

Materials and Methods

Vesicles encapsulating sucrose. Fatty acid vesicles were prepared from oleic acid and myristoleic acid as described (*S1*), at a concentration of 40-80 mM. All fatty acids and fatty acid derivatives were obtained from Nu-chek Prep, Inc, Elysian, MN. POPC (Avanti Polar Lipids, Inc.; Alabaster, AL) vesicles were prepared by rotary evaporation of a stock POPC/chloroform solution followed by resuspension of the thin film and five freeze-thaw cycles. Low osmolarity vesicles were obtained by resuspension in 0.2 M bicine, pH 8.5, titrated with NaOH. For oleic acid and POPC, high osmolarity vesicles were obtained by resuspension in 1 M sucrose, 0.2 M bicine, pH 8.5. For myristoleic acid, high osmolarity vesicles were obtained by resuspension in 0.5 M sucrose, 0.2 M bicine, pH 8.5, because lysis was determined to occur at a lower applied osmotic gradient. Dilutions of myristoleate vesicles were made using buffers containing myristoleate at the critical aggregate concentration ($cac = 4 \text{ mM}$) (*S2*), to avoid dissolution of the vesicles. The cac of oleate vesicles is much lower than the experimental concentrations ($\sim 80 \text{ }\mu\text{M}$) (*S1*). Vesicle preparations were extruded through 100 nm polycarbonate filters using the Mini-Extruder system (Avanti Polar Lipids, Inc.). If necessary, vesicles were purified from unencapsulated solutes by size-exclusion chromatography.

Detection of membrane rupture. Vesicles were prepared by encapsulating an impermeable, labeled solute in addition to the osmolyte (when appropriate) and 0.2 M bicine, pH 8.5. The tracer solute was ^3H -sucrose (PerkinElmer Life and Analytical Sciences; Boston, MA) for oleate vesicles, calcein for myristoleate and MA:GMM vesicles, and pyranine for POPC vesicles. All fluorescent dyes were obtained from Molecular Probes, Inc. (Eugene, OR). Leakage of internal vesicular contents was detected by size exclusion chromatography on a gravity-flow Sepharose 4B column (Sigma-Aldrich; St. Louis, MO). Fractions were analyzed by fluorescence or scintillation counting. To estimate lysis tensions, the encapsulated volumes of isotonic extruded vesicles and vesicles diluted into hypotonic solution were determined following the procedure of Mui et al. (S3), using ^{14}C -sorbitol (Amersham Biosciences; Piscataway, NJ) or ^3H -sorbitol (American Radiolabeled Chemicals, Inc.; St. Louis, MO) as a slowly permeable solute (equilibration time ~4 hours in oleate vesicles, ~1.5 hours in POPC vesicles).

Mixed composition vesicles encapsulating RNA. Neat myristoleic acid (MA) and glycerol monomyristoleate (GMM) were mixed in a 2:1 molar ratio and resuspended in 0.2 M bicine, pH 8.5 (low osmolarity vesicles) or the same buffer containing an appropriate solute (high osmolarity vesicles). 5'-UMP (Sigma-Aldrich; St. Louis, MO) and tRNA from *E. coli* (Roche Applied Science; Indianapolis, IN) were used as solutes without further preparation, and buffers containing these solutes were adjusted to pH 8.5. Oligonucleotides were obtained from bulk torula yeast RNA (MP Biomedicals, Inc.;

Irvine, CA) by hydrolysis of 133 mg/mL solution of RNA in 0.44 M NaOH, for 18 hours. The reaction was stopped by addition of 1 M bicine solution to a final concentration of 0.2 M bicine, adjusted to pH 8.5. The size distribution of the resulting hydrolysis products was determined by HPLC on a Nucleopac PA-100 column (Dionex; Sunnyvale, CA), which indicated the presence of <10% mononucleotides. MA:GMM vesicle suspensions were mixed by vortexing and freeze-thawed 5-10 times to homogenize lipid mixing and stabilize the vesicles (S4). Vesicles were then extruded through 100 nm pore filters as described above. Dilutions of MA:GMM vesicles were made using buffers containing 1 mM MA:GMM to avoid dissolution of the vesicles. Preparations made with bulk RNA or tRNA were further purified by size exclusion chromatography, in the presence of a counter-osmolyte (5'-UMP or sucrose), to decrease the possibility of vesicle aggregation as well as optical absorbance from impurities in the RNA.

