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Mg^{2+}-driven selection of natural phosphatidic acids in primitive membranes†

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Biological membranes are composed exclusively of phospholipids comprising glycerol-1-phosphate or glycerol-3-phosphate. By contrast, primitive membranes would have likely been composed of heterogeneous mixtures of phospholipids, including non-natural analogues comprising glycerol-2-phosphate, as delivered by prebiotic synthesis. Thus, it is not clear how the selection of natural phospholipids could have come about. Here we show how differences in supramolecular properties, but not molecular properties, could have driven the selection of natural phosphatidic acids in primitive membranes. First, we demonstrate that at the molecular level it is unlikely that any prebiotic synthesis or hydrolysis pathway would have enabled the selection of natural phosphatidic acids. Second, we report that at the supramolecular level, natural phospholipids display a greater tendency to self-assemble in more packed and rigid membranes than non-natural analogues of the same chain length. Finally, taking advantage of these differences, we highlight that Mg^{2+}, but not Na^{+}, K^{+}, Ca^{2+} or Zn^{2+}, drives the selective precipitation of non-natural phosphatidic acids from heterogeneous mixtures obtained by prebiotic synthesis, leaving membranes proportionally enriched in natural phosphatidic acids. Our findings delineate a plausible pathway by which the transition towards biological membranes could have occurred under conditions compatible with prebiotic metal-driven processes, such as non-enzymatic RNA polymerization.

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Introduction

Phospholipids are major constituents of biological membranes.[1] Their emergence on early Earth would thus have marked a pivotal step on the path towards primitive cells.[2] Prebiotic synthesis would have produced heterogeneous mixtures of isomeric phospholipids, but only the natural phospholipids comprising glycerol-1-phosphate (**G1P**) and glycerol-3-phosphate (**G3P**) were retained in biological membranes while their non-natural analogues comprising glycerol-2-phosphate (**G2P**) were presumably lost to chemical evolution. Still, it is not clear how these natural phospholipids were selected.

Prebiotic chemists have sought to identify mechanisms by which many of the natural building blocks of life could have been selected. Yet, investigations have focused almost exclusively on nucleosides and peptides. Canonical RNA and DNA nucleosides could have arisen by selective prebiotic synthesis[3–5] or because of their greater hydrolytic and photochemical stability relative to non-canonical isomers.[6–8] Selection could also have resulted from the increased stability of duplexes comprising natural oligonucleotides, compared to non-natural analogues.[9] Similarly, the canonical amino acids could have resulted from selective synthesis,[10] the preferential incorporation of natural over non-natural amino acids into peptides,[11] or by retention due to the enhanced foldability of oligopeptides containing natural amino acids compared to those containing non-natural analogues.[12] The idea that selection processes could have operated at both the molecular and supramolecular levels is central to every scheme.

Two recent reports have begun to address the selection of natural phospholipids. In work on the emergence of amino alcohol bearing phospholipids, our group[13] described a general prebiotic synthesis and hydrolysis scheme that is selective for natural phospholipid headgroups comprising **G1P** over those comprising **G2P**. Then, in work advancing the prebiotic chemistry of cyclophospholipids, Krishnamurthy and co-workers[14] showed that liposomes formed of glycerol monodecanoate and phospholipid comprising **G1P** are more tolerant of pH changes and metal ions, such as those required for prebiotic RNA chemistry,[15] than those formed of phospholipid comprising **G2P**.

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These studies raise further questions, particularly in light of the systems chemistry approach in which mixtures of reactants and products must be considered and their properties compared. First, it is not clear if prebiotic synthesis and hydrolysis processes could have led to the selection of natural over non-natural diacyl phospholipids. Prebiotic acylation reactions of individual glycerol phosphates have been reported,[16,17] but no direct comparison has been made of the reactivity of **G1P** and **G2P** with activated fatty acids, nor of the hydrolytic stability of the corresponding phospholipids. Second, it is not clear if differences in membrane properties could have led to the selection of natural phospholipids from heterogeneous mixtures containing both natural and non-natural forms. Hybrid liposomes often behave differently from those made of individual lipids and so their behavior is difficult to predict.

Here we explore processes operating at the molecular and supramolecular levels that could have led to the selection of natural phosphatidic acids from heterogeneous mixtures on early Earth. We show that hydrolytically stable natural and non-natural phosphatidic acids could have formed on prebiotic acylation of mixtures of glycerol phosphates under a variety of conditions, together with a range of amphiphilic coproducts. We then highlight that natural phosphatidic acids have a greater propensity to self-assemble, compared to their non-natural isomers, and that their liposomes display enhanced rigidity and stability towards changes in pH and metal ion concentrations. Finally, harnessing these differences, we demonstrate that on exposure to Mg^{2+}, but not Na^{+}, K^{+}, Ca^{2+} or Zn^{2+}, natural phosphatidic acids could have been selected from liposomes composed of heterogeneous mixtures. Overall, our findings offer a plausible rationale for the selection of natural phospholipids, taking advantage of differences in properties that manifest only at the supramolecular level.

