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Cyclophospholipids Increase Protocellular Stability to Metal Ions

Ö. Duhan Toparlak, Megha Karki, Veronica Egas Ortuno, Ramanarayanan Krishnamurthy, and Sheref S. Mansy**

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 extant life is completely reliant on metal ions for catalysis and the folding and activity of biological polymers. Here, prebiotically plausible monoacyl cyclophospholipids are shown to form robust vesicles that survive a broad range of pH and high concentrations of Mg^{2+}, Ca^{2+}, and Na^{+}. Importantly, stability to Mg^{2+} and Ca^{2+} 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.

1. Introduction

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 meteorites[10] 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 cations typically found in the environment.[14–16] Admixtures with fatty alcohol or glycerol monoesters of fatty acid increase stability to more alkaline conditions but do not sufficiently increase stability to cations.[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.[17,18] Specifically, a mixture of glycerol and nonanoic acid was phosphorylated by DAP **1** to produce cyclophospholipid **4** (**Figure 1**a), which formed vesicles in the same reaction milieu.[18] Phospholipid **4** belongs to a family of naturally occurring lipids with a cyclic phosphate headgroup.[19] We reasoned that the decreased affinity of Mg^{2+} for the cyclic phosphate headgroup, in comparison to a carboxylate or a phosphatidic acid headgroup, 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 harbored early chemical systems on the path toward life.

2. Results and Discussion

2.1. 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.[12] It was previously suggested

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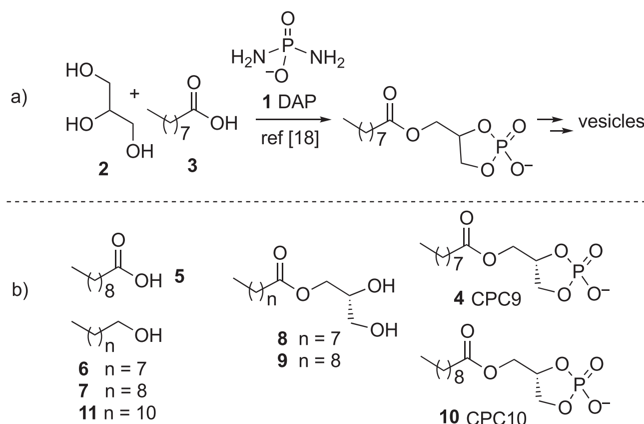

Figure 1. Chemical structures of cyclophospholipids **4** and **10** used in this study. a) The prebiotic reaction of DAP **1** with glycerol **2** and nonanoic acid **3** produces racemic cyclophospholipid, CPC9.[18] b) Chemical structures of compounds used in this study: decanoic acid **5**, fatty alcohols **6**, **7**, **11**; monoglycerides **8**, **9**, and cyclophospholipid CPC10 **10**. **4** and **10** were synthesized according to literature procedure (Figures S1–S8, Supporting Information).[18] Numbers in bold in the manuscript and captions refer to the compounds as indicated.

that such stabilization resulted from decreased charge repulsion between polar headgroups and the strengthening of inter-acyl chain packing.[12,20] By epifluorescence microscopy, we found a dramatic increase in the number of vesicles formed with CPC9 **4** as the mole fraction of the fatty alcohol nonanol **6** increased (**Figure 2**a). 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 40×10^{-3} M.

To better gauge if the observed vesicles were stable enough to retain biological material, fluorescently labeled 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 (Figures S9 and S10, Supporting Information) with running buffer containing the same mixture of lipids above the critical aggregate concentration (Figure S11, Supporting Information). 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.[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 (Figure 2b), consistent with the behavior of previously characterized short-chain fatty acid vesicles.[13,22]

Since longer acyl/alkyl chains generally lead to increased stability of membranes of mixed composition,[23] the ability of longer fatty alcohols and a longer fatty acid to form stable vesicles was investigated. Increasing the alkyl chain of the

