_{pd} \sin(\theta_d) / \sin(\theta_e)$$
[S2]

and

$$\hat{\Sigma}_{de} = \Sigma_{pd} \sin(\theta_p) / \sin(\theta_e), \qquad [S3]$$

which reveal that the two membrane tensions have the same order of magnitude as the interfacial tension Σ_{pd} unless one of the contact angles is exceptionally small or very close to 180°. For the vesicles shown in Fig. 1*B* in the main text, the contact angles vary between $(\theta_p, \theta_d, \theta_e) = (36^\circ, 153^\circ, 171^\circ)$ for panel 1 and $(\theta_p, \theta_d, \theta_e) =$ $(118^\circ, 165^\circ, 77^\circ)$ for panel 3. It then follows from the relations **S2** and **S3** that the membrane tensions $\hat{\Sigma}_{pe}$ and $\hat{\Sigma}_{de}$ were of the same order as $\Sigma_{pd} \sim 10^{-5}$ N/m.

In principle, the tensions $\hat{\Sigma}_{pe}$ and $\hat{\Sigma}_{de}$ lead to a stretching of the membrane. In practice, the corresponding area dilation is extremely small. This can be seen as follows. If the membrane is viewed as a thin elastic sheet, its deformations can be decomposed into stretching and bending modes. This decomposition implies that the tensions $\Sigma_{pe} = \hat{\Sigma}_{pe} - 2\kappa m_{pe}^2$ and $\Sigma_{de} = \hat{\Sigma}_{de} - 2\kappa m_{de}^2$ arising from the constraints on the membrane area are related to the membrane compressibility. For an unstretched

membrane of area A_0 , a tension Σ induces the area dilation $\Delta A = (\Sigma/K_A)A_0$, which depends on the area compressibility modulus K_A . For lipid bilayer membranes, the latter modulus is of the order of 0.2 N/m, and tensions of the order of 10^{-5} N/m lead to the tiny area dilation $\Delta A \cong 10^{-4} A_0$. In fact, the theoretical estimate for the spontaneous curvature m_{pe} of the *pe* membrane implies the tension $\Sigma_{pe} \sim \kappa m_{pe}/R_{sc,pe}$, where the length scale $R_{sc,pe}$ denotes the curvature radius of the spherical pe cap. For the vesicles studied here, the tension Σ_{pe} is then found to be $\Sigma_{pe} \sim 10^{-7}$ N/m and, thus, two orders of magnitude smaller than the tension $\hat{\Sigma}_{pe}$. Therefore, the area dilation of the *pe* membrane segments is even smaller and only of the order of $\Delta A \cong 10^{-6} A_0$. Likewise, we expect that the tension Σ_{de} is also substantially smaller than $\hat{\Sigma}_{de}$ but cannot determine this reduction in the absence of a reliable estimate for the spontaneous curvature m_{de} of the *de* membranes. In any case, these tiny area dilations can be safely ignored, which implies that the total membrane area remains constant during the deflation process and equal to the area A_0 of the initial, spherical vesicle before deflation and phase separation.

Bending Rigidity Measurement Using Fluctuation Analysis. The bending rigidity of G_{M1} -membranes (as in the main text) was measured using fluctuation analysis of vesicles with S_p solution in the interior and exterior. This method consists of studying the shape fluctuations, which the vesicle exhibits in a representative period of time (3).

Vesicles in the native polymer solution were transferred into a chamber made of two microscope slides separated by 1-mm-thick press-to-seal silicone isolator (Molecular Probes). The vesicles were left to relax for 1 d. An inverted microscope Axiovert 135 (Zeiss) equipped with a 40x dry objective was used to visualize the vesicles under phase contrast mode. A floppy quasispherical vesicle without defects was selected. A time sequence of this vesicle was recorded with fast digital camera HG-100 K (Redlake Inc.) for 90 s at acquisition speed of 60 frames per second and exposure time of 160 µs. During the experiments, effort was made to keep the vesicle on focus because of the diffusion of the vesicle. The image time sequences were analyzed to obtain the bending rigidity by home-developed software (4). The result cited in the main text was obtained from measurements on 16 vesicles. The bending rigidity was found to be 1.13×10^{-19} J with standard error of 0.22×10^{-19} J.

