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Phosphorylation, oligomerization and self-assembly in water under potential prebiotic conditions

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Prebiotic phosphorylation of (pre)biological substrates under aqueous conditions is a critical step in the origins of life. Previous investigations have had limited success and/or require unique environments that are incompatible with subsequent generation of the corresponding oligomers or higher-order structures. Here, we demonstrate that diamidophosphate (DAP)—a plausible prebiotic agent produced from trimetaphosphate—efficiently (amido)phosphorylates a wide variety of (pre)biological building blocks (nucleosides/tides, amino acids and lipid precursors) under aqueous (solution/paste) conditions, without the need for a condensing agent. Significantly, higher-order structures (oligonucleotides, peptides and liposomes) are formed under the same phosphorylation reaction conditions. This plausible prebiotic phosphorylation process under similar reaction conditions could enable the systems chemistry of the three classes of (pre)biologically relevant molecules and their oligomers, in a single-pot aqueous environment.

Phosphorylation of biologically relevant molecules under potential prebiotic conditions is an important step in the origins of life and has been investigated (especially for nucleosides) over a wide variety of conditions[1–4] with more recent variations[5,6]. All of these approaches use differing phosphorylation sources and are limited in substrate scope due to their reaction conditions, or require unique non-aqueous environments to enable the phosphorylation process[7,8]. Many of these approaches are therefore incompatible with the next step of generating the corresponding oligomers and higher-order structures (towards RNA or pre-RNA worlds) from these phosphorylated substrates, necessitating spatially separated processes and other discrete mechanisms and chemistries. A universal and efficient phosphorylating agent that would phosphorylate a wide class of (pre)biological molecules in water under similar reaction conditions and enable the formation of higher-order structures would be of significance in the context of prebiotic systems chemistry[9].

We have previously shown that diamidophosphate (DAP, Fig. 1) efficiently phosphorylates a wide variety of prebiotically relevant sugar molecules and their building blocks[10]. A prebiotic synthesis of phosphoenol pyruvate has been demonstrated recently using DAP as the prebiotic phosphorylating reagent[11]. In all cases, the mechanism involves a nucleophilic attack of the NH_2-group of DAP on the free aldehyde moiety to facilitate an intramolecular phosphorylation of the α-hydroxy-aldehydes (Supplementary Fig. 1). Such a mechanism precludes the phosphorylation of hydroxyl groups, which are not next to a carbonyl moiety (for example, nucleosides and glycerol). However, when we observed that DAP alone in water at pH 6, or in the presence of ortho- or pyro-phosphates, produced trimetaphosphate (Supplementary Figs 2–5), this suggested that direct phosphorylation of other nucleophilic groups in aqueous media should be possible by a direct attack on the phosphorous centre, with the protonated NH_2-group of the DAP acting as the leaving group[12].

Results

Phosphorylation of nucleosides/tides and oligonucleotides by DAP. We began by investigating the phosphorylation of nucleosides with DAP. Reacting 0.1 M uridine **1** with DAP in aqueous solution, under a range of conditions (1–25 equiv. DAP, pH 5.5–10, r.t.–50 °C, with and without Zn^{2+} or Mg^{2+} or imidazole and slowly over days to weeks, Supplementary Table 1), we observed the formation of uridine-2′,3′-cyclophosphate (**5**, c-UMP, Fig. 1a) and traces of the 5′-amidophosphate of **5**. While Zn^{2+} or Mg^{2+} accelerated the reaction, Zn^{2+} also lowered the pH, leading to the hydrolysis[13]/condensation of DAP, necessitating the continuous addition of DAP. The addition of imidazole (conjugate acid, $pK_a = 7.05$) circumvented this problem at pH 7, affording similar conversion while decelerating the hydrolysis/condensation of DAP. Cytidine **2** was converted to 2′,3′-c-CMP (**6**, 27%) under similar conditions, while purines **3** and **4** reacted equally well, forming 2′,3′-c-AMP (**7**, 31%) and 2′,3′-c-GMP (**8**, 27%), respectively. Further addition of DAP, over days/weeks, facilitated ongoing conversions to the respective c-NMPs.

