Prebiotic Cell Membranes that Survive Extreme Environmental Pressure Conditions**
Shobhna Kapoor, Melanie Berghaus, Saba Suladze, Daniel Prumbaum, Sebastian Grobelny, Patrick Degen, Stefan Raunser, and Roland Winter*

anie_201404254_sm_miscellaneous_information.pdf
Materials and Methods

Sample preparation: Decanoic acid, decanol and 1-decanoyl-rac-glycerol were purchased from Sigma-Aldrich. All other chemicals and solvents were also obtained from Sigma-Aldrich. The vesicles were prepared by mixing the fatty acid and the other components as neat oils to obtain the desired composition, following dispersion in aqueous 0.2 M bicine buffer solution titrated with NaOH to pH 8.5. This was followed by vigorous vortexing and tumbling overnight at room temperature. Where required, vesicles were subjected to five freeze-and-thaw cycles and/or extruded 11 times through 100 nm pore-sized polycarbonate membrane using an extruder (Avanti Polar lipids, Alabaster, USA). The final total amphiphile concentration (i.e., decanoic acid + decanol) was 80 mM unless noted otherwise. To prevent polycarbonate membrane fouling and concomitant loss of fatty acid material, the hydrated fatty-acid mixtures were disrupted by freeze-and-thaw cycles prior to extrusion. In addition, the extrusion was performed at a temperature above the melting temperature of the amphiphilic mixture (i.e., at room temperature, 25 °C). These steps help prevent loss of material during extrusion, and improve the homogeneity of the size distribution of the suspension finally obtained.[81]

(Cryo) Electron microscopy: Preparation of Negatively Stained Specimens and Electron Microscopy (EM) – Specimens were negatively stained as described previously.[82] Briefly, a 4 µL droplet of 1x diluted sample solution was adsorbed to a freshly glow-discharged 400 mesh copper grid (G2400C, Plano GmbH, Wetzlar) covered with a thin carbon film. The sample was left for 40 s on the grid before excess solution was removed with a filter paper (Whatman no. 4). After washing with three drops of sample buffer (0.2 M bicine buffer, pH 8.5) and once with freshly prepared 0.75% uranyl formate (SPI Supplies/Structure Probe), the specimen was exposed to the staining solution for about 2 min. Excess stain was removed by blotting and the specimen was air-dried. For image acquisition, a JOEL JEM-1400 electron microscope equipped with a LaB$_6$ cathode was used and operated at an acceleration voltage of 120 kV. Digital micrographs were recorded at a corrected magnification of 27,040x using a 4k x 4k CMOS camera F416 (TVIPS) at a pixel size of 5.71 Å/pixel at minimal dose conditions.

Preparation of Vitrified Specimens for Cryo Electron Microscopy (Cryo-EM) – Diluted samples were applied to freshly glow-discharged holey carbon grids (C-Flat 2/1, Protochips). The desired ice thickness was adjusted by blotting for 1 or 1.5 s using a Cryoplunge3 (Cp3, Gatan Inc.) and immediate plunging into liquid ethane. A JOEL JEM 3200FSC electron microscope equipped with a field-emission gun operating at a voltage of 100 kV was used for image acquisition. An omega in-column filter was used with a slit width of 15 eV. Images were recorded at a corrected magnification of 134,320x using an 8k x 8k CMOS camera F816 (TVIPS) at a pixel size of 5 Å/pixel using minimal dose conditions.

Temperature-dependent FTIR spectroscopy: Fatty acid vesicles were prepared in a similar fashion as mentioned above, with the exception of using deuterated bicine buffer, pD 8.5. Temperature-dependent measurements at ambient pressure were performed with a Nicolet 5700 FTIR spectrometer equipped with a liquid nitrogen cooled MCT (HgCdTe) detector, and a sample cell with CaF$_2$ windows separated by 50 µm mylar spacers. Typically, FTIR-spectra of 256 scans were taken with a resolution of 2 cm$^{-1}$, and data processing was performed using GRAMS software (Thermo Electron). An external circulating water thermostat was used for controlling the temperature with an accuracy of ± 0.1 K. In order to avoid erroneous results due to large scattering arising in the non-extruded sample, the fatty acid mixture was extruded through 100 nm pore-sized membranes prior to use.

