Cyclophospholipids Increase Protocellular Stability to Metal Ions

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Model protocells have long been constructed with fatty acids, because these lipids are prebiotically plausible and can, at least theoretically, support a protocell life cycle. However, fatty acid protocells are stable only within a narrow range of pH and metal ion concentration. This instability is particularly problematic as the early Earth would have had a range of conditions, and life as we know it is completely reliant on metal ions for catalysis and the folding and activity of biological polymers. Here we show that prebiotically plausible monoacyl cyclophospholipids form robust vesicles that survive a broad range of pH and high concentrations of Mg2+, Ca2+, and Na+. Importantly, stability to Mg2+ and Ca2+ is improved by the presence of environmental concentrations of Na+. These results suggest that cyclophospholipids, or lipids with similar characteristics, may have played a central role during the emergence of Darwinian evolution.
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Ö. Duhan Toparlak, a Megha Karki, b Veronica Egas Ortuno, b Ramanarayanan Krishnamurthy * b and Sheref S. Mansy, * a

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x
Material and Methods:

General Experimental. Thin layer chromatography (TLC) was performed with a silica gel 60 ÅF254 from Angela Technologies and visualized by UV lamp and/or a stain solution of phosphomolybdic acid (PMMA) in ethanol. Flash Chromatography was performed on a biotage isola. NMR was recorded at 298 K with a Bruker DRX- 600 or AV-600 (600 MHz for 1H and 150 MHz for 31C). 31P-NMR spectra were acquired using a Bruker DPX-400; chemical shifts (δ) in parts per million (ppm), spin multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet), coupling constants (J) in Hertz (Hz), number of protons. Mass spectra was collected with an Agilent ESI-TOF or a ThermoElectron Finnigan LTQ ion trap mass spectrometer. The pH electrode was from Hanna instrument from Spectrum Chemicals and Laboratory products.

Materials. 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid (CAPSO) were from Alfa Aesar. Monoacyl lipids were either from either Nu-Chek Prep or Sigma-Aldrich. Unless otherwise indicated, all other reagents were from Sigma-Aldrich (Merck). Metal ions were provided as NaCl, MgCl₂ and CaCl₂.

Cyclophospholipid synthesis. Reagents and solvents from Sigma-Aldrich, VWR International, Fisher Scientific, and Acros. Cyclophospholipids CPC9 4 and CPC10 10 were synthesized and purified by employing previously reported protocols. Spectroscopic and spectrometric data are presented in the supporting information (Fig. S1–S8).

Vesicle stability assay. Unless otherwise noted, all vesicles were extruded through 100 nm track-etched polycarbonate membranes using an Avanti Polar mini extrusion system (11 passes). Vesicles were prepared either in the presence of 25 µM anionic (not poly-anionic nor dextran-sulfate) 10 kDa dextran conjugated with Alexa Fluor 488 (ThermoFisher Scientific) and purified within 2 h of extrusion. For the encapsulation of oligonucleotide, vesicles were extruded to 200 nm to increase encapsulation efficiency. 10 µM Alexa Fluor 555 DNA oligonucleotide (5'-GGCTCGACTGATGAGCCG-Alexa Fluor 555-3') was hybridized to 10 µM Alexa Fluor 488 labeled oligonucleotide (5'-Alexa Fluor 488-CGCAGAAAACCCGTGTCTCGAGC-3') by heating to 95 °C and cooled to 4 °C at a rate of 0.5 °C/s in the presence of 1 mM MgCl₂. Size exclusion chromatography with sepharose 4b was used to separate vesicles from unencapsulated dye to afford purity of >95%. Columns were pre-equilibrated and run with buffer containing lipid above the critical aggregate concentration (20 mM for cyclophospholipids vesicles and 40 mM for fatty acid vesicles). Fractions were collected with either a FC203B or FC204 Gilson fraction collector. Optical density at 600 nm and fluorescence (λex = 485 nm and λem = 515 nm) measurements were taken with a Tecan Infinite M200 plate reader. Vesicles were then incubated in the dark at 23 °C. For Na⁺, Mg²⁺, Ca²⁺ stability, vesicles were diluted 2-fold into solutions containing empty vesicles and salts. The percent solute retained inside of the vesicles was determined by comparing the vesicle and free dye fractions after a second round of size exclusion chromatography.

Microscopy. Vesicles were prepared the same as above and imaged without extrusion unless otherwise noted. For the Na⁺, Mg²⁺ and Ca²⁺ assays, 100 mM vesicle stock solutions were diluted to a final lipid concentration of 40 mM with buffer and membrane dye (rhodamine 6G, [final] = 10 µM). Vesicle preparations were visualized within 30–60 min. The dsRed channel (λex = 553±18 nm and λem = 605±70 nm) was used with a Zeiss Axio Observer Z1 fluorescence microscope. For the imaging of giant vesicles, the vesicles were prepared as described above but included 0.01 mol% N-(lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine. The vesicles were extruded to 10 µm and purified by size-exclusion chromatography with sepharose 4b. The vesicle fractions were pooled and mixed with salt solutions with empty vesicles (20 mM lipid) and incubated with tumbling for 24 h. The instrumental preset GFP channel (for dextran, λex = 484±25 nm and λem = 525±50 nm) and dsRed channel (for membrane, λex = 553±18 nm and λem = 605±70 nm) were used to image the dextran containing vesicles shown in Fig. 7.