Bending Energy of Cylindrical Nanotubes. The nanotubes consisted of *pe* membrane segments with bending rigidity κ and spontaneous curvature m_{pe} . If the nanotubes are cylindrical in shape with radius R_{cy} , they have the constant mean curvature $M_{cy} = -1/2R_{cy}$ and the bending energy

$$F_{cy} = A_{tu} 2\kappa (M_{cy} - m_{pe})^2 = A_{tu} 2\kappa M_{cy}^2 \left(1 - \frac{m_{pe}}{M_{cy}}\right)^2, \qquad [S4]$$

where A_{tu} denotes the tube area.

In mechanical equilibrium with a large spherical pe cap of curvature radius $R_{sc,pe}$, the mean curvature M_{cy} of the cylindrical tubes satisfies

$$2\hat{\Sigma}_{pe}M_{cy} - 4\kappa M_{cy}^3 \approx 2\hat{\Sigma}_{pe}/R_{sc,pe},$$
[S5]

as follows from the analysis in ref. 5. The solution of this implicit equation leads to the mean curvature

$$M_{cy} \approx -(\hat{\Sigma}_{pe}/2\kappa)^{1/2} - 1/2R_{sc,pe}.$$
 [S6]

When this expression is inserted into the bending energy **S4**, one obtains

$$F_{cy} \approx A_{tu} \hat{\Sigma}_{pe} (1 - m_{pe}/M_{cy})^2.$$
 [S7]

For nanotubes with $m_{pe} = 0$ and $\hat{\Sigma}_{pe} = \Sigma_{pe}$, this free energy is equal to $F_{cy} \approx A_{uu} \Sigma_{pe}$. On the other hand, in the presence of a spontaneous curvature $m_{pe} \neq 0$, the tube curvature M_{cy} and the spontaneous curvature m_{pe} are related via Eq. 2 in the main text, and the tubes then have the bending energy $F_{cy} \approx A_{uu} \kappa / 8R_{sc,pe}^2$. For the vesicles studied here, the latter bending energy is several orders of magnitude smaller than $A_{uu}\hat{\Sigma}_{pe}$.

Derivation of Eqs. 2 and 3. Consider a segment of *pe* membrane separating the PEG-rich phase with osmotic pressure P_p from the external phase with osmotic pressure P_e . If this segment has the shape of a spherical cap, its mean curvature $M_{sc} = 1/R_{sc,pe}$ satisfies the Laplace-like equation

$$P_p - P_e \approx 2\hat{\Sigma}_{pe}/R_{sc,pe}$$
 [S8]

in the limit of large curvature radii $R_{sc,pe}$ with the membrane tension $\hat{\Sigma}_{pe}$ as defined in Eq. 1 of the main text. If another membrane segment forms a cylindrical tube inside the vesicle, the corresponding equation for the mean curvature $M_{cy} = -1/2R_{cy}$ has the form

$$P_p - P_e = 2\hat{\Sigma}_{pe}M_{cy} - 4\kappa M_{cy}^3$$
[S9]

(compare ref. 5). Combining the two Eqs. **S8** and **S9** to eliminate the pressure difference $P_p - P_e$, one obtains Eq. **S5**, which describes the mechanical balance between the two types of membrane segments and implies the expression **S6** for the mean curvature M_{cv} .

For the cylindrical tube, the invariance of the shape energy under infinitesimal scale transformations (see equation 2.11 in ref. 6), implies a second relation for the mean curvature M_{cy} as given by

$$3(P_p - P_e) = 8\hat{\Sigma}_{pe}M_{cy} - 16\kappa m_{pe}M_{cy}^2,$$
 [S10]

for which the second term on the right-hand side depends explicitly on the spontaneous curvature m_{pe} of the *pe* membrane segment. Alternatively, one may obtain an equivalent relation by minimizing the membrane's shape energy with respect to the tube length. Eliminating the pressure difference by combining Eqs. **S9** and **S10** implies the relation

$$\hat{\Sigma}_{pe} = 8\kappa m_{pe} M_{cv} - 6\kappa M_{cv}^2$$
[S11]

for the membrane tension. If expression **S6** is inserted into relation **S11**, one obtains Eq. **3** of the main text. Finally, Eq. **2** of the main text follows from a combination of Eq. **3** and expression **S6**.