Laurdan fluorescence spectroscopy: For the fluorescence measurements, the fatty acids and additional components along with the Laurdan fluorophore (in chloroform) were mixed, followed by the removal of chloroform by a gentle flow of nitrogen gas. Large unilamellar vesicles (the total amphiphile concentration was 80 mM) were prepared by the same procedure as mentioned above to avoid light scattering due to large particles. The final fatty acid to fluorophore molar ratio was kept at 500:1. The fluorescence spectroscopic measurements were performed on a K2 multifrequency phase and modulation
fluorometer (ISS Inc., Champaign, IL). Temperature control with an accuracy of ± 0.1 K was maintained with a circulating water bath. For the pressure-dependent studies, the same equipment was used, coupled with a stainless steel high-pressure vessel (ISS, Champaign, IL). The sample solution was injected into a spherical quartz cell (volume 0.8 mL) and sealed with an O-ring. The cell was then placed in the high-pressure vessel equipped with two quartz windows and connected to a pressure pump and gauge. High quality (18 MΩ) water was used as pressurizing medium. The high pressure cell was connected to a water bath maintaining the required temperature.

The spectral changes of Laurdan were quantified using the general polarization function, defined as $GP = (I_B - I_R) / (I_B + I_R)$. Here, $I_R$ and $I_B$ refer to the fluorescence intensities at 496 nm and 426 nm, which are characteristic for fluid (liquid-crystalline) and ordered (gel) lipid phases, respectively. $GP$ values range from +1 to −1.

**Thermodynamic measurements:** Large unilamellar fatty acid vesicles (extruded) were prepared similar to above, and the final amphiphile concentration was 13 mg/mL. Differential scanning (DSC) and pressure perturbation calorimetry (PPC) - Measurements were carried out on a VP DSC calorimeter from MicroCal with pressurizing accessory (Northampton, MA). The sample cell of the calorimeter was filled with ~0.5 mL of the solution, whereas the reference cell was biffed with the sample buffer. The heating rate was chosen to be 40 °C/h. Before each DSC heating scan, the sample solution was kept at the starting temperature for 20 min. Ultrasound velocimetry and densimetry - The acoustic measurements were performed on an ultrasonic resonator device ResoScan (TF Instruments GmbH, Heidelberg, Germany) using a single-crystal lithium niobate transducer of a fundamental frequency of 9.5 MHz. Both sample and reference cells were filled with 200 μL of the sample and buffer solutions, respectively. Density measurements were carried out using a high-precision density meter, DMA 5000 (Anton Paar, Graz, Austria), based on the mechanical oscillator principle. Determination of the adiabatic compressibility, isothermal compressibility and relative volume fluctuations of fatty acid vesicles - The coefficient of adiabatic compressibility of a medium is a function of the sound speed in the medium, $u$, and the density, $\rho$:

$$\beta_S = -\frac{1}{V} \left( \frac{\partial V}{\partial p} \right)_S = \frac{1}{u^2 \rho}$$

(1)

where $V$, $p$, and $S$ are the volume, pressure, and entropy, respectively. Thus, $\beta_S$ can be obtained directly from combined ultrasound velocity and density measurements.

The partial specific adiabatic compressibility, $K^0_S$, of a solute (here lipid) in diluted solution is given by:

$$K^0_S = \left( \frac{\partial V^o}{\partial p} \right)_S \approx \beta_{S,0} \left[ 2(V^o - [u]) - \frac{1}{\rho_0} \right]$$

(2)

where $\beta_{S,0}$ is the coefficient of adiabatic compressibility of the solvent (i.e., buffer). $[u]$ the velocity number, $V^o$ the partial specific volume of the solute in solution, and $\rho_0$ is the density of the solvent. The sound velocity of the lipid dispersion was determined relative to that in buffer solution at the same temperature in terms of the velocity number, $[u]$, defined as:

$$[u] = \frac{u - u_0}{u_0} c$$

(3)

where $u$ and $u_0$ denote the sound velocity in the solution and solvent, respectively, and $c$ is the specific solute concentration.