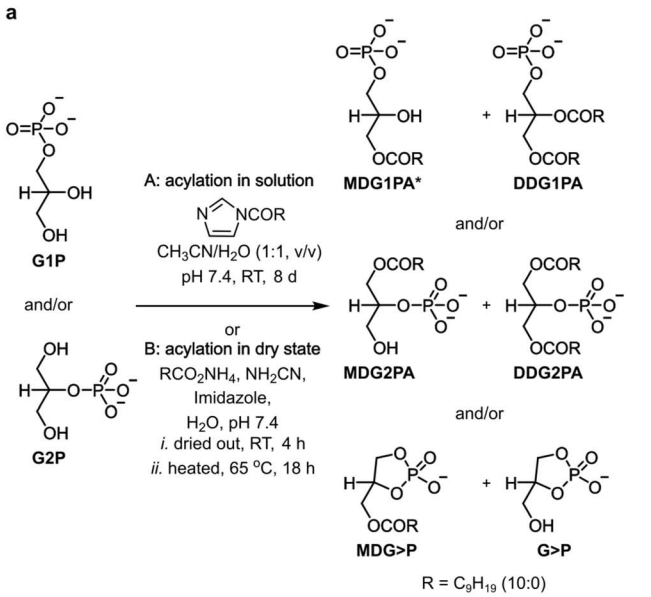
Note: throughout our discussion we treat **G1P** and **G3P** as synonymous and for simplicity refer to them with the acronym **G1P**. The stereospecific numbering (*sn*) scheme for glycerophospholipids is used only where appropriate.

Results and discussion

Prebiotic synthesis delivers mixtures of natural and non-natural phosphatidic acids

Synthesis in solution. Several prebiotic routes to phosphatidic acids have been described,[16–19] and more can be traced by combining steps from multiple reports. For example, glycerol could have been delivered by (photo)reduction of acetaldehyde or dihydroxyacetone,[20–22] and glycerol-1-phosphate (**G1P**) and glycerol-2-phosphate (**G2P**) could have formed on phosphorylation of glycerol.[18,20–25] Phosphatidic acids could have then been produced by acylation of glycerol phosphates with activated fatty acids under various conditions. However, previous investigations[16,17] only addressed the long-chain acylation of single isomers of glycerol phosphate, despite the likely co-occurrence of both **G1P** and **G2P** on early Earth. Thus, it is not clear whether differences in the reactivity of **G1P** and **G2P** towards acylating agents might explain the selection of natural **G1PAs** over non-natural **G2PAs**.

To probe the acylation of glycerol phosphates in solution, each glycerol phosphate was dissolved in water and the pH of each solution was adjusted to 7.4. A solution of *N*-decanoyl imidazole (NDI) in acetonitrile was then added,[16] and each homogeneous mixture was stirred for 8 days at room temperature (Fig. 1a, b and S1†). The progress of each reaction was



b

Entry	Starting material	Conditions	% Phosphatidic acids MDG1PA*	DDG1PA	MDG2PA	DDG2PA
1	G1P	A	20	4	-	-
2	G2P	A	-	-	43	44
3	G1P + G2P	A	10	3	20	22
4	G1P	B	15	4	2	<1
5	G2P	B	2	<1	14	4
6	G1P + G2P	B	10	3	7	3

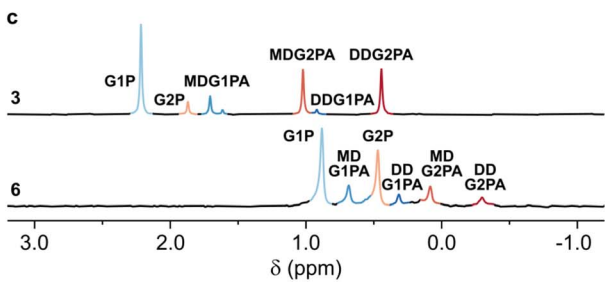


Fig. 1 Prebiotic synthesis of phosphatidic acids. (a) Reactions of **G1P** (10 mM), **G2P** (10 mM), or both (5 mM each) with NDI (100 mM) in solution, or ammonium decanoate (100 mM), cyanamide (100 mM), and imidazole (100 mM) in the dry state, afford natural **G1PAs** and non-natural **G2PAs**. (b) Yields of **G1PAs** and **G2PAs** formed from **G1P**, **G2P**, or both. (c) $^{31}P\{^{1}H\}$ NMR spectra showing the signals for the **G1PAs** and **G2PAs** obtained in reactions described in entries 3 (pH ~7) and 6 (pH ~4 after extraction) of (b). Yields were determined by ^{31}P NMR based on conversion of the respective glycerol phosphate (or sum total glycerol phosphates). The chemical shift of each phosphatidic acid is pH and concentration dependent. *The major positional isomer[26] of MDG1PA is shown in (a) and combined yields of both isomers of MDG1PA are given in (b). See the ESI† for details.

followed by ^{31}P NMR and by TLC.[27] In the reaction of **G1P**, 3(2)-monodecanoyl-glycerol-1-phosphatidic acid (**MDG1PA**, 1.68 and 1.59 ppm) were obtained alongside 2,3-didecanoyl-glycerol-1-phosphatidic acid (**DDG1PA**, 0.89 ppm) in yields of 20% and 4%, respectively (Fig. 1a, b and S1†). By contrast, in the reaction of **G2P**, 1-monodecanoyl-glycerol-2-phosphatidic acid (**MDG2PA**, 1.05 ppm), and 1,3,-didecanoyl-glycerol-2-phosphatidic acid (**DDG2PA**, 0.47 ppm) were obtained in yields of 43% and 44%, respectively (Fig. 1a, b and S2†).