Dry/solid-state and low water-activity prebiotic phosphorylation reactions have been widely investigated[4,6,14–16]. Mixing DAP, imidazole and **1** in the solid state at room temperature with drops of water led to a paste-like material ('paste reaction' conditions) and efficiently produced **5** (~80%, 30 days, Supplementary Table 1). A preparative experiment on a 1 g scale afforded **5** in 65% isolated yield (Supplementary Figs 18–21). To our delight, oligouridylate (up to the tetramer, Fig. 1b) with terminal 2′,3′-cyclophosphate was also observed, indicating that oligomerization was taking place under the same mild phosphorylation conditions. Cytidine **2** was cyclo-phosphorylated under similar conditions (42%, 10 days). However, phosphorylation of purine-nucleosides **3** (<5%) and **4** (17%) was less efficient. Further investigations are under way to optimize the efficacy of the paste reactions and the oligomerization process and to

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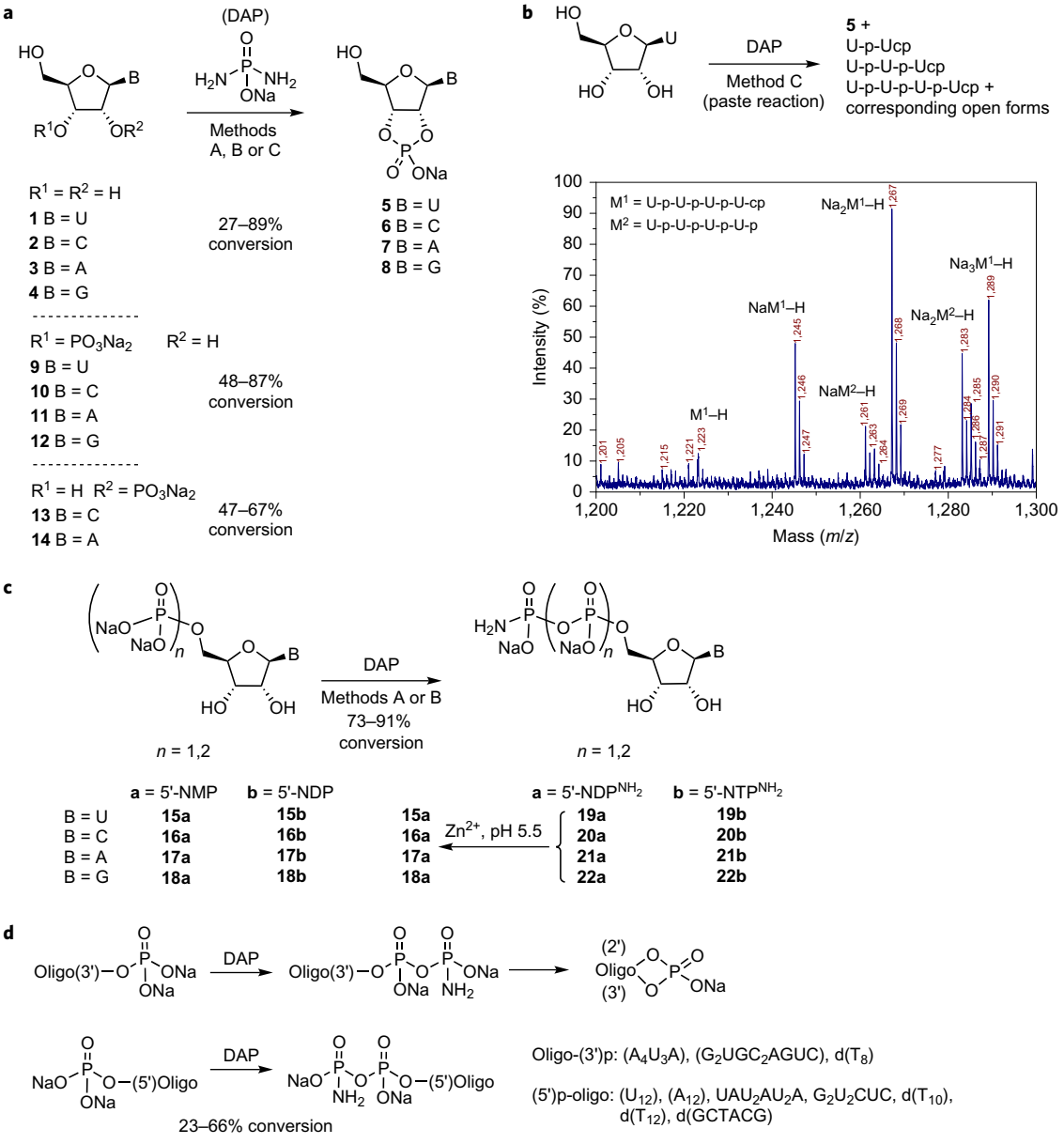