Determination of the critical aggregate concentration (CAC). The CAC was determined by following previously published procedures. Briefly, 100 µL of 200 µM merocyanine 540, 0.2 M HEPES, pH 8.0 was dispensed in the wells of a 96-well plate (Costar 3603, black clear bottom, Corning). Solutions were then diluted two-fold with vesicles of varying concentrations prepared in 0.2 M HEPES, pH 8.0 and incubated at room temperature for 10 min. Absorbance was read with a Tecan Infinite M200 plate reader at 570 nm and 530 nm. The $A_{570nm}/A_{530nm}$ ratio indicated the aggregate state of the lipid, because the absorbance of merocyanine 540 at 570 nm reflects ordered lipid structures, whereas absorbance at 530 nm is correlated to free molecules in solution. No lipid negative controls gave $A_{570nm}/A_{530nm}$ values between 0.5 and 0.65. Measured values above the corresponding negative control were interpreted as points where the lipids formed aggregates.
Tunable Resistive Pulse Sensing (TRPS) Measurements. The experiments were performed with a qNano Gold (Izon Science) instrument. The influence of fatty alcohol content made use of Nanopore NP400 (pore size range = 200–800 nm), and the effect of dextran was assessed with Nanopore NP200 (pore size range = 100–400 nm). For consistency, an identical instrumental setup was used throughout the same-day analyses with current amplitudes of 120 ±10 nA (>100%, as recommended by manufacturer). The parameters were as follows: voltage = 0.34 ±0.06 V, stretch = 46.75 ±0.25 mm, pressure = 7 ±3 mbar. For statistical significance, 500–1000 particles per run were collected with a particle rate maximum of 4000 particles/min. Multiple dilutions (of different samples) and readings (of the same sample) were analyzed and reported as technical replicates (±SD). The instrument and nanopore were calibrated with standard calibration particles from the manufacturer (Izon Science) with mean a diameter of either 210 nm for NP200 or 340 nm for NP400. For dextran analysis, vesicles were prepared as indicated above. For the analysis of the effect of fatty alcohol content, vesicles were prepared from a single stock of 50 mM CPC10 solution at pH 8.0 with 0.2 M HEPES and left tumbling for 24 h. This stock of CPC10 was then aliquoted into new glass vials containing varying amounts of dodecanol. The solution was then adjusted to a final total lipid concentration of 25 mM in a final volume of 200 µL. Prior to TRPS measurements, the vesicle size was reduced to below 500 nm with centrifugal spin column filters (Ultrafree–MC–Durapore 0.45 µm with PVDF membrane, Millipore) more than 5 times. Samples were diluted for each measurement to stay within the manufacturer’s recommended particle rate range (100–3000 particles/min) to avoid overestimation of the concentration. For the effect of dodecanol experiments, the final total lipid concentrations in the flow cell were as following, with respect to dodecanol: 0 mol% = 25 mM, 9 mol% = 5 mM, 16 mol% = 2.5 mM, and 33 mol% = 1 mM. For the analysis of dextran containing vesicles, the final total lipid concentration was 1 mM. The absolute concentration was then determined by taking into account the dilution factor of each sample.
Supplementary Figures and Tables:

Fig. S1. $^1$H NMR spectrum of CPC9 4 in D$_2$O.$^1$
Fig. S2. $^{13}$C NMR spectrum of CPC9 4 in D$_2$O.$^1$
Fig. S3. (H-decoupled) $^{31}$P NMR spectrum of CPC9 4 in D$_2$O (162 MHz).
Fig. S4. ESI negative mode mass spectrum of CPC9 4.¹
Fig. S5. $^1$H NMR spectrum of CPC10 10 in D$_2$O. $^1$H NMR (600 MHz, D$_2$O) δ 4.31 (ddd, $J = 12.0, 9.4, 6.3$ Hz, 1H), 4.25 (dd, $J = 12.1, 4.3$ Hz, 1H), 4.22 – 4.15 (m, 1H), 4.01 (td, $J = 9.6, 6.8$ Hz, 1H), 2.37 (t, $J = 7.5$ Hz, 2H), 1.56 (t, $J = 7.4$ Hz, 2H), 1.32 – 1.16 (m, 12H), 0.81 (t, $J = 6.7$ Hz, 3H).
Fig. S6. $^{13}$C NMR spectrum of CPC10 10 in D$_2$O. $^{13}$C NMR (150 MHz, D$_2$O) δ 73.42, 65.45, 63.76, 33.23, 31.14, 28.69, 28.52, 28.28, 24.02, 21.92, 13.20.
Fig. S7. (H-decoupled) $^{31}P$ NMR spectrum of CPC10 10 in D$_2$O (162 MHz).
Fig. S8. ESI negative mode mass spectrum of CPC10 10. Expected $[M] = 307.1347$, found $[M] = 307.1347$. 
**Fig. S9.** Description of vesicle stability assay. 

**a)** Schematic representation of the process. 

1. **Initial encapsulation**
2. **1st round of SEC**
   - Gravity flow size exclusion chromatography to remove unencapsulated solutes
3. **Chromatography analysis**
   - Yellow shaded fractions are collected and pooled vesicle fractions for subsequent experiments.
   - Green shaded area indicates additional vesicle containing fractions.
   - Red shaded area indicates the free dye fractions.
4. **2nd round of SEC**
   - To assess vesicle-trapped-dye and free-dye fractions
5. **Incubate with salts**

**b)** A typical example for 1st round of size exclusion chromatography to purify vesicles from non-encapsulated solutes.