Since the acylation of **G2P** proceeds to a greater extent than the acylation of **G1P**, we wondered if, in a reaction starting from a mixture of glycerol phosphates, **G2P** would outcompete **G1P** for NDI and thus suppress the formation of **G1PAs**. However, in the event, acylation of a 1 : 1 mixture of glycerol phosphates gave **G1PAs** and **G2PAs** in combined yields of 13% and 42%, respectively, or approximately half the yields obtained in experiments starting from **G1P** or **G2P** (Fig. 1a–c and S3†). This result shows that in solution **G1P** and **G2P** undergo acylation independently of one another. Nevertheless, as the yield of **DDG2PA** is approximately 10 times that of **DDG1PA** under our conditions, we infer **DDG1PA** would only have been produced as the major phosphatidic acid if the initial ratio of **G1P : G2P** exceeded 10 : 1. The literature on prebiotic phosphorylation of glycerol[21–25] suggests that this requirement would rarely have been met, however, especially when hydrolysis of glycerol cyclic phosphate is taken into account.[13,20,28]

The acylation of glycerol phosphates in solution occurs by a two-step phosphate-assisted mechanism in which intermolecular formation of a mixed anhydride intermediate precedes intramolecular transfer of the acyl group to a neighboring hydroxyl group (Fig. S4†).[16] Acyl transfer to the proximal primary hydroxyl group of **G2P** is more efficient than acyl transfer to either the proximal secondary hydroxyl group or the distal primary hydroxyl group of **G1P**. Thus, given the preferential formation of **G2PAs** in these reactions in solution, we sought to determine whether acylation reactions in the dry state are subject to the same bias.

Synthesis in the dry state. To probe the acylation of glycerol phosphates in the dry state, each glycerol phosphate was dissolved in water and ammonium decanoate, cyanamide, and imidazole were added before the pH of the mixture was adjusted to 7.4 (Fig. 1a and b).[17] Each mixture was allowed to evaporate at room temperature and the dried mixture was heated at 65 °C for 18 h. The products were extracted into acetonitrile/water/D_2O (5 : 4 : 1, v/v/v), and the formation of phosphatidic acids was verified by ^{31}P NMR and TLC as before. In contrast to the results obtained in solution, a broader range of products was produced in the dry state. On acylation of **G1P**, **MDG1PA** and **DDG1PA** were obtained in yields of 15% and 4%, respectively, alongside **G2P** (2%), **MDG2PA** (2%), **DDG2PA** (1%), glycerol-1,2-cyclic phosphate (**G>P**, 16%), and 3-monodecanoyl glycerol-1,2-cyclic phosphate (**MDG>P**, 6%) (Fig. 1b and S5†). The positional isomers, **G2P** and **MDG2PA** probably form on hydrolysis of **G>P** and **MDG>P**, respectively,[13,29] or by phosphoryl migration under the ultimately acidic conditions of the reaction.[26] Similarly, the reaction of **G2P** afforded **MDG2PA** and **DDG2PA** in yields of 14% and 4%, respectively, along with the same array of products as obtained in the reaction starting from **G1P** (Fig. 1b and S6†). Finally, the reaction of a 1 : 1 mixture of **G1P** and **G2P** gave comparable amounts of **G1PAs** and **G2PAs** (combined yields of 13% and 10%, respectively) (Fig. 1a–c and S7†).

It is difficult to be certain of the mechanism of the acylation reaction in the dry state, but it is possible it involves direct attack of the acylating agent on the hydroxyl groups of each glycerol phosphate. This proposal would account for the comparable yields of phosphatidic acids obtained on acylation of each glycerol phosphate in the dry state. To test this idea, we subjected *sn-glycero*-3-phosphocholine (**G3PC**)[30] to acylation in the dry state and in solution. This substrate was chosen as its phosphodiester group is not expected to participate in the reaction. On reaction of **G3PC** with ammonium decanoate, cyanamide, and imidazole in the dry state, 1(2)-monodecanoyl-*sn*-glycero-3-phosphocholine (**MDG3PC**, −0.20 and −0.38 ppm) and 1,2-didecanoyl-*sn*-glycero-3-phosphocholine (**DDG3PC**, −0.68 ppm) were obtained in a yields of 10% and 11%, respectively, together with glycero-2-phosphocholine (5%), and traces (<1%, combined) of **MDG>P** and **G>P** (Fig. S8†). By contrast, the reaction of **G3PC** with NDI in solution produced only **MDG3PC** in 4% yield (Fig. S9†). These results show that a free phosphate group is not required for acylation in the dry state, but is required for efficient acylation in solution.

Mechanism aside, our data show that dry state acylation reactions of **G1P** and **G2P** proceed without bias and afford **G1PAs** and **G2PAs** in yields proportional to the amount of each glycerol phosphate present. This result is significant as under certain conditions[21,22] **G1P** is the major product obtained on prebiotic phosphorylation of glycerol. Moreover, acylation in the dry state occurs under arguably more realistic early Earth conditions (the activated fatty acid is generated *in situ* and the reaction requires no organic solvent). However, even in a best-case scenario where only **G1P** was available, prebiotic acylation of glycerol phosphates in the dry state would have produced both natural **G1PAs** and non-natural **G2PAs**.

