

# Photochemically driven redox chemistry induces protocell membrane pearling and division

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**Prior to the evolution of complex biochemical machinery, the growth and division of simple primitive cells (protocells) must have been driven by environmental factors. We have previously demonstrated two pathways for fatty acid vesicle growth in which initially spherical vesicles grow into long filamentous vesicles; division is then mediated by fluid shear forces. Here we describe a different pathway for division that is independent of external mechanical forces. We show that the illumination of filamentous fatty acid vesicles containing either a fluorescent dye in the encapsulated aqueous phase, or hydroxypyrene in the membrane, rapidly induces pearling and subsequent division in the presence of thiols. The mechanism of this photochemically driven pathway most likely involves the generation of reactive oxygen species, which oxidize thiols to disulfide-containing compounds that associate with fatty acid membranes, inducing a change in surface tension and causing pearling and subsequent division. This vesicle division pathway provides an alternative route for the emergence of early self-replicating cell-like structures, particularly in thiol-rich surface environments where UV-absorbing polycyclic aromatic hydrocarbons (PAHs) could have facilitated protocell division. The subsequent evolution of cellular metabolic processes controlling the thiol:disulfide redox state would have enabled autonomous cellular control of the timing of cell division, a major step in the origin of cellular life.**

origin of life | vesicles

One model for the nature of the earliest and simplest cells, or protocells, is that they consisted of a self-replicating membrane compartment (vesicle) that encapsulated a self-replicating genetic polymer (1). The emergence of any genetically encoded function that enhanced protocell reproduction or survival would have marked the beginnings of Darwinian evolution and, thus, of biology itself. We have been attempting to test various aspects of this model for the origin of cellular life through the laboratory construction and characterization of model protocells (2, 3). A necessary and complementary aspect of this program is the exploration of environmental scenarios in which external inputs of matter and energy could drive the replication of the genetic material of a protocell (4), as well as protocell membrane growth and division (5, 6). For example, we recently showed that activated nucleotides, added to a suspension of model protocells, could cross the vesicle membrane and copy encapsulated nucleic acid templates (4). We have also shown that large multilamellar fatty acid vesicles can grow by absorbing fatty acid molecules from added fatty acid micelles (6). This growth pathway begins with the emergence of a thin membranous filament from the side of the initially spherical vesicle; the filament grows and gradually incorporates the contents and membranes of the parent vesicle, which is transformed into a long filamentous vesicle. Under modest shear forces, the filamentous vesicle divides into multiple daughter vesicles (6). The emergence of more robust forms of cellular life requires mechanisms that can drive growth and division independently of environmental perturbations. As a potential example, we recently showed that fatty acid vesicles that contain a small proportion of phospholipids can grow into filamentous

vesicles by absorbing fatty acid molecules from neighbouring vesicles that lack phospholipids (2). Thus, the evolution of an internal metabolic pathway for phospholipid synthesis could drive the growth of a primitive cell in such a manner as to be predisposed for division in response to environmental fluid shear forces.

While the spontaneous division of model protocells occurs readily after growth as a result of environmental shear forces, this process is not readily amenable to evolutionary modification into an internally controllable process. Here we describe an alternative process for protocell division based on simple chemical and physical mechanisms. Because this process is based upon a change in the redox state of small thiol containing molecules, the evolution of an internal redox metabolism would allow cells to control the timing of their cell division independently of environmental fluctuations.