Yellow shaded fractions are collected and pooled vesicle fractions for subsequent experiments. Green shaded area indicates additional vesicle containing fractions. Red shaded area indicates the free dye fractions.
**Fig. S10.** Representative examples of size-exclusion chromatography at 24 h to assess the percentage of dye retained inside of vesicles. Stable (left, 2:1 CPC10:dodecanoic acid 11) and unstable (right, 2:1 CPC10:decanoic acid 5) vesicles retained 90% and 60%, respectively, of fluorescently labelled 10 kDa dextran. The green shaded region indicates the fractions from a sepharose 4b column containing vesicles, and the red shaded region indicates free dextran.
**Fig. S11.** Critical Aggregate Concentration (CAC) measurements of CPC9 4 and nonanoic acid 6 in the absence or presence of 9.1 mol% decanol. The absorption of 100 µM merocyanine 540 was monitored to indicate the aggregation state. Absorbance ratios above 0.65 indicate aggregates.
Fig. S12. Long-term stability of model protocells with respect to increasing fatty alcohol length. Every composition tested was with the same 2:1 ratio of ionic lipid to fatty alcohol. Fluorescently labeled dextran (~10 kDa) leakage at 24 h in 0.2 M HEPES, pH: 8.0. Circles, decanoyl cyclophospholipid 10 (CPC10); squares, decanoic acid 5. Error bars indicate ±SD of n = 2.
Fig. S13. Long-term stability of model protocells with respect to increasing fatty alcohol (dodecanol 11) mol%. Fluorescently labeled dextran (~10 kDa) leakage at 24 h, in 0.2 M HEPES, pH: 8.0. Filled circles, nonanoyl cyclophospholipid 4 (CPC9); open circles, nonanoic acid 3.
Fig. S14. Effect of dextran on vesicle formation of 2:1 CPC10 10:dodecanol 11. The total lipid concentration was 40 mM. The membrane dye was 10 µM Rhodamine 6G. The scale bars indicate 10 µm. The presence of dextran did not detectably disrupt the formation of vesicles.
Fig. S15. Effect of dextran on 2:1 CPC10:10:dodecanol vesicles for total vesicle/particle concentration. The values were obtained using TRPS with a qNano (Izon Science). The "with dextran" sample contained 25 µM dextran. The statistical test was with a two-tailed student’s t-test. No statistically significant difference was observed between the two conditions.
**Fig. S16.** Epifluorescence microscopy of nonanoic acid 3- and CPC9 4-based vesicles at different pH. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was rhodamine 6G. Solution conditions are listed in Supplementary Table 1. NOH, nonanol 6; NA, nonanoic acid 3; GMN, nonanoyl monoglyceride 8.
Fig. S17. Epifluorescence microscopy of decanoic acid 5- and CPC10 10-based vesicles at different pH. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was 10 µM rhodamine 6G. Solution conditions are reported in Supplementary Table 1. CPC10 10, decanoyl cyclophospholipid; DA, decanoic acid 5; LOH, dodecanol 11 (lauryl alcohol); GMD, decanoyl monoglyceride 9.
Fig. S18. Model protocell stability to pH. Retention of fluorescently labelled dextran after 24 h at each indicated pH. Filled circles, 2:1 CPC9 4:dodecanol 11; open circles, 2:1 nonanoic acid 3:dodecanol 11; open triangles, 4:1:1 nonanoic acid 3:dodecanol 11:decanoyl monoglyceride 9.
Fig. S19. Chromatogram of anionic AlexaFluor488 conjugated dextran (10 kDa) at pH 5. The dextran polymer was incubated at room temperature for 24 h in sodium acetate buffer at pH 5 (Supplementary Table 1) and subsequently purified by FPLC, with 4 times column volume (~96 mL in total). The first peaks is the stable Alexa Fluor 488-dextran conjugate (and dextran impurities), whereas free Alexa Fluor 488 dye is expected to elute after 48 mL (2x column volume).
Fig. S20. (H-decoupled) $^{31}$P NMR spectra (162 MHz) of CPC10 10 (50mM; pH 10.49, CAPSO buffer) after a) 0, b) 24, c) 72 and d) 96 h of incubation at room temperature.
Fig. S21. ESI negative mode mass spectrum of CPC10 \(10\) at pH \(10.49\) (50 mM total lipid in CAPSO buffer) after 96 h of incubation at room temperature.
Fig. S22 (H-decoupled) $^{31}$P NMR spectra (162 MHz) of CPC10 10 at pH 10.49 (CAPSO buffer) after 24 h of incubation at room temperature at a concentration of a) 50 mM and b) 12.5 mM.
Fig. S23. (H-decoupled) $^{31}$P NMR spectra (162 MHz) of CPC10 10 at pH 10.49 (50 mM total lipid in CAPSO buffer) after 72 h of incubation at room temperature in a) H$_2$O and b) CD$_3$OD.
Fig. S24. (H-decoupled) $^{31}$P NMR spectra (162 MHz) (left) and ESI negative mode mass spectrum (right) of CPC10 10 in hydrochloric acid/potassium chloride solution at pH 2.3 over a period of 24 h of incubation.
**Fig. S25.** $pK_a$ determination of cyclophospholipid 4 (CPC9) in solution. The amount of NaOH at the 100% equivalence point was 5.5 mmol and 50% was 2.25 mmol. The pH range (between 2.15 – 2.38) near the 50% equivalence point was fit to a straight line ($y = 0.0838x + 1.8479$, $r^2 = 0.996$) to calculate the $pK_a$ = ~2.27.
Fig. S26. Epifluorescence microscopy of nonanoic acid 3- and CPC9 4-based vesicles with increasing [Na⁺]. Vesicles were in 0.2 M HEPES, pH 8.0. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was 10 µM rhodamine 6G. CPC9 4, nonanoyl cyclophospholipid; NA, nonanoic acid 3; NOH: nonanol 6; GMN: nonanoyl monoglyceride 8.
Fig. S2. Epifluorescence microscopy of decanoic acid 5- and CPC10 10-based vesicles with increasing [Na\(^+\)]. Solutions were in 0.2 M HEPES, pH 8.0. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was 10 µM rhodamine 6G. CPC10 10, decanoyl cyclophospholipid; DA 5, decanoic acid; LOH dodecanol 11 (lauryl alcohol); GMD, decanoyl monoglyceride 9.
Fig. S28. Representative micrographs of cyclophospholipid CPC9 4-based vesicles showing excess vesicle formation due to hydrophobic effect in the presence of Na⁺ ions. Here the concentration of Na⁺ was 2.2 M at pH 7.0. Vesicle composition was 2:1 CPC9 4:nonanol 6, total lipid concentration was 40 mM, membrane dye was 10 µM rhodamine 6G. Excessive vesicle formation was clear at all z-positions.
Fig. S29. Epifluorescence microscopy of 2:1 decanoic acid 5:dodecanol 11 (DA:LOH) to Mg$^{2+}$. Solutions were in 0.2 M HEPES, pH 8.0. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was 10 µM rhodamine 6G.
Fig. S30. Epifluorescence microscopy of decanoic acid 5- and CPC10 10-based vesicles to increasing [Mg$^{2+}$]. Solutions contained 0.2 M HEPES, pH 8.0. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was 10 µM rhodamine 6G. CPC10 10, decanoyl cyclophospholipid; DA, decanoic acid 5; LOH, dodecanol 11 (lauryl alcohol); GMD, decanoyl monoglyceride 9.
Fig. S31. Representative micrographs of cyclophospholipid vesicles in the presence of 25 mM Mg$^{2+}$ at pH 8.0. The vesicle composition was 2:1 CPC10:dodecanol 11 (upper row) and 2:1 CPC9:nonanol 6 (lower row). The total lipid concentration was 40 mM, and the membrane dye was 10 µM rhodamine 6G. Scale bars indicate 10 µm.
**Fig. S32.** Epifluorescence microscopy of 2:1 CPC10 4:dodecanol 11 in the presence of Ca²⁺. Solutions contained 0.2 M HEPES, pH 8.0. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was 10 µM rhodamine 6G.
**Fig. S33.** Linear fit details of dextran retention data for 2:1 CPC10 dodecanol 11 vesicles in the presence of (a) Na\(^+\) or (b) Mg\(^{2+}\) ions with 200 mM Na\(^+\). Data are the same as in Fig. 4 and Fig. 5.
Fig. S34. Epifluorescence microscopy of 2:1 CPC9 4:nonanol 6 with increasing [Mg$^{2+}$] and [Na$^+$]. Solutions contained 0.2 M HEPES, pH: 8.0. The total lipid concentration was 40 mM. Scale bars indicate 10 µm. The membrane dye was 10 µM rhodamine 6G. Vesicle formation was observed up to 50 mM Mg$^{2+}$ and 1.2 M Na$^+$, whereas immediately, crystals and aggregates were formed at 75 mM Mg$^{2+}$ and 1.2 M Na$^+$. 
## Table S1. Buffers used in this study.