Hydrolysis. Our group recently demonstrated that hydrolysis provides a mechanism for the selection of complex natural phospholipids and their headgroups.[13] Thinking along similar lines, we wondered if **G2PAs** might be more susceptible to hydrolysis than **G1PAs** and if hydrolysis processes would enable the selection of natural phosphatidic acids. Accordingly, the C10-lipids **DDG1PA** and **DDG2PA** were synthesized[31,32] and subjected to hydrolysis at room temperature, pH 10, either in homogeneous solution in acetonitrile/carbonate buffer (1 : 1, v/v) or in the form of liposomes in carbonate buffer. The extent of hydrolysis of each lipid was greater in solution than in liposomes at every time point, as observed for other primitive amphiphiles,[33] but differences between the extents of hydrolysis of **DDG1PA** and **DDG2PA** in solution and in liposomes were minimal (Fig. S10†). For example, after 11 days in solution 87% of **DDG1PA** and 87% of **DDG2PA** were hydrolyzed, while in liposomes 35% of **DDG1PA** and 34% of **DDG2PA** were hydrolyzed. Similar hydrolysis experiments were performed at pH 7 (imidazole buffer) and pH 4 (acetate buffer), but slow hydrolysis (data not shown) and precipitation of the lipid (see below) prevented us from observing any differences. These results

suggest that, in contrast to the possible emergence of amino alcohol bearing phospholipids,[13] it is not likely that the selection of natural phosphatidic acids on early Earth resulted from hydrolysis.

Natural and non-natural phosphatidic acids exhibit different supramolecular properties

As **G1PAs** and **G2PAs** form stable liposomes in aqueous buffers,[14,16] we sought to identify whether differences in their respective supramolecular properties could have led to the selection of natural **G1PAs**. Microscopy observations showed that **DDG1PA** and **DDG2PA** form liposomes of similar size and lamellarity in phosphate buffer (50 mM, pH 7.5) (Fig. 2a).[14,16] Yet, the biophysical properties of these membranes remain unknown. Previous NMR and neutron diffraction studies on 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (**DPG3PC**) and its isomer 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine (**DPG2PC**) showed that the two phospholipids differ in their gel-to-liquid transition temperature (41.5 °C for **DPG3PC** *vs.* 37.5 °C for **DPG2PC**), probably due to the different conformations of their glycerol backbones (perpendicular and parallel to the membrane surface for **DPG3PC** and **DPG2PC**, respectively).[34] We thus investigated whether natural and non-natural phosphatidic acids also acquire a different spatial organization within their respective bilayer. Accordingly, liposomes prepared from **DDG1PA** and **DDG2PA** labelled with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were studied by fluorescence anisotropy. DPH is an apolar dye that localizes in the hydrophobic region of the lipid bilayer and so fluorescence anisotropy measurements provide information on the freedom of motion of the dye within the membrane, the viscosity of the environment in which the dye is embedded, and hence the fluidity of the membrane.[35] Our data show that **DDG1PA** and **DDG2PA** form liposomes with membranes as fluid as those composed of unsaturated fatty acids, such as myristoleic acid (**MA**) (Fig. 2b), but that **DDG1PA** membranes are more rigid than **DDG2PA** membranes. Moreover, when **DDG1PA** and **DDG2PA** liposomes were labelled with Laurdan, an amphiphilic dye sensitive to lipid packing and order within the bilayer,[36] generalized polarization studies showed that the **DDG1PA** membranes are more ordered and better packed than **DDG2PA** membranes, indicative of stronger hydrophobic interactions between the acyl chains (Fig. 2c and S11†). The fluorescence anisotropy and generalized polarization measurements were made at 25 °C, a temperature expected to be above the gel-to-fluid phase transition temperature of both lipids. These findings confirm that natural and non-natural phosphatidic acids acquire different conformations within their respective bilayers