Figure 1 | DAP-mediated phosphorylation of nucleosides/tides demonstrating the potential to generate and progress through the successive levels of nucleotides and oligonucleotides under similar conditions. a–d, Phosphorylation of nucleosides and 2′- and 3′-mononucleotides to give cyclophosphates (**a**), uridine under paste-reaction conditions, also forming oligonucleotides (**b**), 5′-nucleotides (**c**) and 5′- and 3′-phosphorylated oligonucleotides (**d**) by diamidophosphate (DAP). The reactions were carried out by method A (aqueous, pH 5.5), method B (aqueous, pH ≈ 7) with or without Mg^{2+} or Zn^{2+} or imidazole, or by method C (paste reaction). For details see Supplementary Information and Supplementary Tables 1–11.

analyse the nature of the phosphodiester linkages (2′,5′ vs 3′,5′) of oligomers.

The phosphorylation proceeds through nucleophilic attack of the 2′,3′-*cis*-hydroxyl groups on the protonated DAP, leading to a putative monophosphoramidate (2′-NMP^{NH_2} or 3′- NMP^{NH_2}) intermediate, which then cyclizes to give the 2′,3′-cyclophosphate derivatives **5**–**8**. When imidazole is present, the protonated DAP is converted to the amidophosphoimidazolide (Supplementary Fig. 7), which reacts further. Support for these intermediates was obtained from ^{31}P-NMR using ^{15}N-labelled reactants (Supplementary Figs 7–13). This phosphorylation chemistry, in principle, can be extended to other alternative (prebiotically plausible) nucleosides that have the adjacent *cis*-hydroxyl configuration[17] (a work that is ongoing).

The hydrolysis of nucleoside-2′,3′-cyclophosphates to the 2′/3′-phosphates, and reactivation to regenerate 2′,3′-cyclophosphates, is of importance in the prebiotic formation of oligonucleotides[18]. The reaction of 3′-nucleoside monophosphates (3′-NMPs, **9**–**12**) and 2′-monophosphates (2′-CMP and 2′-AMP, **13**–**14**) with DAP in water formed the corresponding 2′,3′-cyclophosphates efficiently under various conditions (Fig. 1a). The corresponding 3′- and 2′-amidodiphosphate derivatives are observed as intermediates, on the pathway to c-NMPs formation.

The 5′-nucleoside monophosphates (5′-NMPs, **15a**–**18a**) were amidophosphorylated in water at pH 7 by DAP (Fig. 1c and Supplementary Tables 7 and 8), with 77–90% conversions to the corresponding 5′-nucleoside-amidodiphosphates (5′-NDP^{NH_2}, **19a**–**22a**) within 5 days. The 5′-nucleoside diphosphates (5′-NDPs, **15b**–**18b**) were similarly efficiently converted to the nucleoside-amidotriphosphates (5′-NTP^{NH_2}, **19b**–**22b**; Fig. 1c and Supplementary Table 9), with up to 10% of the corresponding