## Results

During the course of our imaging studies of vesicle growth and division, we observed an unexpected artifact: Long thread-like oleate vesicles containing an encapsulated fluorescent dye quickly (in approximately 5 s) round up into large spherical vesicles under intense illumination (Fig. S1 and Movie S1). The vesicles contained 2 mM HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, a water-soluble, membrane-impermeable fluorescent dye) (7) and were illuminated by an EXFO 120 W metal halide lamp. We reasoned that since most fluorescent dyes, such as HPTS, generate reactive oxygen species (ROS) under illumination, this phenomenon might be caused by the radical-mediated oxidation and fragmentation of the internal buffer solute, bicine, leading to increased internal osmolarity, as previously seen in the case of spherical vesicles that explode under similar conditions (8). The fact that the sphere-to-filament transition is reversible shows directly that growth into a filamentous form does not involve topological changes in vesicle structure. In an effort to obtain better images of filamentous vesicles, we added 10 mM dithiothreitol (DTT) to a vesicle suspension to scavenge ROS, in an attempt to block the rounding-up artifact. To our surprise, intense illumination in the presence of 10 mM DTT caused the thread-like fatty acid vesicles to go through pearling and subsequent division (Fig. 1 and Movie S2). The long thread-like membrane first transformed into a periodic string of smaller ellipsoidal vesicles connected by narrow necks, and the smaller vesicles eventually separated into independent vesicles that moved apart by Brownian motion.

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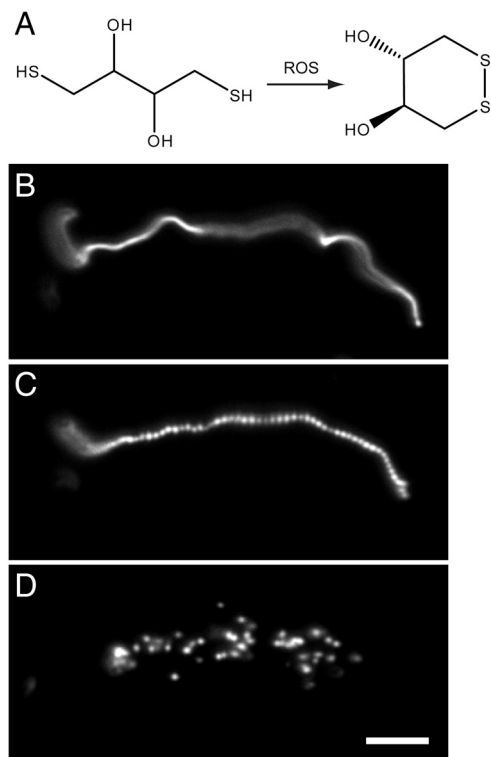
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**Fig. 1.** Oleate vesicle pearling and division. (A) Radical-mediated oxidation of DTT. (B) An oleate vesicle (containing 2 mM HPTS, in 0.2 M Na-glycinamide, pH 8.5, 10 mM DTT) 30 min after the addition of five equivalents of oleate micelles. (C and D) Under intense illumination (for 2 s and 12 s, respectively), the long thread-like vesicle went through pearling and division (Movie S2). Scale bar, 10  $\mu$ m.

We also show that the pearling and division can happen not only in fully grown, filamentous vesicles, but also at intermediate growth stages. We have previously shown that oleate vesicles, when fed with small quantities of micelles (one equivalent), can transform into sphere-tail shapes and divide under gentle fluid shear forces (6). We tested whether the addition of DTT could cause oleate vesicles at sphere-tail stages (containing 2 mM HPTS, in 0.2 M Na-bicine, pH 8.5, 30 min after the addition of one equivalent of oleate micelles) to divide without requiring external mechanical forces. With 10 mM DTT in the solution and under intense illumination, pearling and division of the filamentous portions of vesicles were indeed observed (Fig. S2 A and B; Movies S3 and S4).