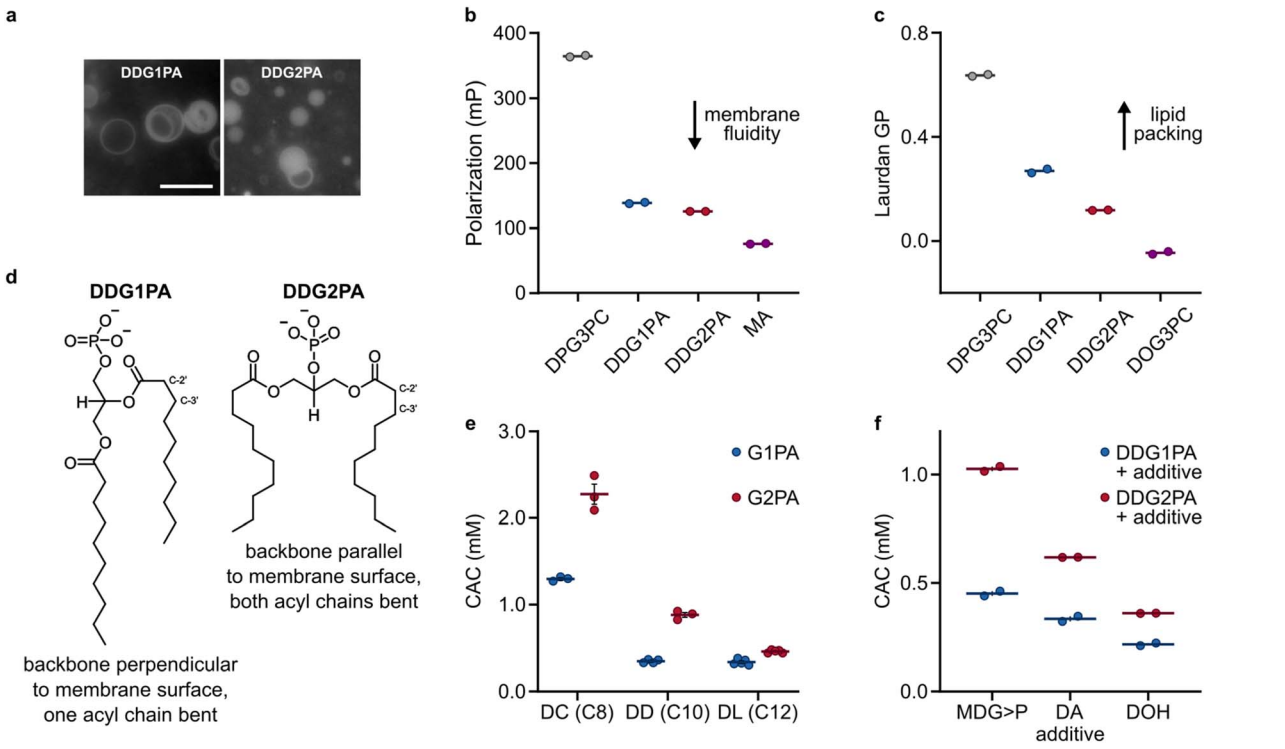


Fig. 2 Self-assembly and supramolecular properties of **G1PAs** and **G2PAs** in pure or mixed systems. (a) Fluorescence microscopy images show that **DDG1PA** and **DDG2PA** (25 mM, containing 1 mol% NBD-PE) form liposomes of similar size and lamellarity. The scale bar represents 10 μm. (b) DPH fluorescence anisotropy (polarization) of **DDG1PA** and **DDG2PA** membranes compared with those of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (**DPG3PC**) and **MA**. Measurements were made at 25 °C. (c) Laurdan generalized polarization (GP) of **DDG1PA** and **DDG2PA** membranes compared with those of **DPG3PC** and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (**DOG3PC**). Measurements were made at 25 °C. (d) Structural and conformational differences between **DDG1PA** and **DDG2PA** in membranes. (e) CACs of **G1PAs** and **G2PAs** of different chain length. (f) CACs of mixtures of **DDG1PA** or **DDG2PA** and other C10-amphiphiles. Data show the mean ± SEM, $n \geq 2$. See the ESI† for details.

(Fig. 2d), as shown previously for long chain phosphatidylcholines.[34]

To determine whether natural phosphatidic acids exhibit a greater propensity to self-assemble compared to their non-natural analogues, we synthesized the C8-lipids, 2,3-dicapryloyl-glycerol-1-phosphatidic acid (**DCG1PA**) and 1,3-dicapryloyl-glycerol-2-phosphatidic acid (**DCG2PA**), and the C12-lipids, 2,3-dilauroyl-glycerol-1-phosphatidic acid (**DLG1PA**) and 1,3-dilauroyl-glycerol-2-phosphatidic acid (**DLG2PA**) (Fig. S12–S17†).[31] With this series of isomeric phospholipids of different chain length in hand, we measured the critical aggregation concentration (CAC) of each species, *i.e.* the minimal concentration of lipid needed to form supramolecular aggregates (*e.g.* liposomes). Liposomes composed of **G1PA** or **G2PA** were prepared by lipid film rehydration in phosphate buffer at pH 7.5, and absorbance spectra were recorded at different lipid concentrations using the solvatochromic probe merocyanine 540, as described previously.[33] Interestingly, we found that without exception the CAC of each natural **G1PA** is lower than that of each non-natural **G2PA** of the same chain length (Fig. 2e). This trend was conserved when **G1PA** and **G2PA** liposomes were prepared in other buffers or pure water at different pH values (Fig. S18†). Furthermore, the CACs of mixtures containing **DDG1PA** and other prebiotic amphiphiles, specifically decanoic acid (**DA**), decanol (**DOH**), and 3-monodecanoyl-glycerol-1,2-cyclic phosphate (**MDG>P**), are consistently lower than those of analogous mixtures containing **DDG2PA** (Fig. 2f). Finally, to see if CACs of natural phospholipids of other types are also lower than those of their non-natural isomers, we measured the CACs of 2,3-didecanoyl-*rac*-glycerol-1-phosphocholine (**DDG1PC**) and 1,3-didecanoyl-glycerol-2-phosphocholine (**DDG2PC**). These lipids were prepared from their respective phosphatidic acid precursors (Fig. S19 and S20†).[37] In line with our observations for phosphatidic acids, we found that **DDG1PC** has a CAC approximately half that of **DDG2PC** (38 ± 5 μm *vs.* 71 ± 2 μm, respectively) (Fig. S21†). Taken together, these results suggest that the tendency of natural phospholipids to self-assemble at lower concentrations than non-natural analogues of the same chain length is conserved across different headgroup types.