The densities of the solution and the buffer solution were used for evaluation of the partial specific volume $V^o$ of the solute by:
\[ V^o = \frac{1}{\rho_0} - \frac{\rho - \rho_0}{\rho_0} \cdot c. \]

(4)

The partial specific isothermal compressibility of solute is related to the measured partial specific adiabatic compressibility, \( K_S^o \), as follows:

\[ K_T^o = K_S^o + \frac{T \alpha_0^2}{\rho_0 C_p,0} \left( 2 \frac{e^o}{\alpha_0} - \frac{C_p^o}{\rho_0 C_p,0} \right) \]

(5)

where \( \alpha_0 \) and \( C_{p,0} \) are the thermal expansion coefficient and the specific heat capacity of the solvent, and \( e^o = (\partial V / \partial T)_p \) is the thermal expansion, and \( C_p^o \) the partial specific heat capacity of the solute at constant pressure, which are obtained using the calorimetric methods PPC and DSC, respectively \[^{[54]}\].

Mean square volume fluctuations of the solute molecules are directly proportional to their intrinsic isothermal compressibility, approximately given by:

\[ \langle \Delta V^2 \rangle = RTK_T = RT \rho_T^o V^o M \]

(6)

where \( \rho_T^o = K_T^o / V^o \) is the coefficient of isothermal compressibility of the solute (of molar mass \( M \)), which reflects the dynamic properties of the solute (here it is assumed that the intrinsic part of the isothermal compressibility is largely identical to the measured partial isothermal compressibility of the solute). The relative volume fluctuations of the solute can then be obtained by

\[ \sqrt{\frac{\langle \Delta V^2 \rangle}{V^2}} = \sqrt{\frac{RT \rho_T^o}{MV^o}}. \]

(7)

**Small-angle X-ray scattering (SAXS):** The pressure-dependent SAXS experiments were carried out at the high brilliance beamline ID02 at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The X-ray energy was 12.4 keV, which corresponds to a wavelength \( \lambda \) of 0.1 nm, with a flux of \( 4 \cdot 10^{13} \) photons/s. The pressure-dependent scattering curves were recorded with a CCD-detector with a sample to detector distance of 1 m, covering a range of momentum transfers, \( q = (4\pi/\lambda) \sin \theta \) (where \( \theta \) is the scattering angle), from 0.02 to 3.30 nm\(^{-1}\). The exposure time was between 0.05 and 0.5 s. The measurements were carried out in a high-pressure X-ray cell, which is equipped with two flat diamond windows of 1 mm thickness (type Iia quality, Drukker, Cuijk, The Netherlands). Further technical details of the set-up can be found elsewhere. \[^{[55]}\] High hydrostatic pressures up to 250 MPa were applied at 298 K using water as pressurizing medium. Temperature control (with an accuracy of \( \pm 0.2 \) K) was achieved by a computer-controlled water circulation system from a thermostat through the temperature-controlled jacket of the pressure cell. The time for thermal equilibration before each measurement was set to 15 min. For the measurements, a total of 1 mL of the sample was filled into the pressure cell, yielding a concentration of 3 % (w/w). The scattering intensity curves were corrected for background using the pure solvent, taking into account the different absorption factors, and plotted against the magnitude of the scattering vector, \( q \), by a MATLAB based software, provided by the ESRF.