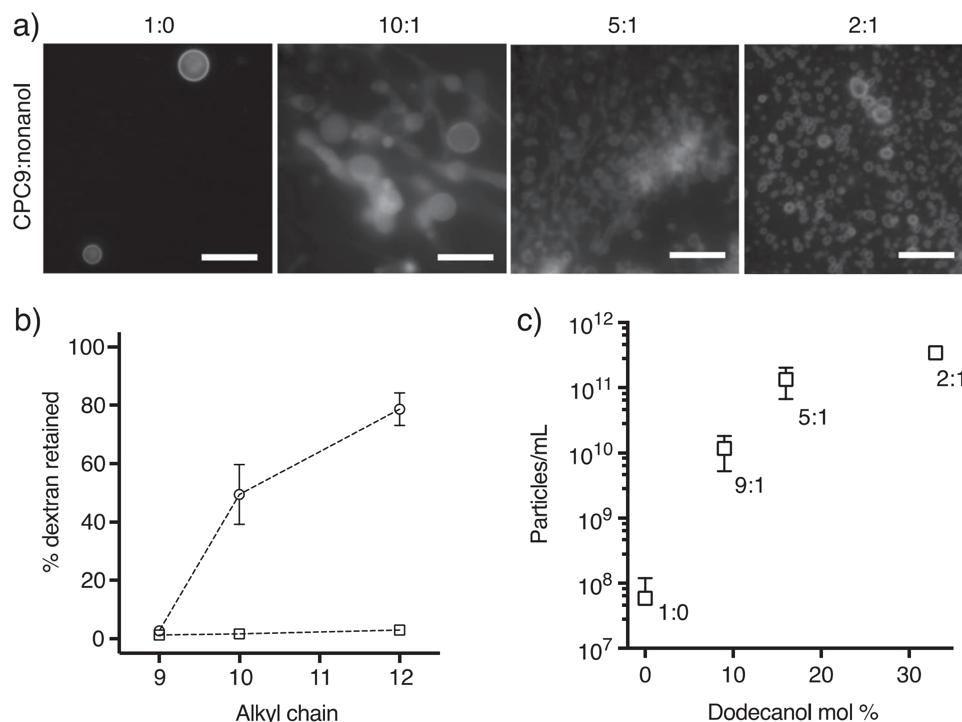

Figure 2. Effect of hydrophobic chain heterogeneity. a) Epifluorescence microscopy of CPC9 **4** admixtures with increasing nonanol **6** content. The total lipid concentration was 40×10^{-3} M. The membrane dye was rhodamine 6G, and scale bars indicate 10 µm. b) Long-term stability of model protocells with respect to increasing fatty alcohol length. Every composition tested was with the same 2:1 ratio of ionic lipid to fatty alcohol. Fluorescently labeled 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 changes with increasing dodecanol **11** content, analyzed by TRPS. Ratios are given for CPC10 **10**:dodecanol **11** ($n \geq 3$ technical replicates).

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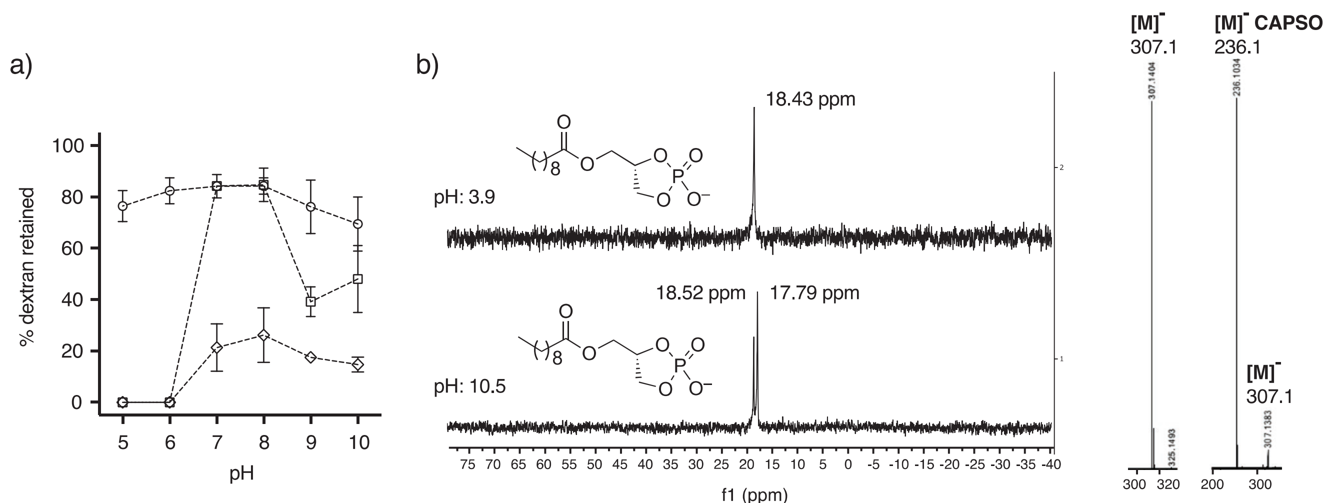