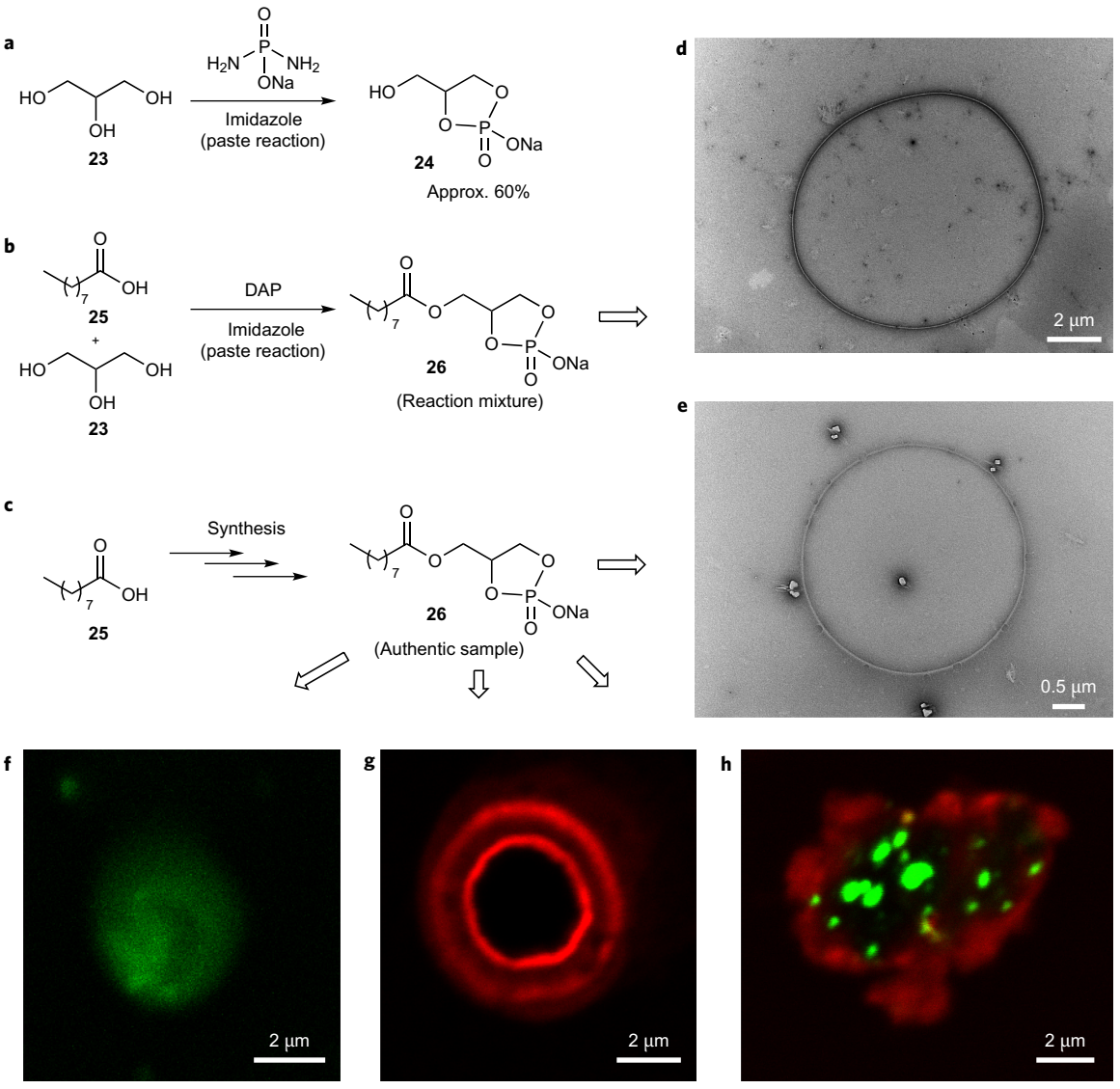

Figure 2 | DAP-induced phosphorylation and esterification of glycerol with short-chain fatty acids give rise to simple mimics of phospholipids, leading to formation of protocell-like structures under the same reaction conditions, signifying the single-pot transition of simple building blocks to higher-order self-assemblies. a,**b**, Phosphorylation reaction of DAP with glycerol (**a**) and with nonanoic acid and glycerol (**b**) under paste-reaction conditions. **c**, Synthesis of authentic cyclophospholipid **26**. **d**, TEM image of a sample (15 mg in 1 ml water) from the crude reaction in **b**, showing the formation of vesicle-like structures with a diameter of ~9.2 μm. **e**, TEM image of a sample (1 mg in 1 ml water) of authentic phospholipid **26** from Fig. 2c showing the formation of vesicle-like structures with a diameter of ~3.5 μm. **f**–**h**, Confocal laser scanning microscopy fluorescence images of vesicles prepared with authentic phospholipid **26** (1 mg in 0.1 ml water) with dye encapsulation. In **f**, green fluorescence indicates hydrophilic pyranine dye encapsulated within the cavity of the liposome. In **g**, red fluorescence indicates rhodamine B dye labelling the bilayer phospholipid membrane of the liposome. In **h**, a fluorescence merged image is shown of a phospholipid vesicle prepared with both rhodamine B dye and pyranine dye. For details see Supplementary Information.