Temperature-dependent data were obtained with a SAXSess mc\(^2\) small-angle X-ray scattering instrument from Anton Paar GmbH (Graz, Austria), equipped with a sealed tube X-ray generator Iso-Debyeflex3003 from GE Inspection Technologies (Ahrensburg, Germany; Cu-K\(_{\alpha}\) radiation (\( \lambda = 0.154 \) nm, 40 kV, 50 mA). The sample was measured in the slit configuration using a 1 mm quartz capillary provided with the TCS 120 temperature-controlled sample holder unit from Anton Paar (Graz, Austria). The exposure time of the sample to the X-rays was 30 min per measurement. While recording the temperature-dependent scattering data, temperature was adjusted using a Julado F25 heating circulator (Seelbach, Germany) and the actual cell temperature was monitored by the TCS control unit for TCS sample stages from Anton Paar (Graz, Austria). Detection was performed via 2D-imaging plates, which
were read out using the Storage Phosphor System Cyclone® Plus by Perkin Elmer (Waltham, Massachusetts). The latter was operated by the software Optiquant. The 2D images obtained were transformed to an intensity profile, $I(q)$, using the software 2D-SAXSquant. The data were collected for $q$-value between 0.12 and 4.00 nm$^{-1}$. The raw data obtained were normalized to the primary beam intensity and corrected for the background using the pure solvent (0.2 M bicine buffer) in SAXSquant 3.1 software provided with the SAXSess mc$^2$ system. Further, the data were desmeared to compensate for instrumental broadening effects (“slit-smearing”) using the GNOM software \[S6\]. The latter was also used to calculate the according pair distance distribution functions, $p(r)$, using the indirect Fourier Transformation. SAXS measurements were carried out for several mixtures including the decanoic acid: decanol, 1:1 and 1:2 molar ratio, with different preparation protocols.

**Dynamic light scattering:** These measurements were performed with the Zetasizer nano instrument (Malvern). A 4 mW He-Ne laser (633 nm wavelength) with a fixed detector angle of 173° was used. The temperature was controlled within a tolerance of ± 0.02 K. 1 mL of the extruded fatty acid sample was placed into the light scattering cell, and the measurement was started after 10 min to allow for temperature equilibration. The sizes of the aggregates were measured by recording the intensity time autocorrelation function, $\delta_2(t)$. For each temperature, the average values of 10 measurements were used for data fitting. The autocorrelation function of the scattered intensity was analyzed using the cumulant method \[S7\] to yield the distribution of z-average diameters ($D_z$) using the Stokes-Einstein relation. The same fatty acid mixture but without extrusion, exhibited a high polydispersity index (between 0.6 and 1.0) due to a wide size distribution (diameters between 93 nm and 1000 nm), thus restricting the use of this sample preparation for further analysis. Therefore, for the DLS experiments extruded samples comprising decanoic acid : decanol (2:1 molar ratio, pH 8.5) were used.

**Supplementary Figures**

![Fig. S1. Electron microscopy of the prebiotic membrane mixtures. (A-C) Representative micrograph area of negatively stained samples comprising decanoic acid : decanol (1:1 molar ratio, pH 8.5), tumbled overnight and non-extruded showing the presence of heterogeneous structures (denoted by an arrowhead) and vesicles (denoted by an asterisk). Scale bar, 200 nm.](image-url)
Fig. S2. Temperature-dependent phase behavior of model prebiotic membrane mixtures of different composition. DSC scans for different prebiotic lipid mixtures at 1 bar. Inset: DSC scan for unilamellar vesicles of the phospholipid bilayer system DPPC for comparison.

Fig. S3. Volumetric properties (PPC data) of different prebiotic lipid mixtures. PPC scans (temperature dependence of the thermal expansion coefficient) for different prebiotic membrane mixtures. Inset: PPC scan for unilamellar vesicles of the phospholipid bilayer system DPPC for comparison.
Fig. S4. Dynamic characteristics of different prebiotic lipid mixtures as a function of temperature. (A) Isothermal compressibilities, (B) adiabatic compressibilities and (C) relative volume fluctuations. For comparison, respective data for a model phospholipid bilayer, DPPC, are shown.