Figure 3. Stability of cyclophospholipids to pH. a) Retention of fluorescently labeled dextran after 24 h at each indicated pH. Circles, 2:1 CPC10 **10**:dodecanol **11**; squares, 2:1 decanoic acid **5**:dodecanol **11**; diamonds, 4:1:1 decanoic acid **5**:dodecanol **11**:decanoyl monoglyceride **9** (n = 2). Error bars are ±SD. b) ^{31}P 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 new 18.52 ppm peak observed in the ^{31}P NMR spectrum at pH = 10.5 is from the glycerol-cyclophosphate (Figures S20–S24, Supporting Information). By ESI-MS, no peaks corresponding to the hydrolytic opening of the cyclophosphate moiety were observed at pH = 3.9 (left) and pH 10.5 (right).

fatty alcohol from nonanol to decanol greatly improved stability. The stability of 2:1 CPC9 **4**:decanol **7** (Figure 2b) and 2:1 CPC10 **10**:decanol **7** (Figure S12, Supporting Information) 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 labeled dextran for 24 h. Dodecanol **11** is synthesized by Fischer–Tropsch reactions in comparable yields to nonanoic **3** and decanoic acids **5**.[11] The stability of CPC9 **4**-based vesicles also directly correlated with the mole fraction of fatty alcohol present (Figure S13, Supporting Information). 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 1.2×10^{10} ($\pm 0.2 \times 10^{10}$) particles, whereas 3.4×10^{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 (Figure 2c). To confirm that dextran was not disrupting the vesicles, 2:1 CPC10 **10**:dodecanol **11** vesicles were further probed by epifluorescence microscopy and TRPS. Vesicles were clearly observed by microscopy (Figure S14, Supporting Information), 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 $4.2 \times 10^{11} \pm 2.5 \times 10^{11}$) (Figure S15, Supporting Information). 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 200×10^{-3} M Na^+ (Figure 2b and Figure S12 and Table S1, Supporting Information).

2.2. 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, 40×10^{-3} M 2:1 CPC9 **4**:nonanol **6** and 2:1 CPC10 **10**:dodecanol **11** were dispersed in solutions at different pH with a constant Na^+ concentration of 200×10^{-3} M and evaluated by epifluorescence microscopy. Vesicles clearly formed between pH 4 and pH 10 (Figures S16 and S17, Supporting Information). 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 labeled 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 (**Figure 3**a). In contrast, 2:1 decanoic acid **5**:dodecanol **11** vesicles did not leak dextran at pH 7 and pH 8 (Figure 3a), and 4:1:1 decanoic acid 5:dodecanol **11**:decanoyl monoglyceride **9** vesicles were comparatively unstable (Figure 3a). 2:1 CPC9 4:dodecanol **11** were stable between pH 7 and pH 10 (Figure S18, Supporting Information). Control experiments demonstrated that there was no hydrolysis of the fluorophore from the dextran at low pH (Figure S19, Supporting Information). Additionally, the cyclic phosphate headgroup of CPC10 **10** was not hydrolyzed at low (pH ≈ 3.9) or high (pH = 10.5) pH at 24 h (Figure 3b) at room temperature. At the extreme basic pH of 10.5, the ^{31}P NMR showed two peaks (≈18 ppm) that developed over 24–96 h (Figure S20a, Supporting Information). We initially ascribed these two peaks to the cyclic phosphate moiety existing in different