2′,3′-cyclophosphates. At pH 5.5 with Zn^{2+}, the 5′-NDP^{NH_2} (**19a**–**22a**) hydrolyse back to the starting 5′-NMP (**15a**–**18a**).

This 'phosphorylation followed by hydrolysis' raises the possibility of using DAP as a (re)activating agent for 5′-, 2′- and 3′-nucleoside phosphates in water for ligation/oligomerization reactions, providing an alternative to the current methods of activation in prebiotic oligomerization and replication studies[19]. Phosphorylations of selected oligonucleotides with 5′-phosphate and 3′-phosphate terminal ends were briefly investigated and found to produce the respective oligo-5′-DP^{NH_2} and the corresponding oligo-2′,3′-cyclophosphate (via the oligo-3′-DP^{NH_2}), paralleling observations made for mononucleotides (Fig. 1d and Supplementary Tables 10 and 11). Investigations probing the scope of DAP for template-mediated ligation/oligomerization are ongoing.

Phosphorylation of glycerol and fatty acids by DAP leading to (self-assembly) vesicles. Because *cis*-disposed 2′,3′-hydroxyl groups of nucleosides are converted to cyclophosphates, glycerol **23** was reacted with DAP with the purpose of accessing the building blocks of phospholipids (Fig. 2a). Mixing **23** with DAP and imidazole in water at r.t. led to the formation of glycerol-1,2-cyclophosphate **24** (~12%), with the paste reaction demonstrating efficient conversion (~60%) via the amidophosphorylated intermediate (**24i**; Supplementary Table 12). Heating the paste reaction (50 °C) accelerated the reaction, but also led to the formation of the diglycerol- phosphodiester, indicating that the cyclophosphate is attacked by another molecule of glycerol. Based on studies by Deamer[20], we probed the ability for DAP to mediate ester bond formation by reacting nonanol with ammonium acetate under paste-reaction conditions and found that DAP increased the