Vesicle Preparation, Deflation, and Observation. Giant vesicles were prepared in S_p (4.05 wt% PEG and 2.22 wt% dextran) or S_d (2.10 wt% PEG and 7.44 wt% dextran) by the method of electroformation (7). The explored membrane compositions are given in Table S1. Lipid stock solution in chloroform $(25 \sim 30 \ \mu L)$, 2 mg/mL) was spread on conductive glass substrates coated with indium tin oxide (ITO). The lipid films were dried in a vacuum desiccator for at least 3 h. A rectangular Teflon frame of thickness 1.6 mm served as a chamber spacer between two opposing glass substrates. The chamber was sealed with grease. The coated ITO surfaces acted as electrodes. Approximately 2 mL of polymer solution was injected into the chamber through a 0.22-um filter. Afterward, an ac field of $1.5 \sim 2.2$ V (peak-to-peak, according to the resistance of the ITO glasses) and 10 Hz was immediately applied using a function generator (Agilent 33220A 20 MHz function/arbitrary waveform generator). The electroformation

continued for 2 to 3 h. After formation, the vesicle solution was transferred into a small tube.

To ensure homogeneity of the polymer solution, vesicles containing S_p were prepared at 60 °C in an oven. In this case, the chamber, the filter, the syringe, the needle, and the S_p were preheated before the polymer solution was injected into the chamber. After the formation, the chamber was taken out of the oven and cooled to room temperature (\cong 23 °C). Vesicles containing S_d were prepared at room temperature.

After preparation, the vesicles were diluted in an isotonic solution (22 mOsmol/kg) containing 4.41 wt% PEG and 1.45 wt% dextran. The phase separation of the polymer solution in the vesicles was induced by injecting a certain amount of hypertonic solution. The latter was prepared by dissolving 0.1 mol sucrose in 1 L of S_p , yielding a solution with 3.27 wt% sucrose, 3.92 wt% PEG, 2.14 wt% dextran, and osmolarity of 146 mOsmol/kg. The deflation was done stepwise. The osmolarity was measured with an osmometer (Gonotec Osmomat 030). The compositions of the polymer solutions were selected so that the density of the external one is lower than the overall density of the vesicle (see also Fig. S6). In this way, the vesicles sediment at the chamber bottom to be observed with an inverted microscope. In addition, the density of the external medium must be lower than the dextran-rich phase but higher than the PEG-rich phase, which ensures that the vesicles always "stand" on the chamber bottom with the PEG-rich phase pointing upward (Fig. 1B in

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the main text). The latter criterion is especially important for observing nonspherical vesicles containing two phases. If not fulfilled, the vesicles may lie sidewise, producing overlapping projections of the two phases (see, e.g., refs. 8 and 9). All vesicles were diluted in the same external medium and deflated with the same hypertonic solution.

For the confocal microscopy observation, a $63 \times$ water immersion objective, N.A. 1.20, was used at pinhole of 111.4 µm (Leica TCS SP5). Due to spherical aberrations, the vertical cross sections of the vesicles are distorted in the *z* direction. This was corrected by adjusting the vesicle contours to circle or circular segments because the side-view observation via the horizon-tally aligned microscope (where no spherical aberrations are present) showed that the vesicles have the geometry of a sphere or an assembly of spherical caps.

The fluorescent dye was excited with a diode-pumped solidstate laser at 561 nm. The emission signal was collected at 570–650 nm. Top-view observations, which are typically used in studies of giant vesicles, provide only limited information about the morphology of vesicles enclosing two liquid phases. Therefore, most images were obtained via side-view observation using a horizontally aligned inverted microscope (Axiovert 135, Zeiss) equipped with a 40× long-distance objective, or performing *xz* scans with the confocal microscope. The observation chamber used with the horizontally aligned microscope is illustrated in Fig. S7.

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Fig. 51. Tube formation in a PEG-membrane vesicle prepared in S_d . The vesicle is observed from the side with phase contrast and fluorescence microscopy. Before deflation (*A*), the vesicle is spherical and rests at the chamber bottom (black zone in the lower area of the images). After the addition of the hypertonic solution into the chamber, the vesicle volume decreases because of the loss of water leading to the release of the tension (or excess area). As a result, the vesicle adopts a sessile-shape (*B*), which is the result of gravity and the available excess area. When the polymer concentration crosses the binodal, phase separation is initiated (last snapshot in *B*; the small droplets are the newly formed PEG-rich phase). At the same time, the vesicle becomes spherical again, which indicates elevation of the membrane tension due to tube formation. Now the tension can overcome gravity. After the phase separation is completed (*C*), the vesicle is spherical again. Tubes are observed in the vesicle interior; see arrows in *C*. (*A* and *C*) The *Left* image is obtained with phase contrast, and the *Right* image is from fluorescence microscopy. The big spherical dense part in the *Left* image in *C* is the dextran-rich phase, and the light part is the PEG-rich phase. (*B*) Phase contract images. The minutes on each frame indicate the time after the addition of the hypertonic solution. The osmolarity of the external medium is increased by 28% (r = 1.28). Scale bar, 20 µm.