| pH  | Short name | Full name                                | Final Concentration | Total [Na⁺] after pH adjustment[^a] |
|-----|------------|------------------------------------------|---------------------|-----------------------------------|
| 4.0 | Acetate    | Sodium acetate (0.18) / Acetic acid (0.82) | 0.2 M / 0.2 M       | 60 mM                             |
| 5.0 | Acetate    | Sodium acetate (0.7) / Acetic acid (0.3)  | 0.2 M / 0.2 M       | 140 mM                            |
| 6.0 | MES        | 4-Morpholineethanesulfonic acid           | 0.2 M               | 90 mM                             |
| 7.0 | HEPES      | 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid | 0.2 M                  | 50 mM                             |
| 8.0 | HEPES      | 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid | 0.2 M                  | 160 mM                            |
| 9.0 | CAPSO      | 3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid | 0.2 M             | 50 mM                             |
| 10.0| CAPSO      | 3-(Cyclohexylamino)-2-hydroxy-1-propanesulfonic acid | 0.2 M             | 160 mM                            |

[^a]: Changes from batch to batch due to small variations in each preparation; therefore, approximate values are given.
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Author contributions

R.K. and S.S.M. conceived of the research project. Ö.D.T. and S.S.M. designed the experiments with vesicles, and Ö.D.T. carried-out the experiments with vesicles. R.K., M.K., and V.E.O. planned the synthesis and characterization of cyclophospholipids and glycerides, and M.K. and V.E.O. performed the synthesis and chemical characterization of the lipids. Ö.D.T and S.S.M. wrote the original draft, and all authors participated in the revisions. All authors contributed to data analysis and approved the final version of the manuscript.
Cyclophospholipids increase protocellular stability to metal ions

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Abstract

Model protocells have long been constructed with fatty acids, because these lipids are prebiotically plausible and can, at least theoretically, support a protocell life cycle. However, fatty acid protocells are stable only within a narrow range of pH and metal ion concentration. This instability is particularly problematic as the early Earth would have had a range of conditions, and life as we know it is completely reliant on metal ions for catalysis and the folding and activity of biological polymers. Here we show that prebiotically plausible monoacyl cyclophospholipids form robust vesicles that survive a broad range of pH and high concentrations of Mg2+, Ca2+, and Na+. Importantly, stability to Mg2+ and Ca2+ is improved by the presence of environmental concentrations of Na+. These results suggest that cyclophospholipids, or lipids with similar characteristics, may have played a central role during the emergence of Darwinian evolution.

Introduction

Main text

A major problem in our understanding of how protocells could have emerged is the lack of identified prebiotically plausible lipids that form robust vesicles capable of surviving the environments of the early Earth. Typically, mixtures of fatty acids, fatty alcohols, and the glycerol monoesters of fatty acids, i.e. monoglycerides, have been used to construct model compositions of protocellular membranes.¹,² This is, in part, because RNA molecules retain their activity within fatty acid-based model protocells,³,⁴ and such protocells can grow, divide,⁵–⁷ and acquire nutrients from the environment.⁸,⁹ Fatty acids are found in carbonaceous meteorites¹⁰ and are abiotically synthesized by Fischer-Tropsch chemistry.¹¹ However, fatty acid vesicles are unstable; they only form over a narrow range of pH,¹²,¹³ and rapidly disassemble in the presence of low concentrations of divalent cations,⁴,¹⁴ and precipitate at the concentration of monovalent

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Admixtures with fatty alcohol or glycerol monoesters of fatty acid increase stability to more alkaline conditions but do not sufficiently increase stability to cations.\textsuperscript{4,12,14} Such characteristics appear at odds with the conditions of the early Earth and greatly limit the regions where the Earth's first cells could have emerged. Therefore, the identification of prebiotically plausible membrane compositions that can withstand a wide variety of chemical conditions, including the concentrations of metal ions necessary for the folding and activity of biomolecules, would be advantageous.