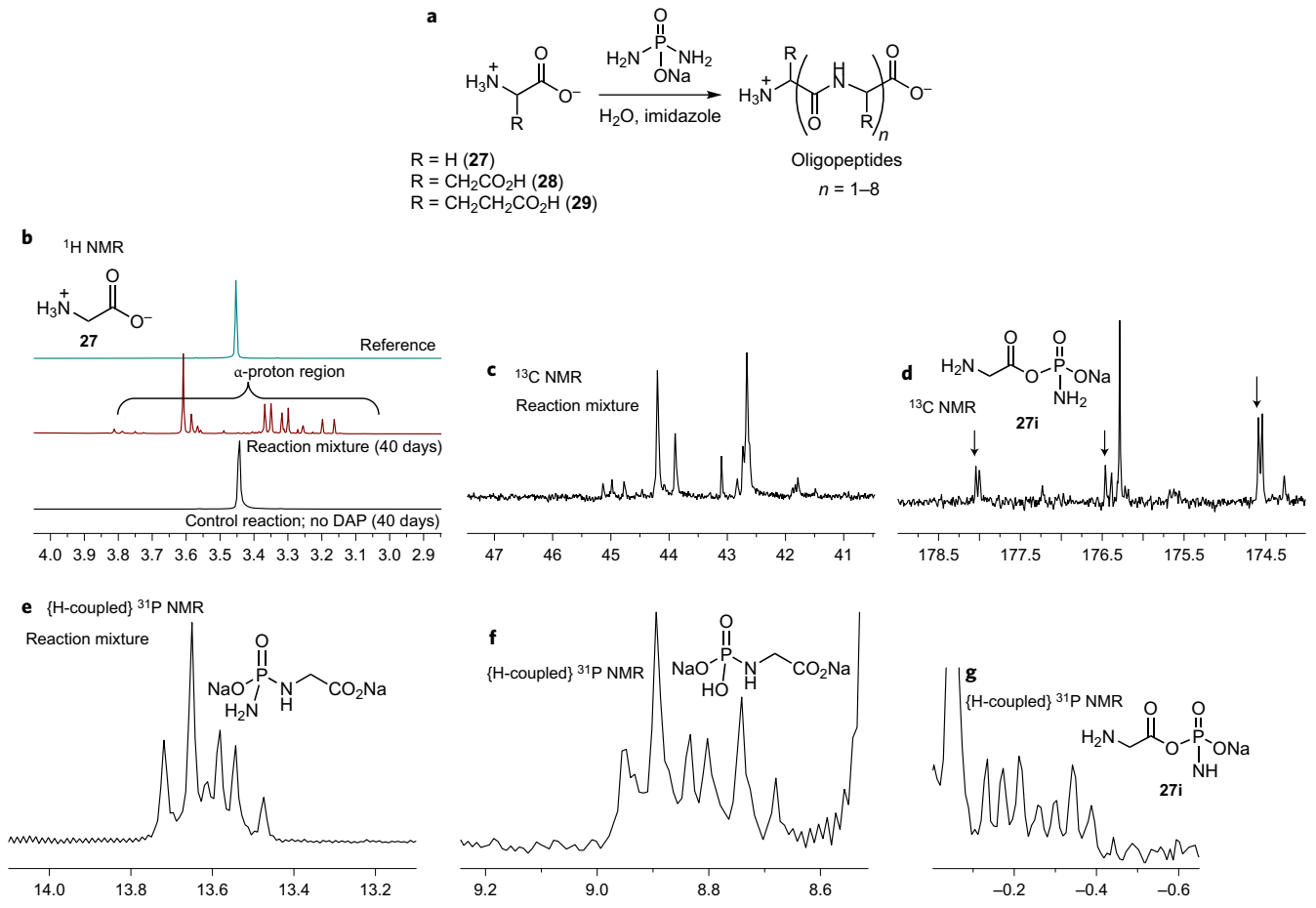

Figure 3 | DAP phosphorylates amino acids in water while activating them towards the formation of oligopeptides in the same reaction setting. **a**, Reaction of DAP with glycine, aspartate or glutamate leading to oligopeptides. **b**–**g**, Reaction of glycine with DAP leading to (quantitatively) phosphorylated species that are intermediates on the way to oligopeptide formation, as seen by ^{1}H-NMR (**b**), ^{13}C-NMR (**c**,**d**) and ^{1}H-coupled-^{31}P-NMR (**e**–**g**) of the reaction mixture. For conditions and details see Supplementary Information.

efficiency of ester bond formation when compared to the control (Supplementary Fig. 117 and Supplementary Table 13). Encouraged by these observations, we performed a one-pot paste reaction with nonanoic acid **25** (ref. 20) and glycerol in the presence of DAP and imidazole (Fig. 2b). Analysis of the crude reaction mixture suggested the formation of a cyclophospholipid **26**, the structure of which was confirmed by comparison with synthesized authentic **26** (Fig. 2c and Supplementary Figs 118–123). Such cyclophospholipid structures (for example, cyclophosphatidic acid) are known in extant biology, but in a diagnostic and pharmacological context[21].

Mixing a portion of this crude paste reaction containing **26** in water (pH of ~8.5) resulted in an opaque solution, which became clear upon sonication. This was filtered (0.2 μm) and analysed by dynamic light scattering (DLS) and transmission electron microscopy (TEM) techniques, revealing the formation of micelles and vesicle-like bilayer and multilamellar structures in the range of 30–110 nm (Supplementary Figs 124 and 137). TEM analysis of the opaque solution itself (without sonication and filtration) displayed liposome-like structures with diameters around 280 nm (Supplementary Fig. 125). In addition, we were surprised to also find TEM images implying the formation of giant bilayer vesicle-like structures with diameters of 9.2 μm on average (Fig. 2d), with some structures nearing 20 μm (Supplementary Fig. 126). The control reaction lacking DAP, but containing only nonanoic acid and glycerol (Supplementary Fig. 133), or each of the components alone (for example, nonanoic acid alone), did not show the formation of these 'vesicle-like' structures (Supplementary Figs 132–135). The opaque solution generated from the synthetic cyclophospholipid **26** in water was analysed by TEM, which showed 'vesicle-like' structures (Fig. 2e) comparable to those obtained from the crude paste-reaction mixture. This demonstrates that cyclophospholipid **26**, generated in the crude paste-reaction mixture, is indeed capable of forming these vesicle-like structures, perhaps even better in heterogeneous mixtures with glycerol[20]. To prove that the structures are indeed liposomes, we performed dye-inclusion experiments[20], and confocal microscopy images showed that the red rhodamine dye was retained within the bilayer, while the green pyranine dye was encapsulated within the cavity of the liposome (Fig. 2g,f). Preparing the liposomes with both dyes afforded a merged-image suggesting an 'uneven-enclosed' structure (Fig. 2h), probably arising from changes in internal osmotic pressure[22] and interlayer distances[23] when both dyes are present. Preliminary reactions of octanoic acid with DAP under the paste-reaction conditions gave rise to smaller vesicle-like structures (Supplementary Fig. 131). That a library of prebiotically plausible fatty acids, available through meteoritic delivery[24] or other prebiotic means[20], could form stable phospholipid liposomes such as giant vesicles by phosphorylation in the presence of glycerol, while unprecedented, does align with the advantage provided by the physical and chemical heterogeneity in vesicles in the context of protocells[25].