Fig. 52. The deflation of an S_d vesicle with G_{M1} membrane as in the main text showing the morphological evolution and tube localization. (*Upper*) Side-view phase contrast images. (*Lower*) Side-view fluorescence images. The numbers indicate the osmolarity ratio r. The system was left to equilibrate for about 4 h after each consecutive deflation step. At r = 0, the vesicle encloses the homogeneous S_d solution. In the phase contrast images with r > 1.0, the lower dense part is the dextran-rich phase and the upper part is the PEG-rich phase, which at r = 1.17 is too small to be visualized in phase contrast image. At $r \ge 1.28$, the vesicle membrane is wetted by both liquid phases. At $r \ge 1.58$, the vesicle has a dumbbell-like shape. The fluorescence images show the location of the tubes. The tubes are in the PEG-rich phase at r = 1.17 and 1.28. They are at the interface at r > 1.28. Scale bar, 20 μ m.



Fig. S3. Membrane tube localization in an S_p vesicle with G_{M1} membrane as in the main text (see Table S1). The vesicle was deflated along the trajectory in Fig. 1A in the main text. The snapshots are confocal fluorescence images (vertical *xz* cross-sections) of a vesicle at different osmolarity ratios *r*, indicated by the numbers on the snapshots. The system was left to equilibrate for about 2 h after each consecutive deflation step. At *r* = 1.51, the tubes are adsorbed at the interface between the dextran-rich phase (lower vesicle part) and the PEG-rich phase as indicated by the arrow. Scale bars, 20 µm.



Fig. 54. Membrane tube localization in a vesicle consisting of S_p and PEG-membrane deflated along the trajectory in Fig. 1*A* in the main text. The snapshots are confocal fluorescence images (vertical *xz* cross-sections) of vesicles at different osmolarity ratios *r*, indicated by the numbers on the snapshots. The system was left to equilibrate for about 2 h after each deflation step. The lower vesicle part contains the dextran-rich phase. One part of the tubes is adsorbed at the interfaces between the dextran-rich and the PEG-rich phases (indicated by the arrows), and another part of them is in the PEG-rich phase. At *r* < 2.69, the tubes are located only in the PEG-rich phase. Scale bars, 20 μ m.



Fig. S5. Schematic illustration of the image fitting under low suction pressure (A) and under high suction pressure (B).



Fig. S6. Densities of the S_p solution (blue star), dextran-rich phase (solid triangles), PEG-rich phase (solid circles), and external solutions (open squares) as a function of the osmolarity ratio r at 24.2 °C. The densities of the coexisting phases were measured in bulk polymer solutions, which had the exact polymer concentration as in the deflated vesicles. The polymer concentration inside the vesicle at each deflation step can be obtained from the volume change of the vesicle. The bulk polymer solutions were left to phase separate at 24.2 °C for 5 d before probes of each of them was taken for the density measurements. The overall dextran/PEG weight ratio was fixed to 0.55 (same as in S_p) in all solutions.



Fig. S7. Schematic illustration of the chamber for side-view observation using horizontally aligned microscope. The sketched objects are not in scale. The objective is located in front of the chamber. The condenser and the illumination are located behind the chamber. (*A*) Chamber used for vesicle deflation. The chamber was filled with the isotonic external medium (cyan) and vesicles (red). The osmolarity of the external medium was increased by addition of the hypertonic solution (blue) through one of the holes using a pipette. (*B*) Chamber used for vesicle aspiration. After introducing the micropipette, the open part of the chamber is sealed with grease (light green) to avoid evaporation. The micropipette was filled with the same solution as the external medium (cyan).

Table S1. Membrane composition	s explored in vesicle	s with tube	formation
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Membrane	L		
G _{M1} membrane (as in the main text)	G _{M1} ganglioside 4.0%	DOPC 95.9%	DPPE-Rhod 0.1%
PEG membrane	DOPE-PEG 4.0%	DOPC 95.9%	DPPE-Rhod 0.1%
DOPS membrane	DOPS 27.9%	DOPC 72.0%	DPPE-Rhod 0.1%

DOPC, dioleoylphosphatidylcholine; DPPE-Rhod, dipalmitoylphosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt); DOPE-PEG, dioleoylphosphatidylethanolamine-N-[methoxy (polyethylene glycol)-2000] (ammonium salt); DOPS, dioleoylphosphatidylserine (sodium salt)

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