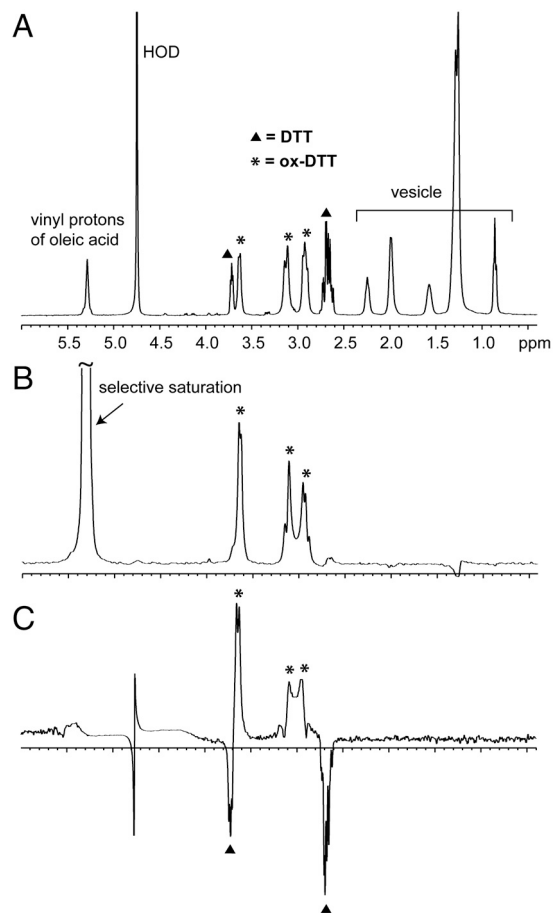
To understand the mechanisms responsible for the pearling and division of thread-like fatty acid vesicles, we examined oleate vesicles labeled with different dyes, in either the internal aqueous space or in the membrane itself. When we prepared vesicles containing 2 mM calcein (bis[*N,N*-bis(carboxymethyl)aminomethyl]fluorescein) in the internal aqueous space and added 10 mM DTT 30 min after the addition of five equivalents of oleate micelles, vesicle pearling and division were again observed upon illumination. In contrast, we found that long thread-like oleate vesicles labeled with a low concentration of membrane-localized dye [0.5 mol% Rh-DHPE (Lissamine rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine)] did not go through pearling and division (Fig. S3 A and B). This may be because there are fewer dye molecules associated with the vesicles in the latter case, since in a control experiment where the encapsulated water-soluble fluorescent dye was adjusted to a lower concentration (0.05 mM) comparable to that of the membrane dye used, no vesicle pearling and division were observed. We also observed that with equal concentrations (2 mM) of HPTS inside and outside of the filamentous vesicles (labeled with 1 mol% Rh-DHPE

for imaging), vesicle pearling and division still occurred (Fig. S4). This result indicates that a cross-membrane concentration gradient of the fluorescent dye is not a prerequisite for the pearling and division phenomenon.

It has been shown that vesicle pearling and division can occur as a result of laser tweezers induced surface tension changes which induce a Rayleigh instability in vesicle membranes (9). Recent experiments have also shown that encapsulated cationic nanoparticles can induce vesicle pearling (10). We hypothesized that the photochemically induced oxidation of thiols into its oxidized form, *trans*-4,5-dihydroxy-1,2-dithiane, resulted in an increased hydrophobicity of the compound (logP 0.12 for DTT vs. logP 0.52 for *trans*-4,5-dihydroxy-1,2-dithiane), and consequently increased interaction of the molecule with the membrane. We used NMR methods to examine the interaction of DTT and its oxidation product with fatty acid vesicle membranes. Because the proton chemical shifts of both DTT and *trans*-4,5-dihydroxy-1,2-dithiane overlap with the strong background peaks of bicine buffer at 2–4 ppm (and other organic buffers such as tricine and TRIS), which are difficult to selectively suppress, we used oleate vesicles prepared without buffer (15 mM oleate in 7.5 mM NaOD solution in D<sub>2</sub>O, pD approximately 8.5) for the following experiments. We added 15 mM DTT and 15 mM *trans*-4,5-dihydroxy-1,2-dithiane to vesicles prepared as above and used two independent NMR methods to examine the interaction of these small molecules with the oleate membranes. We first used saturation transfer difference (STD) spectroscopy, a widely used method for examining the interaction of small ligands with large receptors (11, 12). Following selective saturation irradiation of the oleate vinyl protons, we observed saturation transfer to the CH protons of *trans*-4,5-dihydroxy-1,2-dithiane but not to the CH protons of DTT (Fig. 2 A and B). Thus, only the oxidized form of DTT appears to interact with oleate membranes. As a further test, we used the waterLOGSY method, another sensitive NMR method commonly used for screening ligand-receptor interactions (13). Again, only oxidized and not reduced DTT was observed to interact with the oleate bilayer membrane (Fig. 2C).