Recently, a prebiotically plausible small molecule, diamidophosphate (1, DAP), was found to phosphorylate nucleosides, amino acids, and glycerides.\textsuperscript{17,18} Specifically, a mixture of glycerol and nonanoic acid was phosphorylated by DAP to produce cyclophospholipid 4 (Fig. 1a), which formed vesicles in the same reaction milieu.\textsuperscript{18} Phospholipid 4 belongs to a family of naturally occurring lipids with a cyclic phosphate head group.\textsuperscript{19} We reasoned that the decreased affinity of Mg\textsuperscript{2+} for the cyclic phosphate headgroup, in comparison to a carboxylate or a phosphatidic acid head group, would render the vesicles more resistant to hard divalent metal ions. We, therefore, sought to determine the stability of model protocells built with prebiotically plausible, short-chain, saturated lipids with a cyclic phosphate headgroup. We find that vesicles built from cyclophospholipids are stable over a broad range of pH and salinity, suggesting that such vesicles could have harboured early chemical systems on the path towards life.

**Figure 1.** Chemical structures of cyclophospholipids 4 and 10 used in this study. a) The prebiotic reaction of DAP 1 with glycerol 2 and nonanoic acid 3 produces cyclophospholipid 4, CPC9.\textsuperscript{18} b) Chemical structures of compounds used in this study: decanoic acid 5, fatty alcohols 6, 7; monoglycerides 8, 9, and cyclophospholipid CPC10 10. 4 and 10 were synthesized according to literature procedure (Fig. S1–S8).\textsuperscript{18}
Results and Discussion

Fatty alcohols stabilize cyclophospholipid vesicles

To start with, we observed that fatty alcohols enhanced the formation of cyclophospholipid vesicles, consistent with previous work with fatty acid vesicles.\textsuperscript{12} It was previously suggested that such stabilization resulted from decreased charge-repulsion between polar head-groups and the strengthening of inter-acyl chain packing.\textsuperscript{12,20} By epifluorescence microscopy, we found a dramatic increase in the number of vesicles formed with CPC9 4 (Fig. 1a) as the mole fraction of the fatty alcohol nonanol 6 increased (Fig. 2a). While only a few vesicles were detected in the absence of nonanol 6, many well-defined vesicles were observed with 2:1 CPC9 4:nonanol 6 at the same total lipid concentration of 40 mM.

To better gauge if the observed vesicles were stable enough to retain biological material, fluorescently labelled 10 kDa dextran was encapsulated within 2:1 CPC9 4:nonanol 6 vesicles, purified by size-exclusion chromatography, and incubated at room temperature for 24 h. Subsequently, the fraction of dextran released from the vesicles was quantified by another round of size-exclusion chromatography (Fig. S9–S10) with running buffer containing the same mixture of lipids above the critical aggregate concentration (Fig. S11). A large hydrophilic molecule, such as dextran, would not be expected to cross the membrane. Therefore, the identification of extravesicular dextran would indicate a major loss of structural integrity of the vesicle, which is why dextran is often used to assess the stability of vesicles.\textsuperscript{3,21} Despite the clear observation of vesicles by epifluorescence microscopy, the chromatography data revealed that 2:1 CPC9 4:nonanol 6 vesicles were not stable. After 24 h, more than 95% of the dextran was lost (Fig. 2b), consistent with the behaviour of previously characterized short-chain fatty acid vesicles.\textsuperscript{13,22}

Since longer acyl/alkyl chains generally lead to increased stability of membranes of mixed composition,\textsuperscript{23} the ability of longer fatty alcohols and a longer fatty acid to form stable vesicles was investigated. Increasing the alkyl chain of the fatty alcohol from nonanol to decanol greatly improved stability. The stability of 2:1 CPC9 4:decanol 7 (Fig. 2b) and 2:1 CPC10 10:decanol 7 (Fig. S12) increased 18-fold and 11-fold, respectively, with respect to their nonanol 6 counterparts. The most stable vesicle compositions tested were 2:1 CPC9 4:dodecanol 11 and 2:1 CPC10 10:dodecanol 11, which retained 79% ±9% and 85% ±5%, respectively, of the fluorescently labelled dextran for 24 h. Dodecanol 11 is synthesized by Fischer-Tropsch reactions in comparable yields to nonanoic 3 and decanoic acids 5,\textsuperscript{11} The stability of CPC9 4-based vesicles also directly correlated with the mole fraction of fatty alcohol present (Fig. S13).
The stabilizing influence of dodecanol 11 on CPC10 10-based vesicles was confirmed by Tunable Resistive Pulse Sensing (TRPS). Solutions containing 9:1 CPC10 10:dodecanol 11 contained $1.2 \times 10^{10} (\pm 0.2 \times 10^{10})$ particles, whereas $3.4 \times 10^{11} (\pm 1.8 \times 10^{10})$ particles were detected in solutions containing 2:1 CPC10 10:dodecanol 11 at the same total lipid concentration (Fig. 2c). To confirm that dextran was not disrupting the vesicles, 2:1 CPC10 10:dodecanol 11 vesicles were further probed by epifluorescence microscopy and tunable resistive pulse sensing. Vesicles were clearly observed by microscopy (Fig. S14), and the concentration of vesicles was not perturbed by the presence of dextran (without dextran $3.4 \times 10^{11} \pm 9.5 \times 10^{10}$ vs. with dextran $4.2 \times 10^{11} \pm 2.5 \times 10^{11}$) (Fig. S15). Importantly, both cyclophospholipid vesicles, i.e. CPC9 4 and CPC10 10, greatly outperformed vesicles built with comparable short-chain fatty acids. For example, nearly all of the dextran was lost from 2:1 nonanoic acid 3:nonanol 6, 2:1 nonanoic acid 3:dodecanol 11, and 2:1 decanoic acid 5:decanol 7 vesicles under the employed experimental conditions, which contained 200 mM Na⁺ (Fig. 2b, Fig. S12, Table S1).