Small-Angle X-ray Scattering (SAXS) and Dynamic Light Scattering (DLS)

To explore the structural properties of the lipid system in more detail, scattering techniques were applied. Small-angle x-ray scattering (SAXS) is an excellent method of choice for structural elucidation of systems of colloidal size, typically up to 100 nm, and was used to obtain further insights into the pressure- and temperature-induced structural changes of the prebiotic lipid mixture. As an example, Fig. S5 A exhibits SAXS patterns of the decanoic acid:decanol system upon compression at 298 K. The absence of Bragg reflections indicates, in agreement with the TEM data, the absence of multilamellarity of the vesicles present in the sample. At ambient pressure, the distance-distribution function \( p(r) \) (Fig. S5 B), which can be calculated from the scattering intensity data (see the Supplementary Material), depicts not only spherical particles with radii of 20 nm and more, but also a significant population of small-sized particles (i.e., micelles), pointing again to the co-existence of vesicles and micelles in the prebiotic lipid mixture.
Fig. S5. Effect of temperature and pressure on the structural characteristics of the prebiotic lipid mixture. (A) Pressure dependent SAXS profiles for the prebiotic lipid mixture comprising decanoic acid: decanol (2:1 molar ratio, pH 8.5) in the range from 0.1 MPa to 250 MPa (step size 10 MPa) at 298 K. (B) The corresponding pair distance distribution functions, $p(r)$. (B, inset) Zoom-in of the changes within the micellar sub population upon compression. Pressure increases the amount of vesicular structures of about 40-100 nm size. (C) Temperature dependent SAXS profiles in the range from 278 K to 348 K (step size 5 K) at ambient pressure (0.1 MPa). (D) Temperature dependent changes in the diameter, z-average (black) and polydispersity index (red) of decanoic acid: decanol (2:1 molar ratio, pH 8.5) at ambient pressure (DLS-data).

Upon pressurization, the scattering intensities at small angles were found to increase, indicating formation of more and larger vesicles at the expense of small-sized micelles. This behavior is clearly evident by the pressure-dependent changes in the $p(r)$ functions, which show a strong decrease in the micellar population under pressure (Fig. S5 B, inset; some selected fits used for the calculation of $p(r)$ are depicted in Fig. S6). Hence, we can conclude that pressurization leads to a redistribution of the population of micelles (M) and vesicles (V), i.e., a shift of the M-V equilibrium, favoring vesicular particles under pressure.

Temperature-dependent SAXS profiles of the same system at ambient pressure also show an increase in the scattering intensity at small angles, indicating an increase in larger particles (Fig. S5 C).

Owing to the size limitation of the SAXS technique, complementary dynamic light scattering studies were carried out as well. Figure S5 D exhibits an increase in the average size of the vesicles with increasing temperature, reaching values for the diameter $D$ of about 160 nm at 328 K. The diameters determined by DLS are characteristic for spherical aggregates with water inside, typical for vesicular structures. Within this temperature range, the polydispersity index also increases, but remains rather low, pointing to a large fraction of vesicles of similar size. The marked increase in the particle size around 323 K correlates well with the second transition measured via DSC, supporting the claim that this transition
most likely depicts formation of larger sized particles or aggregates without significant associated conformational change of the individual lipid molecules.

**Fig. S6. Structural characteristics of the prebiotic lipid mixture upon compression.** Selected pressure dependent SAXS profiles (open circles) for the prebiotic lipid mixture comprising decanoic acid : decanol (2:1 molar ratio, pH 8.5) and corresponding fits for the calculation of the $p(r)$ functions (solid lines) at 25 °C. The curves have been vertically shifted for clarity.