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environments (e.g., bilayers, micelles, or monomers). However, later work suggested that the newer second peak could belong to the carboxyl-ester hydrolyzed product, glycerol-1,2-cyclophosphate. This interpretation was supported by electrospray ionization-mass spectrometry (ESI-MS). Apart from the molecular ion peak corresponding to the CPC10 **10**, a relatively small molecular ion peak for glycerol-cyclophosphate was also observed (Figure S21, Supporting Information), and confirmed by comparison with the authentic spectra of glycerol-1,2-cyclophosphate (Figure S20b, Supporting Information). The relative intensities of the two peaks corresponding to the cyclophosphate species in ^{31}P NMR spectra at pH 10.5 (Figure 3b) have to be reconciled with the fact that glycerol-cyclophosphate is more soluble than CPC10 **10** in water and may not be representative of the actual ratio of the mixture. This hypothesis is supported by a) the presence of the molecular ion mass of CPC10 **10** in the sample at pH 10.5 after 96 h (Figure S21, Supporting Information); b) the changes in the relative intensities of the two peaks upon dilution of the NMR sample (Figure S22, Supporting Information); and c) by acquiring ^{31}P NMR on the same 72 h sample after lyophilization and resuspension in methanol, which showed a single peak (Figure S23, Supporting Information) ascribed to CPC10 **10**. Nevertheless, despite the carboxyl–ester bond cleavage, our functional data indicate that protocells consisting of cyclophospholipid species can still retain the inner contents much more efficiently than fatty acid counterparts. 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 (Figure S24, Supporting Information).

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 to 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 nontitratable 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 pK_a 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 pK_a of the cyclic phosphate headgroup was measured to be 2.3 (Figure S25, Supporting Information), which was similar to the pK_{a1} of dodecyl phosphate.[21] However, large changes in pK_a are common between free and membrane localized lipids,[27,28] and so the pK_a may be different when embedded in a membrane.

2.3. 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 broad 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 and 2.2 M Na^+ (Figures S26–S28, Supporting Information), although nonvesicular aggregates were also observed at high concentrations of Na^+. For comparison, the concentration of Na^+ in modern day seawater is between 0.4 and 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^+ (Figures S26 and S27, Supporting Information).

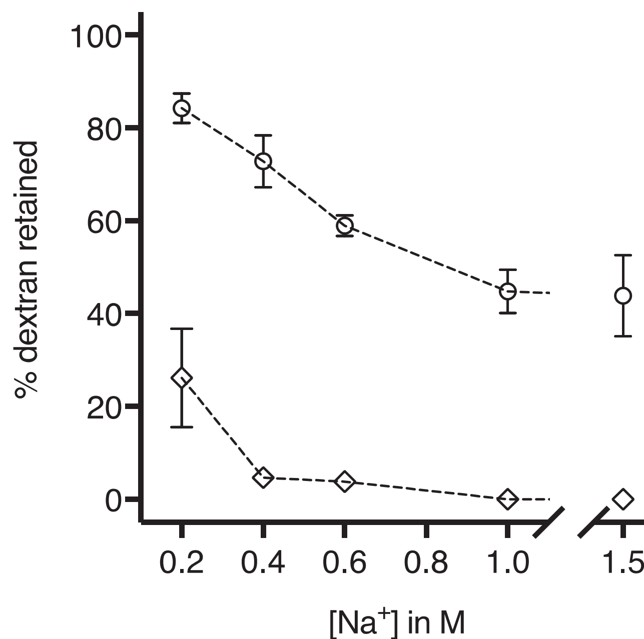

Figure 4. Model protocell stability to Na^+. Retention of fluorescently labeled dextran after 24 h at each indicated concentration of Na^+ at pH 8.0. Circles, 2:1 CPC10 **10**:dodecanol **11**; diamonds, 4:1:1 decanoic acid **5**:dodecanol **11**:decanoyl monoglyceride **9** (n = 2). Error bars are ±SD.