Figure 2. Effect of hydrophobic chain heterogeneity. a) Epifluorescence microscopy of CPC9 4 admixtures with increasing nonanol 6 content. The total lipid concentration was 40 mM. The membrane dye was rhodamine 6G, and scale bars indicate 10 µm. b) Long-term stability of model protocells with respect to increasing fatty alcohol length. Every composition tested was with the same 2:1 ratio of ionic lipid to fatty alcohol. Fluorescently labelled dextran retention at 24 h, pH: 8.0. Circles, CPC9 4; squares, nonanoic acid 3 (n = 2). Error bars are ±SD. c) Total particle (CPC10 10-based vesicles) concentration change with increasing dodecanol 11 content, analysed by TRPS. Ratios are given for CPC10 10:dodecanol 11. (n ≥ 3 technical replicates).
Cyclophospholipid vesicles are stable to changes in pH

To determine if vesicles composed of cyclophospholipids were more stable to changes in pH than fatty acid vesicles, 40 mM 2:1 CPC9:nonanol 6 and 2:1 CPC10:dodecanol 11 were dispersed in solutions at different pH with a constant Na\(^+\) concentration of 200 mM and evaluated by epifluorescence microscopy. Vesicles clearly formed between pH 4 and pH 10 (Fig. S16–S17). Conversely, 2:1 decanoic acid 5:dodecanol 11 formed vesicles only between pH 7 and 10. Since monoglycerides are frequently employed to increase the stability of fatty acid vesicles, a ternary mixture of fatty acid, fatty alcohol, and monoglyceride was tested. A 4:1:1 decanoic acid 5:dodecanol 11:decanoyl monoglyceride 9 mixture also formed vesicles, but only between pH 7 and pH 10.

To confirm the increased stability of vesicles composed of cyclophospholipid to pH in comparison to fatty acid, the retention of fluorescently labelled 10 kDa dextran was assessed. Remarkably, 2:1 CPC10:10:dodecanol 11 retained greater than 75% of the dextran at all pH values tested after 24 h of incubation (Fig. 3a). In contrast, 2:1 decanoic acid 5:dodecanol 11 vesicles did not leak dextran at pH 7 and pH 8 (Fig. 3a), and 4:1:1 decanoic acid 5:dodecanol 11:decanoyl monoglyceride 9 vesicles were comparatively unstable (Fig. 3a). 2:1 CPC9:4:dodecanol 11 were stable between pH 7 and pH 10 (Fig. S18). Control experiments demonstrated that there was no hydrolysis of the fluorophore from the dextran at low pH (Fig. S19). Additionally, the cyclic phosphate headgroup of CPC10 10 was not hydrolysed at low (pH ≈ 3.9) or high (pH = 10.5) pH at 24 h (Fig. 3b) at room temperature. At pH 10.5, the \(^{31}\)P-NMR showed two peaks (~18 ppm) that developed over 24–96 h. These two peaks were tentatively attributed to the cyclic phosphate moiety existing in different environments, e.g. bilayers, micelles, or monomers (Fig. S20). This interpretation was supported by ESI-MS (Fig. S21), and the changes in the relative intensities of the two peaks upon dilution of the NMR sample (Fig. S22). That there was no other hydrolytic cleavage taking place was confirmed by acquiring \(^{31}\)P-NMR on the same 72 h sample after lyophilization and resuspension in deuterated methanol. The sample showed a single peak (Fig. S23). Hydrolysis of the cyclophosphate moiety could be achieved by incubation at pH 2.3 within 24 h, as observed by \(^{31}\)P-NMR and ESI-MS (Fig. S24).

A likely explanation for the formation and stability of cyclophospholipid vesicles over a broader range of pH than for fatty acid vesicles could be attributed the lower pK\(_a\) of the cyclic phosphate headgroup. For example, pure fatty acid vesicles form at the pK\(_a\) of the headgroup of the bilayer-associated acid (~ 7–9)\(^{21,23–25}\) because of the stabilizing interactions between the protonated and deprotonated forms of the carboxylate.\(^{26}\) Similarly, pure dodecyl phosphate forms vesicles at the first pK\(_a\) of the phosphate headgroup.
near pH 2.21 Although vesicles are not formed when fatty acids or alkyl phosphates are fully protonated, fully deprotonated, or possess more than a single negative charge, the incorporation of a non-titratable hydrogen-bond donor, such as a fatty alcohol, in the membrane allows for the formation of vesicles under more alkaline conditions.25 Since cyclic phosphates typically have a lower pKₐ than carboxylates, a mixture of cyclophospholipids with fatty alcohol would be expected to form vesicles at all pH values where deprotonated lipids exist. In bulk aqueous solution, the pKₐ of the cyclic phosphate headgroup was measured to be 2.3 (Fig. S25), which was similar to the pKₐ of dodecyl phosphate.21 However, large changes in pKₐ are common between free and membrane localized lipids,27,28 and so the pKₐ may be different when embedded in a membrane.

Figure 3. Stability of cyclophospholipids to pH. a) Retention of fluorescently labelled dextran after 24 h at each indicated pH. Circles, 2:1 CPC10:10:dodecanol 11; squares, 2:1 decanoic acid 5:dodecanol 11; diamonds, 4:1:1 decanoic acid 5:dodecanol 11:decanoyl monoglyceride 9 (n = 2). Error bars are ±SD. b) 31P NMR (left) and ESI mass (right) spectra of CPC10 10 after 24 h incubation at 23 °C, pH = 3.9 (Sodium acetate buffer) and pH = 10.5 (CAPSO buffer). The two peaks observed in the 31P-NMR spectrum at pH = 10.5 are likely due to different lipid packing environments (Fig. S20–24). By ESI-MS, no peaks corresponding to the hydrolytic opening of the cyclophosphate moiety or hydrolytic cleavage of the ester-bond were observed.
Cyclophospholipid vesicles are stable to the presence of monovalent and divalent metal ions