FRET-based assay. This assay measures the distance-dependent energy transfer between non-exchanging fluorescent phospholipids; as the membrane grows larger, the separation between probe molecules increases and the FRET signal decreases. To prepare FRET-labeled vesicles, membrane-incorporated dyes, NBD-PE (*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; excitation at 430 nm, emission at 530 nm) and Rh-DHPE (Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; emission at 586 nm) were added to a solution of the appropriate oil in methanol before rotary evaporation and resuspension in buffer (S2). Vesicles were then extruded through 100 nm pore filters as described above. A standard

curve of FRET signal vs. dye concentration (up to 0.8 mol%) was prepared for each membrane composition. Fluorescence was then used to follow changes in membrane surface area, as described (S1,S2).

Vesicle competition. Vesicles were diluted into 0.2 M bicine, pH 8.5 (low osmolarity buffer), to the desired concentration (0.5-5 mM acyl chains). The final concentration of osmolyte in the extra-vesicular solution was ≤ 0.04 osmol/L. In the case of fatty acid vesicles, the kinetics of exchange were too fast to be captured by manual mixing. Therefore, two populations of vesicles, one labeled by FRET dyes and one unlabeled, were loaded in separate syringes of an RX-2000 rapid mix accessory (Applied Photophysics, Leatherhead, UK) of a Cary Eclipse fluorimeter (Varian, Inc., Mulgrave, Australia). Stopped-flow mixing was performed according to the manufacturer's instructions. In the case of MA:GMM and POPC vesicles, exchange was slow enough that manual mixing could be performed without significant loss of kinetic data. Fluorescence data were converted to vesicle surface area by a FRET standard curve and fit by single exponential decay equations by non-linear regression analysis.

Transfer rates of fluorescently labeled fatty acids. To prepare R18-labeled vesicles, R18 was added to a solution of the appropriate oil in methanol before rotary evaporation and resuspension in buffer, and 100 nm vesicles were further prepared as described above. Fluorescence (f) was determined by excitation at 540 nm and emission at 610 nm. Vesicles were solubilized in 1% Triton X-100 to determine the maximal (dequenched)

fluorescence value (f_T). The ratio f/f_T was determined for a range of R18 concentrations (S5), and vesicles containing 3 mol% R18 gave an appropriate self-quenching signal and were used in the assay. R18-labeled and unlabeled vesicles were mixed in equal ratio, as described above, and the fluorescence data were fit by single exponential decay equations.

Vesicles labeled with BODIPY 550/510 C12 and BODIPY 558/568 C12, a fatty acid-based FRET pair, were prepared in a manner analogous to vesicles labeled with NBD-PE and Rh-DHPE. A standard curve was generated for dye concentration vs. FRET signal (excitation at 475 nm, emission at 516 nm). To determine the rate of probe transfer, donor vesicles were prepared with 0.05 mol% BODIPY 500/510 C12 and 0.1 mol% BODIPY 558/568 C12. Acceptor vesicles were prepared with 0.05 mol% BODIPY 500/510 C12 only. Donor and acceptor vesicles were mixed in equal ratio and the data were fit by single exponential decay equations as described above.

Supporting Online Text

The prebiotic role of vesicle compartments

One function of the membrane may have been to protect the genome from environmental dangers. In addition, encapsulation of the genome in a replicating compartment creates spatial segregation among different genomes. This segregation would be essential for enabling Darwinian evolution at the level of genome replication, allowing superior mutant replicases to copy their own genotype preferentially and possibly preventing the accumulation of parasitic genomes (*S6, S7*).

Vesicle competition

If the osmotically swollen vesicle obtains additional membrane from other vesicles, then this process may be seen as a competition among vesicles for a limited resource.

Fatty acid vesicles as a prebiotic model

Fatty acids are simple amphiphiles that have been found in meteorites (*S8, S9*) and can be synthesized under simulated prebiotic conditions (*S10*). They spontaneously assemble into vesicles at intermediate pH (*S11, S12*). Such vesicles have been used as a model for primitive cells (*S13*), as they form autocatalytically (*S14*) and can be made to grow and divide in the laboratory (*S2*). Fatty acid vesicles grow larger upon the addition of fatty acid micelles, prepared at high pH. Surface area changes can be monitored by

fluorescence resonance energy transfer (FRET) between non-exchanging membrane-incorporated dye molecules (S2).