We have shown that fatty acid vesicles of other lipid compositions, such as decanoate:decanol (2 : 1), can grow into filamentous vesicles when fed with two equivalents of decanoate micelles and one equivalent of decanol emulsion (6). With 10 mM DTT in the solution, intense illumination of decanoate:decanol (2 : 1) vesicles containing 2 mM HPTS led to pearling and division (Fig. S2 C and D). Since both decanoic acid and decanol have saturated hydrocarbon chains that are unlikely to react with ROS or DTT, this experiment suggests that oxidation of the carbon-carbon double bond in fatty acids is unlikely to be the cause of vesicle pearling. We also tested whether phospholipid vesicles can go through pearling and division with DTT in the solution. We found that POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) vesicles containing 2 mM HPTS, after the addition of 0.5 M sucrose (to produce thread-like vesicles by osmotically driven water efflux) and under intense illumination, did not go through pearling and division (Fig. S3 C and D), presumably due to less dramatic changes in the membrane surface tension in phospholipid vesicles.

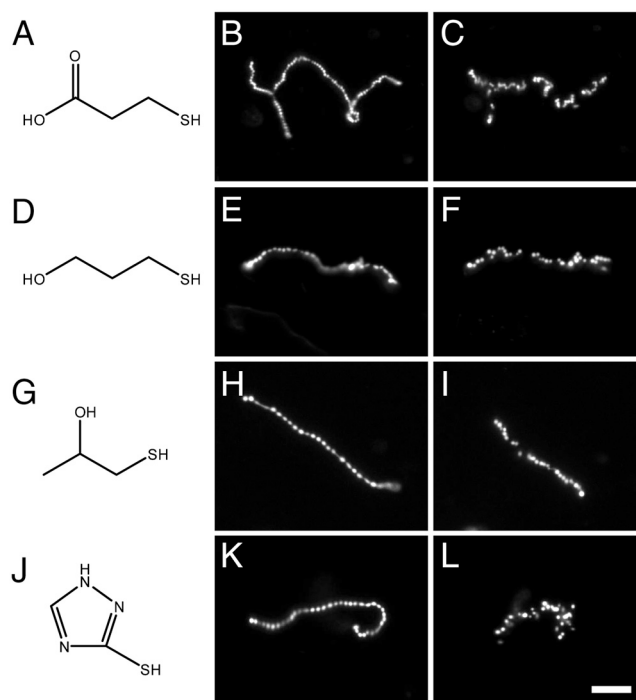
To further understand the effect of thiols on vesicle pearling and division, we asked whether other thiols can also cause this phenomenon. Thiols, such as 3-mercaptopropionic acid (10 mM), 3-mercapto-1-propanol (50 mM), 1-mercapto-2-propanol (50 mM), and 3-mercapto-1,2,4-triazole (50 mM), all caused thread-like oleate vesicles containing 2 mM HPTS to undergo pearling and division (Fig. 3). At lower thiol concentrations (2 mM 3-mercaptopropionic acid, 10 mM 3-mercapto-1-propanol, 10 mM 1-mercapto-2-propanol, and 10 mM 3-mercapto-1,2,4-triazole), vesicle pearling was observed but without subsequent division. Recent evidence suggests that the thiol-containing amino acid cysteine may have been prebiotically available, as



**Fig. 2.** NMR experiments to characterize the association of oxidized but not reduced DTT with oleate bilayer membranes. (A) Reference spectrum: conventional 1D  $^1\text{H}$  spectrum of oleic acid vesicles (15 mM oleate, 7.5 mM NaOD in 100%  $\text{D}_2\text{O}$ , pH approximately 8.5), with 15 mM DTT and 15 mM *trans*-4,5-dihydroxy-1,2-dithiane added. Peaks corresponding to DTT (solid triangles) and its oxidized form *trans*-4,5-dihydroxy-1,2-dithiane (ox-DTT; stars) were assigned by spiking with the two compounds respectively. (B) STD (saturation transfer difference) spectrum of the above mixture in 100%  $\text{D}_2\text{O}$ . The vinyl protons of oleic acid were selectively irradiated. Positive peaks reveal the interaction of *trans*-4,5-dihydroxy-1,2-dithiane molecules with oleate molecules of the bilayer membrane (stars). The absence of detectable peaks for DTT molecules suggests that DTT does not interact significantly with the oleate membranes. (C) WaterLOGSY (water ligand observed via gradient spectroscopy) spectrum of the above mixture in 10%  $\text{D}_2\text{O}$ . Positive peaks show the interaction of oxidized DTT molecules with the oleate bilayer membranes (stars), while negative peaks suggest the absence of DTT association with bilayer membranes (solid triangles).