Differences in supramolecular properties enable the selection of natural phosphatidic acids in primitive membranes

In a prebiotic scenario where **G1P** and **G2P** were acylated in the same environment, both natural **G1PAs** and non-natural **G2PAs** would have formed and co-assembled into mixed liposomes. The selection of natural phospholipids would therefore have necessitated the loss of the non-natural isomers from liposomes, either by dilution in the aqueous medium or by precipitation, as a result of their different supramolecular properties.

CAC-driven selection. To determine if the different propensities of **G1PAs** and **G2PAs** to self-assemble could have enabled the selection of **G1PAs** over **G2PAs**, we measured the CACs of binary mixtures of **DDG1PA** and **DDG2PA** (Fig. S22†). Our results show that CACs decrease linearly with the proportion of **DDG1PA**, suggesting that **DDG1PA** and **DDG2PA** co-assemble into mixed liposomes with properties intermediate between those made of their individual constituents. Thus, due to the stabilizing effect of **DDG1PA** on **DDG2PA** membranes, these results rule out a selection pathway based solely on the lower CACs of natural *versus* non-natural phosphatidic acids. Nevertheless, the observation that **G1PAs** and **G2PAs** have different spatial organizations within the lipid bilayer (Fig. 2) encouraged us to investigate their behavior when subjected to changes in pH and metal ion concentrations.

pH-driven selection. To test the pH tolerance of the lipids, 5 mM **DDG1PA** or **DDG2PA** were dispersed in 50 mM phosphate buffer, and the presence of supramolecular assemblies was determined at different pH values by dynamic light scattering (DLS) and UV-vis spectrophotometry, using the merocyanine 540 assay. Both lipids form liposomes between pH 2 and 9, but form micelles above pH 10 (Fig. S23†). By contrast, both lipids form liposomes at pH 10 in carbonate buffer (Fig. S10†), further illustrating that different buffers influence the self-assembly of primitive lipids in unpredictable ways.[33] Intrigued by these findings, we looked at the effect of other buffers, namely 50 mM carbonate (pH 10.0), HEPES (pH 7.5) and acetate (pH 4.0), on the stability of **DDG1PA** and **DDG2PA** liposomes. Stable liposomes formed in all of these buffers, but after incubating overnight at room temperature in acetate buffer **DDG2PA** precipitated while **DDG1PA** liposomes remained dispersed (Fig. S24†). Precipitation of **DDG2PA** was also observed on incubating overnight in phosphate buffer or in water and so this behavior does not appear to be buffer-specific. We suggest that the intermolecular hydrogen bonding network between **DDG2PA** headgroups is more easily disrupted by protonation compared to that between **DDG1PA** headgroups. On protonation, the neutralized **DDG2PA** dissociates from the membrane and precipitates. Curiously, when a mixture of **DDG1PA** and **DDG2PA** (1 : 1, 5 mM each) was used to form liposomes, no precipitate was observed. Thus, the stabilizing effect of **DDG1PA** in mixed liposomes, previously inferred from CAC measurements (Fig. 2), probably results from its ability to maintain a hydrogen bonding network with **DDG2PA** headgroups at pH 4.[38,39] Therefore, despite the contrasting pH tolerance of the individual lipids, our observations seemingly rule out a purely pH-driven selection process for natural phospholipids from heterogeneous mixtures.

Metal ion-driven selection. The intolerance of fatty acid liposomes to metals[2,40] and the simultaneous need of Mg^{2+} and Fe^{2+} for non-enzymatic RNA polymerisation[15] and proto-metabolic processes[41,42] have long been considered significant obstacles on the path to early cells.[2] The differential stability of natural **G1PA** and non-natural **G2PA** liposomes towards Mg^{2+} has been previously reported,[14] but no quantitative data are available for mixed liposomes in the presence of divalent cations.

To establish the relative Mg^{2+}-tolerance of the isomeric phosphatidic acids, liposomes made of 5 mM **DDG1PA** or **DDG2PA** were assembled in 50 mM phosphate buffer and titrated with $MgCl_2$. Our data show that **DDG2PA** liposomes are particularly sensitive to Mg^{2+} and precipitation of the lipid was

quantified by ^{31}P NMR (99% loss after addition of 5 mM Mg^{2+}) (Fig. S25†). By contrast, **DDG1PA** liposomes are largely unaffected by Mg^{2+} (16% loss after addition of 5 mM Mg^{2+}) (Fig. S26†). Similar observations were made by epifluorescence microscopy using a higher lipid concentration (25 mM), where precipitation of **DDG2PA** was observed at 7.5 mM $MgCl_2$ (Fig. 3a). The concentration of Mg^{2+} required to induce precipitation is proportional to the concentration of the lipid.