The retention of fluorescently labeled 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 26% of the dextran from 0.2 M NaCl and above (**Figure 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 and 1 M Na^+. High concentrations of Na^+ promoted the formation of nonvesicular aggregates, as noted above, which may have led to the co-elution of lipid aggregates with dextran. Similar behavior was observed by others with fatty acid vesicles.[33] However, high concentrations of K^+ did not noticeably induce the formation of nonvesicular aggregates and thus showed a linear dependence of vesicle stability between 0.4 and 1.5 M K^+ (Figure S29, Supporting Information).

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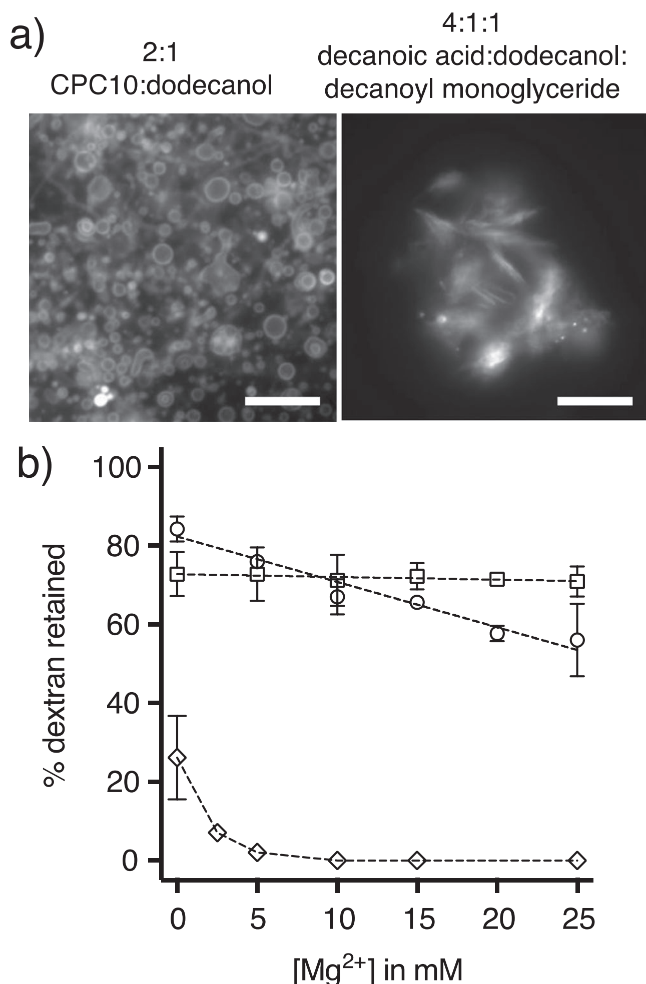

Figure 5. Model protocell stability to Mg^{2+}. a) Epifluorescence microscopy at pH 8.0 in the presence of 25×10^{-3} M Mg^{2+}. Cyclophospholipids formed vesicles, whereas fatty acids formed crystals. The membrane dye was rhodamine 6G. Scale bars indicate 10 µm. b) Retention of fluorescently labeled dextran after 24 h at pH 8.0 with 25×10^{-3} M Mg^{2+}. Circles, 2:1 CPC10 **10**:dodecanol **11** with 0.2 M Na^+; squares, 2:1 CPC10 **10**: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 5×10^{-3} M of Mg^{2+}. Similarly, 4:1:1 decanoic acid **5**:dodecanol **11**:decanoyl monoglyceride **9** vesicles were aggregated at Mg^{2+} concentrations above 10×10^{-3} M (Figures S30 and S31, Supporting Information). In contrast, the cyclophospholipid vesicles (i.e., 2:1 CPC10 **10**:dodecanol **11**) were observed in solutions with up to 25×10^{-3} M Mg^{2+} (**Figure 5**a and Figures S31 and S32, Supporting Information). For comparison, the concentration of Mg^{2+} in modern day seawater is ≈50×10^{-3} M.[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 5×10^{-3} M Mg^{2+}, the cyclophospholipid vesicles retained more than 50% of the entrapped dextran at 25×10^{-3} M Mg^{2+} (Figure 5b).