Having demonstrated that the vesicles containing cyclophospholipid were more stable over a range of pH compared to fatty acid vesicles, we next asked whether this increased stability extended to high concentrations of Na\(^+\) and Mg\(^{2+}\). Epifluorescence microscopy showed that 2:1 CPC9 4:nonanol 3 and 2:1 CPC10 10:dodecanol 11 vesicles were stable between 0.2 M Na\(^+\) and 2.2 M Na\(^+\) (Fig. S26–S28), although non-vesicular aggregates were also observed at high concentrations of Na\(^+\). For comparison, the concentration of Na\(^+\) in modern day seawater is between 0.4 – 0.5 M.\(^{29}\) In contrast to cyclophospholipid vesicles, all of the fatty acid vesicles tested began to aggregate beyond 0.6 M Na\(^+\), consistent with previous studies.\(^{14-16,30-32}\) The incorporation of monoglyceride did not improve the stability of fatty acid vesicles to Na\(^+\) (Fig. S26–S27).

The retention of fluorescently labelled 10 kDa dextran after 24 h was more sensitive to the stability of the vesicles than could be observed by microscopy. Vesicles formed by a 4:1:1 decanoic acid 5:dodecanol 11:decanoyl monoglyceride 9 mixture never retained more than 16% of the dextran from 0.15 M NaCl and above (Fig. 4). Cyclophospholipid vesicles were much more stable but did show a linear dependence on the concentration of Na\(^+\). Although greater than 85% of the dextran was retained by 2:1 CPC10 10:dodecanol 11 vesicles at 0.2 M Na\(^+\), approximately 10% more dextran was lost per 0.2 M increase of Na\(^+\) between 0.2 M and 1 M Na\(^+\).
Figure 5. Model protocell stability to Mg$^{2+}$. a) Epifluorescence microscopy at pH 8.0 in the presence of 25 mM Mg$^{2+}$. Cyclophospholipids formed vesicles, whereas fatty acids formed crystals. The membrane dye was rhodamine 6G. Scale bars indicate 10 µm. b) Retention of fluorescently labelled dextran after 24 h at pH 8.0 with 25 mM Mg$^{2+}$. Circles, 2:1 CPC10:dodecanol 11 with 0.2 M Na$^+$; squares, 2:1 CPC10:dodecanol 11 with 0.4 M Na$^+$; diamonds, 4:1:1 decanoic acid 5:dodecanol 11:decanoyl monoglyceride 9 with 0.2 M Na$^+$. Dashed lines are linear fits for CPC10 10- and visual guides for decanoic acid 5-based vesicles (n = 2). Error bars are ±SD.

The effect of Mg$^{2+}$ on vesicle stability mirrored the results above with cyclophospholipid vesicles outperforming their fatty acid counterparts. For example, epifluorescence microscopy showed Mg$^{2+}$ induced aggregation of fatty acid vesicles composed of 2:1 decanoic acid 5:dodecanol 11 at 5 mM of Mg$^{2+}$. Similarly, 4:1:1 decanoic acid 5:dodecanol 11:decanoyl monoglyceride 9 vesicles aggregated at Mg$^{2+}$ concentrations above 10 mM (Fig. S29–S30). In contrast, the cyclophospholipid vesicles (i.e. 2:1 CPC10 10:dodecanol 11) were observed in solutions with up to 25 mM Mg$^{2+}$ (Fig. 5a, Fig. S30 – S31). For comparison, the concentration of Mg$^{2+}$ in modern day seawater is ca. 50 mM.$^{29}$ The retention of dextran data confirmed the large difference in stability between fatty acid and cyclophospholipid vesicles. While fatty acid vesicles completely lost the encapsulated dextran by 5 mM Mg$^{2+}$, the cyclophospholipid vesicles retained more than 50% of the entrapped dextran at 25 mM Mg$^{2+}$ (Fig. 5b).
Figure 6. Model protocell stability to mixtures of divalent cations at pH 8.0. a) Retention of fluorescently labelled dextran after 24 h. b) Retention of fluorescently labelled DNA oligonucleotide after 24 h. Vesicle compositions were CPC10:10:dodecanol 11. Error bars are ±SEM of n = 3.

In addition to magnesium, the prebiotic Earth was rich in calcium. Since phospholipid membranes can interact with and be disrupted by calcium ions, we next asked if cyclophospholipid membranes could withstand the presence of Ca\(^{2+}\). After 24 h, 2:1 CPC10:10:dodecanol 11 vesicles were morphologically unchanged in the presence of 5 mM Ca\(^{2+}\) and began to crystallize at 10 mM Ca\(^{2+}\) (Fig. S32). The stability to Ca\(^{2+}\) was assessed by measuring the retention of fluorescently labelled dextran. Approximately 50% of the entrapped dextran was retained after 24 h in the presence of 10 mM Ca\(^{2+}\) (Fig. 6a). The concentration of Ca\(^{2+}\) in the contemporary ocean is ca. 10 mM. The observed stability to pH and metal ions is in contrast to that observed with alkyl phosphates and fatty acids. Although mixtures of alkyl phosphate and fatty alcohol form vesicles over a broad range of pH, experiments with alkyl phosphate vesicles were always performed either in deionized water or in the presence of chelators of metal ions.