cysteine oxidation products have been found in the material from a 1958 Miller  $\text{H}_2\text{S}$ -rich spark discharge experiment (14). When we tested cysteine, we observed vesicle pearling but not division. We then considered the possibility that the *cys-cys* dipeptide, di-L-cysteine (15), might allow pearling and division of vesicles after oxidation to generate an intramolecular disulfide bond. When we prepared filamentous oleate vesicles containing 20 mM di-L-cysteine and 2 mM HPTS in the internal aqueous phase, we observed both vesicle pearling and division upon illumination (Fig. 4).

As an initial step toward exploring prebiotically plausible scenarios in which photochemically driven pearling and division might operate, we tested the idea that polycyclic aromatic hydrocarbons (PAHs) might replace the synthetic fluorescent dyes used in the above experiments (16). PAHs are the most abundant polyatomic organic molecules in the universe, have been postulated to play important roles in the origin of life (16, 17), and have

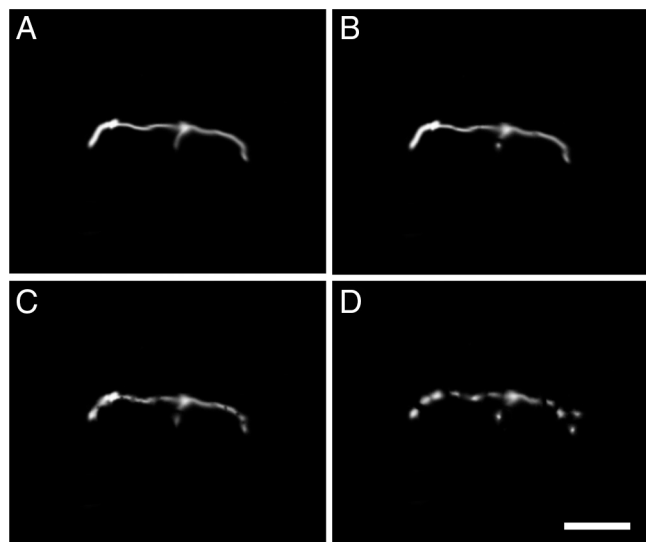


**Fig. 3.** Oleate vesicle pearling and division with various thiols in the solution. (A) 3-mercaptopropionic acid. (B and C) An oleate vesicle (containing 2 mM HPTS, in 0.2 M Na-bicine, pH 8.5, 10 mM 3-mercaptopropionic acid, 30 min after the addition of five equivalents of oleate micelles) went through pearling and division under intense illumination (for 3 s and 15 s, respectively). (D) 3-mercapto-1-propanol. (E and F) An oleate vesicle as above but in 50 mM 3-mercapto-1-propanol, went through pearling and division under intense illumination (for 2 s and 10 s, respectively). (G) 1-mercapto-2-propanol. (H and I) An oleate vesicle as above but in 50 mM 1-mercapto-2-propanol went through pearling and division under intense illumination (for 2 s and 9 s, respectively). (J) 3-mercapto-1,2,4-triazole. (K and L) An oleate vesicle as above but in 50 mM 3-mercapto-1,2,4-triazole went through pearling and division under intense illumination (for 3 s and 13 s, respectively). Scale bar, 20  $\mu\text{m}$ .

been shown to stabilize short-chain fatty acid membranes (18). We therefore asked whether oxygenated derivatives of PAHs, such as 1-hydroxypyrene (while HPTS itself is also a hydroxypyrene derivative, it is unlikely to be prebiotically abundant), can be incorporated into vesicle membranes and act as photosensitizers that can absorb UV radiation, generate ROS, and facilitate the division of protocells. To test this idea experimentally, we dissolved a high concentration of 1-hydroxypyrene (20 mol%), oleic acid and a low concentration of Rh-DHPE (1 mol% for imaging) in a chloroform solution, followed by rotary evaporation and re-suspension in buffer (0.2 M Na-bicine, pH 8.5). After vesicle growth into filamentous form, and in the presence of 15 mM DTT and UV illumination, vesicle pearling and division were indeed observed (Fig. 5 and Movie S5).