In addition to magnesium, the prebiotic Earth was rich in calcium. Since phospholipid membranes can interact with and be disrupted by calcium ions,[34] 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 5×10^{-3} M Ca^{2+} and began to crystallize at 10×10^{-3} M Ca^{2+} (Figure S33, Supporting Information). The stability to Ca^{2+} was assessed by measuring the retention of fluorescently labeled dextran. Approximately 50% of the entrapped dextran was retained after 24 h in the presence of 10×10^{-3} M Ca^{2+} (**Figure 6**a). The concentration of Ca^{2+} in the contemporary ocean is ≈10×10^{-3} M.[35] The observed stability to pH and metal ions is in contrast to that observed with alkyl phosphates[36] and fatty acids.[37,38] 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[25] or in the presence of chelators of metal ions.[21]

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 25×10^{-3} M Mg^{2+} and 10×10^{-3} M 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 25×10^{-3} M Mg^{2+} and 0.4 M Na^+, and 61% ± 4% of the dextran was retained with 10×10^{-3} M Ca^{2+} and 0.4 M Na^+ (Figures 5b and 6a). Protection by Na^+ was possible because the disruptive effects per Na^+ were 20-fold less than per Mg^{2+} (Figure S34, Supporting Information). The data were confirmed with entrapped, fluorescently labeled DNA in place of the dextran (Figure 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 **10**:dodecanol **11** vesicles retained ≈50% of entrapped dextran and 75% ± 3% of fluorescently labeled DNA after 24 h in a solution containing 5×10^{-3} M Ca^{2+}, 20×10^{-3} M Mg^{2+}, and 0.4 M Na^+ (Figure 6b).

Taken together, cyclophospholipid vesicles can withstand high concentrations of metal ions in the absence of chelators, such as citrate (**Figure 7** and Figure S35, Supporting Information).[9] 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.[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.

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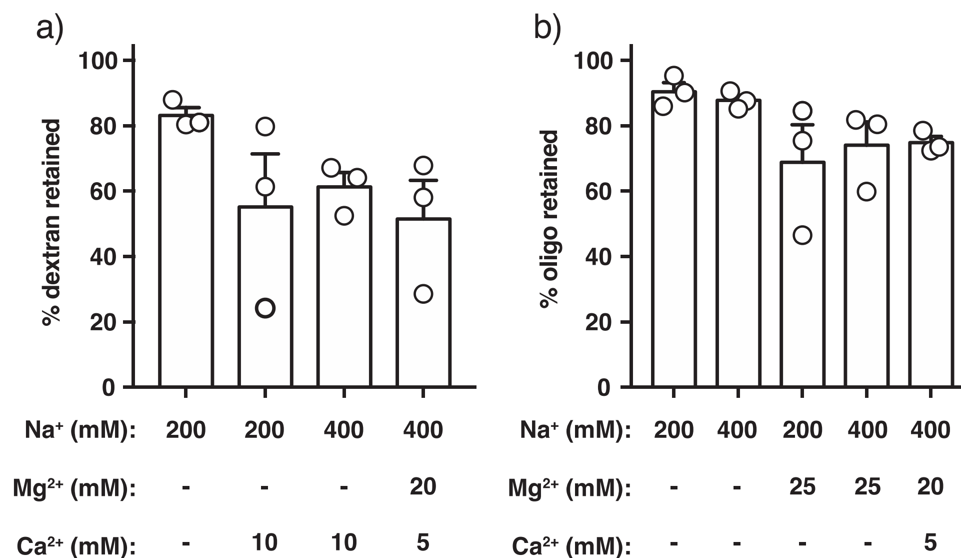

Figure 6. Model protocell stability to mixtures of divalent cations at pH 8.0. a) Retention of fluorescently labeled dextran after 24 h. b) Retention of fluorescently labeled DNA oligonucleotide after 24 h. Vesicle compositions were 2:1 CPC10 **10**:dodecanol **11**. Error bars are ±SEM of $n = 3$.