The prebiotic environment likely contained a complex mixture of molecules that cannot be well represented by solutions containing a single type of metal ion. To better assess the plausibility of cyclophospholipid vesicles surviving environments of complex composition, the effect of mixtures of cations on the retention of entrapped dextran was evaluated after 24 h. The presence of an additional 0.2 M Na\(^+\) increased the stability of 2:1 CPC10:10:dodecanol 11 vesicles to 25 mM Mg\(^{2+}\) and 10 mM Ca\(^{2+}\) by more than 20% and 10%, respectively. That is, 71% ±4% of the dextran remained entrapped within the cyclophospholipid
vesicles in solutions containing 25 mM Mg\textsuperscript{2+} and 0.4 M Na\textsuperscript{+}, and 61% ±4% of the dextran was retained with 10 mM Ca\textsuperscript{2+} and 0.4 M Na\textsuperscript{+} (Fig. 5b and 6a). Protection by Na\textsuperscript{+} was possible because the disruptive effects per Na\textsuperscript{+} were 20-fold less than per Mg\textsuperscript{2+} (Fig. S33). The data were confirmed with entrapped, fluorescently labelled DNA in place of the dextran (Fig. 6b), demonstrating the ability of cyclophospholipid vesicles to hold genetic material. Next, a ternary mixture of metal ions was added to the cyclophospholipid vesicles. 2:1 CPC10 \textsuperscript{10}:dodecanol \textsuperscript{11} vesicles retained ca. 50% of entrapped dextran and 75% ±3% of fluorescently labelled DNA after 24 h in a solution containing 5 mM Ca\textsuperscript{2+}, 20 mM Mg\textsuperscript{2+}, and 0.4 M Na\textsuperscript{+} (Fig. 6a and 6b).

Taken together, cyclophospholipid vesicles can withstand high concentrations of cations in the absence of chelators, such as citrate\textsuperscript{9} (Fig. 7 and S34). Although prebiotically plausible short-chain cyclophospholipid vesicles may have been incapable of surviving long periods of time in seawater, such vesicles would have been capable of surviving conditions of higher salinity than found in many present day hot springs.\textsuperscript{31,32} That is, the increased stability to salinity expands the regions where protocells could have survived, thus increasing the overall likelihood of their emergence.

**Figure 7.** Vesicles composed of 2:1 CPC10 \textsuperscript{10}:dodecanol \textsuperscript{11} encapsulating 25 µM fluorescently labelled dextran after purification and 24 h tumbling in the presence of 0.4 M Na\textsuperscript{+} and 25 mM Mg\textsuperscript{2+} at pH 8.0. The membrane label was 0.01 mol\% N-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine. Dextran was labelled with Alexa Fluor 488. The scale bar indicates 10 µm. Empty vesicles were due to either freshly formed vesicles during tumbling, low initial encapsulation efficiency (~5%), or breaking (and thus leaking of dextran) and subsequent reformation.
Conclusions

Stability to divalent cations has long been considered a problem in our understanding of protocell chemistry. The Earth is rich in metal ions, so biology has evolved in a way that exploits this abundant resource.\(^{38,39}\) For example, \(\text{Mg}^{2+}\) plays structural and catalytic roles necessary for the activity of extant nucleic acids, proteins, and small molecules. Although there are places with low concentrations of metal ions,\(^{31,32}\) even bodies of fresh water would experience transiently high concentrations of salt during the types of wet-dry cycles frequently invoked to aid the dehydration reactions needed for the synthesis of biological polymers.\(^{40,41}\) Previous reports demonstrated that vesicle stability can be improved with admixtures of prebiotically plausible short chain fatty acids with alkyl amines,\(^{30}\) and that some single chain lipids can assemble into vesicles that withstand high salinity.\(^{42}\) However, such lipids are either prebiotically unlikely or cannot be clearly assigned a transitional role between prebiotic chemistry and extant lipids.

The esterification of a glycerol to a fatty acid renders the resulting vesicles more stable to \(\text{Mg}^{2+}\) and \(\text{Ca}^{2+}\),\(^{14}\) in part, because the binding site of the metal ion is weakened. However, vesicles containing mixtures of fatty acid and monoglyceride are still susceptible to changes of pH, because of the loss of polarity that results from the protonation of the fatty acid component of the membrane. Cyclic phosphate headgroups improve both of these features at once. The cyclophospholipid likely has decreased affinity for \(\text{Mg}^{2+}\) and \(\text{Ca}^{2+}\) and a lower pK\(_a\) that allows for vesicle formation over a broader range of pH in the presence of suitable hydrogen-bond donors.

Past attempts to determine environmental conditions compatible with the existence of protocells has tended to focus on either identifying regions of low salinity, the presence of prebiotic chelators of metal ions, or lipid additives that can withstand the effects of \(\text{Mg}^{2+}\) alone. What has been less considered is the effect of \(\text{Na}^+\), which is important because high concentrations of \(\text{Na}^+\) can interfere with the binding of other cations.\(^{43}\) Here we show that vesicles composed of cyclophospholipid and fatty alcohol are more resistant to \(\text{Na}^+\), \(\text{Mg}^{2+}\), and \(\text{Ca}^{2+}\) individually than fatty acid and alkyl phosphate vesicles.\(^{21,25}\) More significantly, the increased stability to \(\text{Na}^+\) gives rise to an increased tolerance to \(\text{Mg}^{2+}\) and \(\text{Ca}^{2+}\). Therefore, not only could such cyclophospholipid systems survive a wider variety of chemical conditions more compatible with what is known about the prebiotic Earth,\(^{44}\) but also cyclophospholipid protocells would be able to persist in conditions ideal for the non-enzymatic polymerization of nucleotides\(^{45,46}\) and the evolution of extant-like nucleic acid and protein folds.
Contemporary diacyl phospholipids can be broken down into component parts that may chart a historical path starting from fatty acids. The coupling of glycerol to the fatty acid gives a lipid that forms vesicles that are more stable to pH and the presence of metal ions. Similarly, phosphorylation of this monoglyceride to give a cyclophospholipid increases the stability to pH and metal ions even further. Such incremental improvements with each discrete chemical step, presumably by energy-dissipative cycling, suggest a path in which environmental selective pressures could lead to modern day lipids. The remaining step needed to convert the monoacyl cyclophospholipid to a contemporary diacyl phospholipid would greatly improve stability at the expense of the ability to acquire nutrients and grow and divide without protein machinery. It may be that cyclophospholipids played a critical role in the space between protein independent and protein dependent (proto)cells.

Conflicts of interest
There are no conflicts to declare.

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