## Discussion

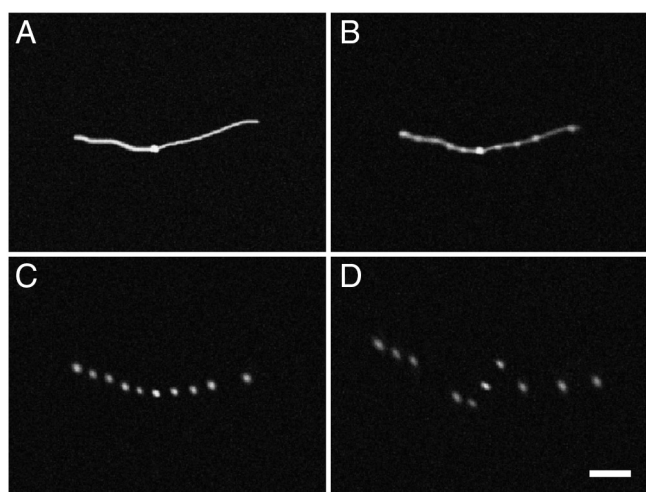
Our observations strongly suggest that oxidized, but not reduced, thiols can associate with membranes, triggering division in our protocell model. While the mechanism by which disulfide-containing compounds cause membrane pearling and division is not entirely clear, it is plausible that disulfide-membrane interactions lead to changes in surface tension. Changes in membrane surface tension due to externally applied force from optical tweezers have been shown to induce pearling in tubular vesicles (9, 19), as well as vesicle expulsion (20). Vesicle pearling has also been demonstrated by insertion of amphiphilic polymers in the outer leaflet of the membrane, inducing changes in spontaneous curvature (21, 22). In addition, lipid phase separation has been



**Fig. 4.** Oleate vesicle pearling and division mediated by the dipeptide di-L-cysteine. (A) An oleate vesicle (containing 2 mM HPTS, in 0.2 M Na-bicine, pH 8.5, 20 mM di-L-cysteine) 30 min after the addition of five equivalents of oleate micelles. (B–D) Under intense illumination (for 3 s, 8 s, and 12 s, respectively), the long thread-like vesicle went through pearling and division. Scale bar, 10  $\mu$ m.

shown to drive vesicle budding and division as a consequence of minimizing interdomain edge energy (23). While our results suggest that the oxidation of dithiothreitol to *trans*-4,5-dihydroxy-1,2-dithiane results in increased interaction of the molecule with the membrane, how such interactions lead to membrane pearling and division remains unclear. Future studies will be required to investigate the location and homogeneity or heterogeneity of disulfide association with the bilayer membrane in order to quantitatively understand the resulting changes in morphology of the vesicle membrane.

The observation that thiols and UV-absorbing PAHs facilitate vesicle pearling and division may have important implications for understanding the origin of cellular life. Thiols are likely to have been abundant in prebiotic hydrothermal systems, such as near-surface hydrothermal vents (24). Oxygenated PAH fragments



**Fig. 5.** Pearling and division of an oleate vesicle containing 1-hydroxypyrene in the membrane. (A) An oleate vesicle (with 20 mol% 1-hydroxypyrene and 1 mol% Rh-DHPE in the membrane, in 0.2 M Na-bicine, pH 8.5, 15 mM DTT) 30 min after the addition of five equivalents of oleate micelles. (B–D) Under intense illumination (for 57 s, 153 s, and 213 s, respectively), the long thread-like vesicle went through pearling and division (Movie S5). Scale bar, 10  $\mu$ m.