3. Conclusion

Stability to metal ions 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.[39,40] For example, 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.[41,42] 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.[43] However, such lipids are either prebiotically unlikely or cannot be clearly assigned a transitional role between prebiotic chemistry and extant lipids.

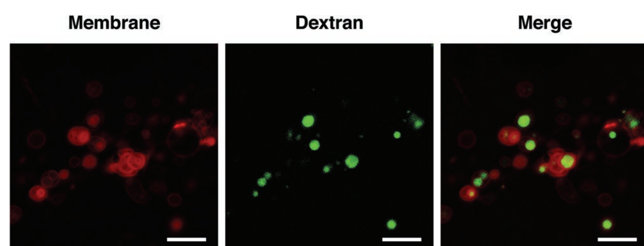

Figure 7. Vesicles composed of 2:1 CPC10 **10**:dodecanol **11** encapsulating 25×10^{-6} M fluorescently labeled dextran after purification and 24 h tumbling in the presence of 0.4 M Na^+ and 25×10^{-3} M Mg^{2+} at pH 8.0. The membrane label was 0.01 mol% *N*-(Lissamine rhodamine B sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine. Dextran was labeled 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.

The esterification of a glycerol to a fatty acid renders the resulting vesicles more stable to Mg^{2+} and 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 Mg^{2+} and 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 have 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 Mg^{2+} alone. What has been less considered is the effect of Na^+, which is important because high concentrations of Na^+ can interfere with the binding of other cations.[44] Here, we show that vesicles composed of cyclophospholipid and fatty alcohol are more resistant to Na^+, Mg^{2+}, and Ca^{2+} individually than fatty acid and alkyl phosphate vesicles.[21,25] More significantly, the increased stability to Na^+ gives rise to an increased tolerance to Mg^{2+} and 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,[45] but also cyclophospholipid protocells would be able to persist in conditions ideal for the nonenzymatic polymerization of nucleotides[46,47] and the evolution of extant-like nucleic acid and protein folds. It should be noted that a recent report showed a stabilizing effect on fatty acid vesicles by prebiotic amino acids.[33] Whether similar stabilization of cyclophospholipid vesicles is possible has yet to be tested.

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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,[48] 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.[49] It may be that cyclophospholipids played a critical role in the space between protein-independent and protein-dependent (proto)cells.

4. Experimental Section

General Experimental: Thin layer chromatography was performed with a silica gel 60 ÅF254 from Angela Technologies and visualized by UV lamp and/or a stain solution of phosphomolybdic acid in ethanol. Flash chromatography was performed on a biotage isolera. NMR was recorded at 298 K with a Bruker DRX-600 or AV-600 (600 MHz for ^{1}H and 150 MHz for ^{13}C). ^{31}P 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 were 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 Nu-Chek Prep or Sigma-Aldrich. Unless otherwise indicated, all other reagents were from Sigma-Aldrich (Merck). Metal ions were provided as NaCl, KCl, $MgCl_2$, and $CaCl_2$.

Cyclophospholipid Synthesis: Reagents and solvents were from Sigma-Aldrich, VWR International, Fisher Scientific, and Acros. Cyclophospholipids CPC9 **4** and CPC10 **10** were synthesized and purified by employing previously reported protocols.[18] Spectroscopic and spectrometric data are presented in the Supporting Information (Figures S1–S8, Supporting Information).

Preparation of Vesicles: Fatty acid vesicles were prepared by oil-dispersion.[8] More specifically, fatty acids and their nonphosphorylated glycerol monoesters were heated to 55 °C. Subsequently, aliquots of the lipid oils (total: ≈10 µmol) were quickly mixed with 100 µL aqueous solution and vortexed for ≈30 s. The resulting solution was then repeatedly heated to 55 °C and vortexed for ≈30 s between five and eight times. The solution was allowed to reach room temperature (23 °C) before use. Cyclophospholipids were handled similarly, except that the powder was added directly to the aqueous solution. Buffer compositions are reported in Table S1 in the Supporting Information.