Vesicle containing sucrose

No leakage of encapsulated ^3H -sucrose from 100 nm diameter oleate vesicles was observed by size exclusion chromatography after 6 days.

Membrane rupture

When extruded vesicles, which are initially non-spherical, are diluted into a hypotonic buffer (i.e. with osmolarity less than that of the vesicle interior), they swell to a spherical shape. If the pressure is high enough, transient membrane rupture releases contents to the exterior, causing the internal osmotic pressure to drop. When the membrane tension decreases below the rupture tension, the membrane reseals at a sustainable level of membrane tension (S3, S15, S16). Increasing the initial applied gradient beyond this point does not increase the final residual gradient (S3).

Calculation of maximum membrane tension

τ^* was calculated from the osmolarity of an oleate vesicle after lysis and resealing. For example, measurements of encapsulated volume showed that extruded oleate vesicles prepared in buffer with sucrose and trace ^3H -sucrose (osmolarity $m = 1.28$ osmol/L) increased in volume by a factor of 1.6 when diluted into hypotonic solution ($m = 0.38$ osmol/L). In addition, 33% of the vesicle contents were lost after dilution into

hypotonic buffer, as determined by size-exclusion chromatography, resulting in a final internal osmolarity of 0.54 osmol/L and a residual osmotic gradient of 0.16 osmol/L. For POPC vesicles, rupture was observed upon applying a gradient of 0.9 M sucrose, which resulted in a residual transmembrane gradient of 0.40 osmol/L. Laplace's law for a sphere was used to calculate membrane tension: $\tau^* = (\Delta\Pi^*)r/2$, where r is the radius of the sphere.

Calculation of membrane loss from extruded vesicle for spherical shape

Because the volume of a vesicle after extrusion is 63% of its maximum volume (spherical shape), the minimum surface area necessary to enclose the contents of an extruded vesicle is $(0.63)^{2/3} = 0.73$ times its original surface area. A non-spherical extruded vesicle could thus lose $1-0.73 = 27\%$ of its original surface area without an increase in membrane tension caused by an osmotic differential.

MA:GMM vesicle stability

No leakage or macroscopic aggregation was observed, and these membranes exhibited a decreased critical aggregate concentration (~ 1 mM) relative to pure myristoleate vesicles (4 mM), indicating greater bilayer stability.

Vesicles containing 5'-UMP

In MA-GMM vesicles (100 nm), the permeability half-time of UMP was found to be 20 hours, as determined by the leakage of encapsulated ^3H -UMP. This timescale was much longer than the duration of each experiment.

Transfer rates of BODIPY-labeled fatty acids

Fatty acids with a fluorescently tagged hydrophobic chain (BODIPY 500/510 C12 and BODIPY 558/568 C12) exhibited much faster transfer rates in both oleate and MA:GMM vesicles ($k \sim 15 \text{ s}^{-1}$), indicating that chain packing in the bilayer is also an important determinant of exchange rates.

Gibbs-Donnan effect with permeable anions

Since the concentration of tRNA itself was relatively low, most osmotic pressure was due to associated counterions. One qualification is that the encapsulation of impermeable RNA polymers would lower the concentration of encapsulated permeable anions due to the Gibbs-Donnan effect, assuming permeable cations and anions were present. It was not necessary to consider this effect in the experiments described here, because no anions were permeable on the experimental timescale (*S17*). Although the Gibbs-Donnan effect may decrease the magnitude of the osmotic pressure gradient generated by RNA polymerization, the essential consequences are not expected to change.

RNA synthesis in phospholipid liposomes

RNA can be synthesized by a Q β replicase system in phospholipid vesicles (S18) and fatty acid vesicles under certain conditions (S19). Analogous RNA synthesis within fatty acid vesicles would presumably increase intravesicular osmotic pressure, thereby causing membrane accretion.

Darwinian evolution at the cellular level

Darwinian evolution at the organismal level requires some means by which the genome can influence the properties and behavior of the cell as a whole. Previously, replicating protocellular RNA has been viewed as a passive component trapped within replicating vesicles.

Regulatory role of inter-vesicle competition

Another consequence of the coupling of vesicle surface area with osmotic pressure is a simple form of negative feedback regulation for the control of internal solute concentration. If the intravesicular solute concentration increases, membrane area increases, which allows intravesicular volume to increase. At equilibrium, the intravesicular solute concentration would return to its original value, which is balanced by the osmotic pressure of the external solution.

Supplemental References

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