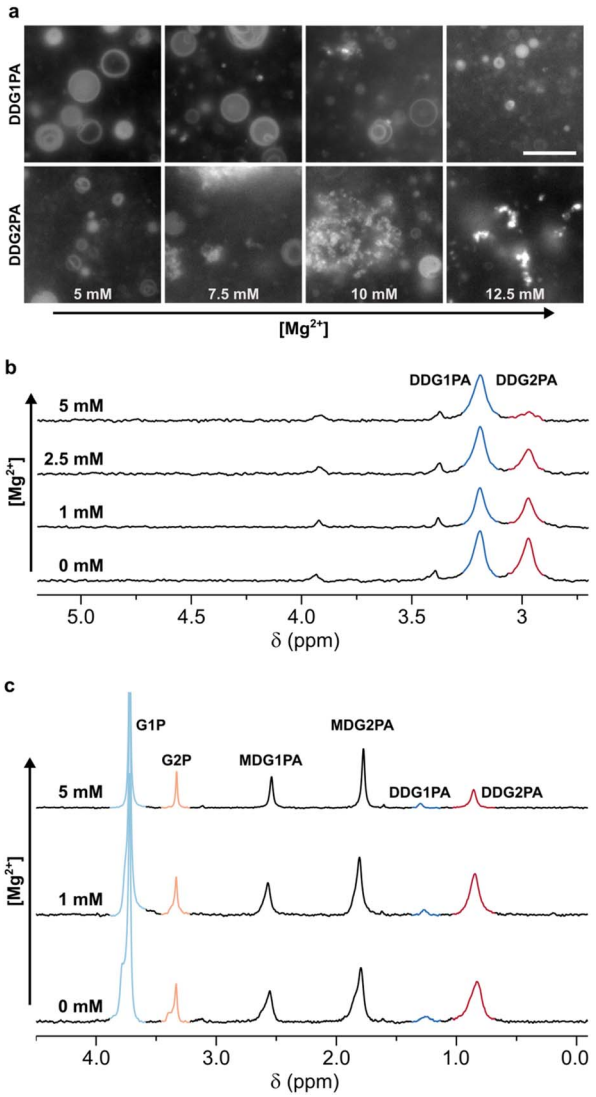


Fig. 3 Mg^{2+}-driven selection of natural **G1PAs** over non-natural **G2PAs**. (a) Microscopy images showing the relative Mg^{2+}-tolerance of **DDG1PA** and **DDG2PA** liposomes (25 mM). The scale bar represents 10 μm. **DDG1PA** liposomes remain intact while **DDG2PA** liposomes disassemble following aggregation and precipitation of the lipid. The concentration of Mg^{2+} required to induce precipitation is proportional to the concentration of the lipid. (b) $^{31}P\{^1H\}$ NMR spectra showing the change in composition of mixed liposomes prepared from **DDG1PA** and **DDG2PA** (1 : 1, 5 mM each) on titration with Mg^{2+}. (c) $^{31}P\{^1H\}$ NMR spectra showing the change in composition of mixed liposomes prepared from **G1PAs** and **G2PAs** obtained by prebiotic synthesis on titration with Mg^{2+}. **DDG2PA** precipitates from mixed liposomes on titration with Mg^{2+}, while **DDG1PA** remains in liposomes. See the ESI† for details.

Analogously, **DDG2PA** liposomes were more susceptible to precipitation by $CaCl_2$ and $ZnCl_2$ than **DDG1PA** liposomes (Fig. S27–S30†). Finally, by comparison, neither lipid was susceptible to precipitation on addition of up to 100 mM NaCl or KCl, as expected for monovalent cations.[43]

To probe the greater tolerance of **DDG1PA** over **DDG2PA** to Mg^{2+}, we measured the change in membrane ζ-potential of liposomes made of **DDG1PA** or **DDG2PA** (5 mM each) on titration with $MgCl_2$. In the absence of Mg^{2+}, the ζ-potentials of **DDG1PA** and **DDG2PA** liposomes were −56 mV and −74 mV, respectively (Fig. S31†). The more negative surface potential of **DDG2PA** suggests that its phosphate group is less hydrated and hence forms weaker hydrogen bonding interactions in the headgroup space of the bilayer compared to **DDG1PA**. Further, it is possible that the phosphate group of **DDG2PA** is more exposed to the bulk medium than that of **DDG1PA**, as might be expected since **DDG2PA** forms less rigid and less well packed membranes than **DDG1PA** (Fig. 2c). On addition of Mg^{2+} to liposomes composed of each lipid, we observed a gradual increase in ζ-potential, demonstrating the electrostatic interactions of Mg^{2+} with the anionic headgroups and its charge shielding effect (Fig. S31†).[43]

Intrigued by the lower tolerance of **DDG2PA** over **DDG1PA** to divalent cations, we tested the stability of mixed liposomes made of **DDG1PA** and **DDG2PA** (1 : 1, 5 mM each, prepared in 50 mM phosphate buffer, pH 7.5) in the presence of 5 mM Mg^{2+}. In the event, the sample remained turbid despite the immediate formation of a precipitate.

To determine the composition of the remaining liposomes, the precipitate was removed by filtration, the liposomes were dissolved by addition of 0.2 M Triton X-100,[44] and the resulting solution was analyzed by ^{31}P NMR (Fig. 3b and S32†). **DDG1PA** was found to be four times more abundant than **DDG2PA** in the filtrate, demonstrating that selective precipitation of the non-natural **DDG2PA** occurred on addition of Mg^{2+}. This finding suggests that a Mg^{2+}-driven selection process could have led to the enrichment of natural phosphatidic acids in mixed liposomes. Moreover, Mg^{2+} appears unique among the metal ions studied as selective precipitation of **DDG2PA** from mixed liposomes could not be effected by titration with Ca^{2+}, Zn^{2+} (Fig. S33 and S34†), Na^+ or K^+ (data not shown).