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 in the presence of 25 × 10^{-6} M 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 × 10^{-6} M Alexa Fluor 555 DNA oligonucleotide (5′-GGCTCGACTGATGAGGCGCG-Alexa Fluor 555-3′) was hybridized to 10 × 10^{-6} M Alexa Fluor 488 labeled oligonucleotide (5′-Alexa Fluor 488-CGCGCCGAAACACCGTGTCTCGAGC-3′) by heating to 95 °C and cooled to 4 °C at a rate of 0.5 °C s^{-1} in the presence of 1 × 10^{-3} M $MgCl_2$. 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 × 10^{-3} M for cyclophospholipid vesicles and 40 × 10^{-3} M for fatty acid vesicles). Fractions were collected with either a FC203B or FC204 Gilson fraction collector. Optical density at 600 nm and fluorescence (λ_{ex} = 485 nm and λ_{em} = 515 nm) measurements were taken with a Tecan Infinite M200 plate reader. Vesicles were then incubated in the dark at 23 °C. For Na^+, K^+, Mg^{2+}, Ca^{2+} stability, vesicles were diluted twofold into solutions containing empty vesicles (with a final total lipid concentration of ≈10 × 10^{-3} M) 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^+, Mg^{2+}, and Ca^{2+} assays, 100 × 10^{-3} M vesicle stock solutions were diluted to a final lipid concentration of 40 × 10^{-3} M with buffer and membrane dye (rhodamine 6G, [final] = 10 × 10^{-6} M). Vesicle preparations were visualized within 30–60 min. The dsRed channel (λ_{ex} = 553 ± 18 nm and λ_{em} = 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 × 10^{-3} M lipid) and incubated with tumbling for 24 h. The instrumental preset GFP channel (for dextran, λ_{ex} = 484 ± 25 nm and λ_{em} = 525 ± 50 nm) and dsRed channel (for membrane, λ_{ex} = 553 ± 18 nm and λ_{em} = 605 ± 70 nm) were used to image the dextran-containing vesicles shown in Figure 7.

Determination of the Critical Aggregate Concentration (CAC): The CAC was determined by following previously published procedures.[13] Briefly, 100 µL of 200 × 10^{-6} M 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 twofold with vesicles of varying concentrations prepared in 0.2 M HEPES, pH 8.0 and incubated at room temperature (23 °C) for 10 min. Absorbance was read with a Tecan Infinite M200 plate reader at 570 and 530 nm. The $A^{570\,nm}/A^{530\,nm}$ ratio indicated the aggregation 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.[50] No lipid negative controls gave $A^{570\,nm}/A^{530\,nm}$ values between 0.5 and 0.65. Measured values above the corresponding negative control were interpreted as points where the lipids formed aggregates.

TRPS Measurements: Data were collected 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^{-1}. 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 × 10^{-3} M CPC10 **10** solution at pH 8.0 with 0.2 M HEPES and left tumbling for 24 h. This stock of CPC10 **10** 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 × 10^{-3} M in a final volume of 200 µL. Prior to TRPS measurements,

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the vesicle size was reduced to below 500 nm with centrifugal spin column filters (Ultrafree–MC–Durapore 0.45 µm with polyvinylidene difluoride membrane, Millipore) more than five times. Samples were diluted for each measurement to stay within the manufacturer's recommended particle rate range (100–3000 particles min^{-1}) 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% = 25×10^{-3} M, 9 mol% = 5×10^{-3} M, 16 mol% = 2.5×10^{-3} M, and 33 mol% = 1×10^{-3} M. For the analysis of dextran-containing vesicles, the final total lipid concentration was 1×10^{-3} M. The absolute concentration was then determined by taking into account the dilution factor of each sample.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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