**Fig. S7. pH and molar ratio dependence of vesicle formation and their stability in decanoic acid and decanoic acid/decanol mixtures.** (A) pH dependence as monitored by following the turbidity at 480 nm of decanoic acid alone, and of an equimolar mixture of deanoic acid + decanol. (B) Vesicle formation as a function of different molar ratios of decanoic acid and decanol, monitored via turbidity at 480 nm.
It is well established that fatty acids with acyl chain lengths of eight or more carbon tend to form vesicles in the pH range near the pK\textsubscript{a} of their terminal carboxyl group. For example, nonanoic and decanoic acid form vesicles around the pK\textsubscript{a} of the acids (pH 6.9 and pH 7.1, respectively). Above the pK\textsubscript{a}, the fatty acids prefer formation of micelles, whereas at a pH below their pK\textsubscript{a}, oil droplets can be detected. Figure S7 A follows this process by plotting the absorbance at 480 nm of a solution of decanoic acid in water and of a solution comprising an equimolar mixture of decanoic acid and decanol in water as a function of solution pH. In accord with previously published records, decanoic acid forms vesicles in a very narrow pH range, from 7.0-7.5, as depicted by an increase in the light scattering. At high pH, the formation of micelles prevails, leading to a decrease of light scattering and hence low absorbance. On the contrary, the mixture of decanoic acid and decanol exhibits a wide pH range (6.0 – 12.0) where vesicle formation takes place. This result is also in line with published reports on similar systems in stressing that inclusion of fatty alcohols of similar chain lengths to fatty acids markedly increases the stability of vesicles over a wide pH range. Based on these results, specifically for decanoic acid and decanol mixtures, a suitable pH range of around 8.5 was employed for all experiments, and, concomitantly, a suitable buffer in that range, i.e. bicine, was used. In addition, bicine buffers are not only stable at low temperatures (as required for the FTIR experiments) but also display minor changes in their pK\textsubscript{a} with varying temperature, making them apt for this study. The temperature-dependent changes in the pK\textsubscript{a} of bicine buffer is rather low (d\text{pK}\textsubscript{a}/dT = -0.018), making it a good choice for this study (R2). Choosing a pH value of around 8.5 offers a quite flexible window within which the temperature-induced changes in the pH of the solution will not affect the vesicle stability (Fig. S7 A) and hence the interpretation of the conclusions drawn. Figure S7 B depicts the ability of decanoic acid/decanol based prebiotic membrane mixtures to form vesicles over a wide range of molar ratios of the two constituents. Addition of decanol even at low amounts triggers the formation of vesicles as revealed by these turbidity measurements, and vesicle formation reaches a saturation level at elevated alcohol concentrations. These data hence provide evidence that also after extrusion, even if the initial molar ratio of the two constituents might slightly change, the propensity of the system to form vesicles is retained.

Fig. S8. Effect of extrusion on the pressure-dependent changes in the fluidity and conformational order of the decanoic acid/decanol system at 298 K. Pressure-dependence of Laurdan GP-values for the prebiotic lipid mixture comprising of decanoic acid : decanol (1:1 molar ratio, pH 8.5) at 298 K, before (A) and after (B) extrusion through 100 nm pore-sized membranes.

No significant changes were imparted into the pressure-dependent changes in the fluidity and conformational order of the system due to the extrusion process. At 298 K, the overall conformational order gradually increases, with a sigmoidal-like shape in the pressure interval between about 60 and 130 MPa for the system after extrusion, and between about 40 and 130 MPa before extrusion. In these pressure ranges, fluid and ordered phases co-exist. In both scenarios, a GP-value is reached above ~130 MPa (GP
= 0.45 ± 0.02), as the system enters a fully ordered conformational state, and is essentially unaffected upon further compression. Thus, before extrusion, due to a larger heterogeneity, the system is slightly more susceptible to pressure-induced changes, i.e., enters the two-phase coexistence region at slightly lower pressures. The general shape of the temperature-pressure phase diagram is not markedly affected by the extrusion process, however.

![Figure S9](image)

**Fig. S9. Effect of extrusion on the temperature-dependent structural characteristics of the prebiotic lipid mixture.** Temperature-dependent SAXS profiles in the range from 278 K to 348 K (step size 5 K) at ambient pressure (0.1 MPa) for the decanoic acid : decanol 2:1 molar ratio, pH 8.5; (A) before extrusion and (B) after extrusion through 100 nm pore-sized membranes.

Figure S9 exhibits the scattering curves of the extruded and non-extruded prebiotic membrane mixture with similar temperature-dependent profile, except that the changes in the small q-range are slightly less pronounced for the extruded compared to the non-extruded sample, probably due to the lower dispersity of this system. In addition, it can be seen that the scattering of the small-sized particle (micelles) fraction decreases with increasing temperature for both conditions, and scattering from large-sized particles (vesicles) increases concomitantly. Hence, these results further bolster the view that the behavioral propensity of the system to respond to temperature (or pressure) remains largely unaffected by the actual ratio between micelles and vesicles, as long as both co-exist.

**References**