The mechanism that underpins the Mg^{2+}-induced selection from mixed membranes can be understood in terms of the electrostatic interactions between Mg^{2+} and the anionic surface of the membrane and the differential binding of the isomeric lipids. By analogy with the effect of Ca^{2+} on natural phospholipids,[45] Mg^{2+} binding to phosphatidic acids would liberate water of hydration from the membrane, leading to tighter packing of the lipids and ultimately precipitation. Our data show that **DDG2PA** membranes have a more negative surface charge than those of **DDG1PA** and suggest that the headgroups of **DDG2PA** are less well hydrated and so more prone to disruption on dehydration. It is also possible that the binding affinity of Mg^{2+} for **DDG2PA** is greater than that for **DDG1PA** and the difference in binding affinities is enough to enable the selective precipitation of **DDG2PA** in the presence of **DDG1PA**, in contrast to the other metal ions tested. Whether the selective

precipitation of **DDG2PA** from mixed membranes suggests that phase separation into isomeric lipid domains occurs prior to precipitation remains an intriguing possibility.

Finally, to demonstrate the plausibility of the Mg^{2+}-driven selection process on lipid mixtures obtained by prebiotic synthesis, we prepared a mixture of **G1PAs** and **G2PAs** by acylation of **G1P** and **G2P** with NDI, as described above (Fig. 1). The mixture of phospholipids obtained after 8 days (**MDG1PA**, **MDG2PA**, **DDG1PA**, and **DDG2PA**) was lyophilized, then gently hydrated with water at pH 7.0 to give liposomes. The imidazole released on reaction of NDI served as the buffer. On titration with $MgCl_2$, **DDG2PA** precipitated and the surviving liposomes became proportionally enriched in **DDG1PA** (^{31}P NMR, Fig. 3c and Fig. S35–S37†). Interestingly, the lysophospholipids **MDG1PA** and **MDG2PA** were unaffected by Mg^{2+} up to a concentration of 5 mM, yet these species could undergo further acylation and thus Mg^{2+}-induced precipitation.

Overall, these results highlight the unique role of Mg^{2+} in driving the selection of **G1PAs** from heterogeneous mixtures of phospholipids under conditions compatible with key metal-driven prebiotic processes like non-enzymatic RNA polymerisation.[15]

Conclusion

Due to the heterogeneity of prebiotic building blocks present on early Earth, efficient mechanisms would have been required for the selection of the now canonical biomolecules. In relation to the emergence of phospholipids, recent studies showed that the selection of natural phospholipid headgroups could have resulted from the preferential hydrolysis of their non-natural analogues[13] and that liposomes composed of non-natural phosphatidic acids are poorly tolerant to pH changes and metal ions.[14,16] However, no prior study addressed whether processes operating at the molecular level (prebiotic synthesis) or the supramolecular level (membrane properties) could have driven the selection of natural phospholipids from heterogeneous mixtures available on early Earth. Our work addresses these questions.

First, we show that acylation of mixtures of glycerol phosphates in solution affords non-natural **G2PAs** in higher yields than natural **G1PAs**, but acylation in the dry state provides **G1PAs** and **G2PAs** in similar yields. These isomeric phospholipids are comparably stable to hydrolysis. Second, we highlight that natural **G1PAs** possess a greater propensity to self-assemble and to pack in ordered bilayers, compared to non-natural **G2PAs**, and that they form liposomes of enhanced rigidity and broader tolerance to pH and metal ions. Finally, we demonstrate that Mg^{2+} drives the selective precipitation of **G2PAs** and, thus, the accumulation of **G1PAs** in liposomes composed of mixtures of phospholipids obtained by prebiotic synthesis. The effect of Mg^{2+} is unique among the metal ions tested. Taken together, our results suggest that properties that manifest at the supramolecular level can be harnessed for the selection of natural phospholipids, while processes that operate at the molecular level (synthesis and hydrolysis) probably cannot.

In summary, our work outlines prebiotic synthesis and selection pathways that could have driven the transition towards biological phospholipid membranes on early Earth. The phosphatidic acids focused on here are minor constituents of biological membranes but serve as important biosynthetic intermediates in pathways leading to complex lipids, such as phosphatidylethanolamines and phosphatidylcholines. Since the transformation of **G1PAs** into both of these lipids under early Earth conditions has already been described,[46,47] our study renders these foundational investigations all the more relevant. Importantly, the requirements of Mg^{2+}-driven non-enzymatic RNA replication and the need for Mg^{2+}-stable biological phospholipid liposomes can be harmonized. In fact, Mg^{2+} is essential in driving the selection of metal ion-resistant natural phospholipids. Beyond, the interactions between Mg^{2+} ions and membranes enriched in natural phosphatidic acids could have led to the co-localization of RNA on the membrane and enabled synergistic effects in prebiotic RNA chemistry[48] as well as RNA-driven recognition and fusion of primitive liposomes.[49]

Data availability

Data are available upon reasonable request from the corresponding author.

Author contributions

K. T. M., Y. L., V. J. B.-T. and D. A. R. carried out the experimental work. D. A. R. and C. B. conceived the project, directed the work and wrote the manuscript with input from all the authors.

Conflicts of interest

There are no conflicts to declare.

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