could be generated by hydrothermal processing of meteoritic kerogen or by pyrolysis of organic materials. Such hydrophobic molecules are likely to have been selectively solubilized and concentrated in primitive membranes. Indeed, hydroxypyrene, a simple oxygenated PAH, has been shown to integrate into and stabilize membranes composed of short chain fatty acids (18). In a high UV surface environment, membrane-localized PAHs would be expected to absorb UV radiation and generate ROS, even under anaerobic conditions (16). Our observations show that in the presence of thiols, generation of ROS could lead to the pearling and subsequent division of filamentous vesicles composed of fatty acids and PAHs, thus providing an independent pathway for protocell division that is distinct from the previously described shear force-dependent pathway (6).

The control of the timing of cell division by thiol redox state provides a mechanism by which an initial dependence on environmental fluctuations could transition to an internally controlled process, through the evolution of cellular metabolism. For example, we have shown that the dipeptide *cys-cys* can result in vesicle pearling and division, whereas the amino acid cysteine by itself cannot. Therefore, metabolic control of dipeptide synthesis and degradation could potentially mediate the control of the timing of cell division in primitive cells. Alternatively, as cellular metabolism evolved to the point that cells derived energy from environmental redox disequilibrium, they could potentially use the control of their internal thiol:disulfide redox state as a means of controlling cell division. Finally, it would be interesting to investigate the possibility that intracellularly produced small molecules, peptides, or structured RNAs that interact with protocell membranes might also lead to pearling and division. Such processes would result in competition between protocells on the basis of faster or more appropriately timed cell division, and thus control of cell size, foreshadowing the rise of more complex and more tightly regulated modes of cell division.

## Materials and Methods

**Vesicle Preparation.** Oleate vesicles were prepared by resuspending a dried film of oleic acid (Nu-Chek Prep) in 0.2 M Na-bicine (Sigma-Aldrich) containing 2 mM HPTS at pH 8.5, to a final concentration of 10 mM oleic acid. Large (approximately 4  $\mu$ m in diameter) monodisperse multilamellar vesicles were prepared by extrusion and large-pore dialysis as described (7). Oleate vesicles containing 1-hydroxypyrene (Sigma-Aldrich) in the membrane were prepared by co-dissolving 1-hydroxypyrene (20 mol%), oleic acid, and Rh-DHPE (1 mol%) in a chloroform solution, followed by rotary evaporation and resuspension in buffer (0.2 M Na-bicine, pH 8.5, 15 mM DTT). Oleate vesicles containing di-L-cysteine were prepared by resuspension of 10 mM oleic acid in 0.2 M Na-bicine (pH 8.5) containing 2 mM HPTS and 20 mM di-L-cysteine. Di-L-cysteine (cysteinylcysteine) was prepared using F-moc solid phase synthesis on a microwave peptide synthesizer as described (15).

**Imaging Vesicle Pearling and Division.** Vesicle growth experiments were performed as described (6), in a buffer solution containing DTT, 3-mercaptopropionic acid, 3-mercaptopropanol, 1-mercaptopropanol, 3-mercaptopropionamide, or di-L-cysteine. Vesicle pearling and division were imaged using a Nikon TE2000S inverted epifluorescence microscope with extra-long working distance (ELWD) objective lenses (Nikon). The illumination source was a metal halide lamp (EXFO) with a  $480 \pm 20$  nm (for HPTS),  $546 \pm 5$  nm (for Rh-DHPE), or  $360 \pm 20$  nm (for UV) optical filter (Chroma).

**NMR Measurements.** All NMR spectra were recorded at 293 K with a Varian 400 MHz NMR spectrometer (Oxford AS-400) equipped with a 5 mm broadband PFG (z-gradient) probe. DTT and *trans*-4,5-dihydroxy-1,2-dithiane (Sigma-Aldrich) at concentrations of 15–20 mM were added to sonicated vesicle suspensions prior to NMR measurements. Oleate vesicles were prepared without buffer by mixing 15 mM oleate with 7.5 mM NaOD (pH approximately 8.5, in 100% D<sub>2</sub>O for STD experiments and in 10% D<sub>2</sub>O for waterLOGSY experiments).

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