Phosphorylation of amino acids by DAP leading to oligopeptides. Motivated by these observations we briefly explored—as a proof of principle—the phosphorylation of carboxylic acids of three

representative prebiotic amino acids **27**–**29** (Fig. 3a) under aqueous conditions[3]. When glycine **27** was reacted with DAP in water, quantitative phosphorylation of the α-amino and α-carboxyl groups was observed (Fig. 3b–g and Supplementary Figs 138–140). Concomitant formation of peptides up to an octamer was observed by mass spectral analysis (Supplementary Figs 141–143). Controls lacking DAP did not show any oligo-glycine formation (Fig. 3b). The acyl-phosphoramidate of glycine **27i** observed by NMR (Fig. 3d,g) is proposed to be the active intermediate[26] that leads to amide bond formation. There may be other possible mechanistic pathways[27,28], the establishment of which need further detailed investigations. Diketopiperazine (DKP) was also detected. With Gly-Gly **30**, the formation of oligomers up to an octamer was observed by mass spectrometry (Supplementary Fig. 144). The reaction of DAP with aspartic acid **28** and glutamic acid **29** (Fig. 3a) also showed the formation of oligopeptides up to tetramers (Supplementary Figs 145 and 147). In the case of **28**, ^{1}H-NMR shows ~23% conversion to higher-order products with minimal DKP (Supplementary Fig. 146), while for **29**, it was ~15% conversion to higher-order products (DKP + peptides, Supplementary Fig. 148). Accurate determination of oligopeptide yields (currently complicated by interference from the various phosphorylated species), optimization of reaction conditions for increasing oligopeptides yields, the nature of mechanistic pathways and elucidation of the nature of connectivity (*α*- versus *β*-) are some of the issues that need to be addressed and are underway. The promising preliminary results of oligopeptide formation in water warrant in-depth and systematic investigations to determine the scope and selectivity of the oligopeptide-forming process and to address the issues associated with random peptide synthesis such as the rapid increase in diversity of even short peptide sequences[29].

Discussion

The similar phosphorylation conditions for all three classes of prebiological molecules (nucleosides, fatty acids and glycerol and amino acids) suggest that they could be combined and conducted in a single pot. Moreover, the commonality of conditions for the oligomerization of different building blocks suggests that productive and mixed chemistries might be possible[30,31], such as cross-catalysing the oligomerization and self-assembly process, and the resulting higher-order structures could, in turn, increase the efficiency of phosphorylation. This approach could create opportunities that go beyond the generation of phosphorylated building blocks, leading to the co-formation and coexistence of building blocks and their oligomers in the same locality, which would be conducive for the emergence of primordial synergistic systems within confined (aqueous) environments towards an RNA or pre-RNA world(s). Although there are similarities with extant biochemical pathways using such P–N activation chemistries[32] (such as *N*-phosphoryl transfers[27,28]), any comparison must be viewed with caution given the pitfalls of extrapolating extant biochemical pathways backwards all the way to prebiotic chemistry and vice versa[33].

It has been conjectured that combining the nitrogenous and oxygenous versions of prebiotic molecules (as in TNA and its nitrogenous versions[34]) could provide a library of alternative opportunities[32]. The results from this work and previous DAP-phosphorylation investigations[10,11,35] demonstrate that such an expansion of the phosphorylation scenario to include the prebiotically plausible nitrogenous version of phosphate (such as DAP) provides an efficient and alternative solution to the prebiotic-phosphorylation problem. Although DAP has been used as a prebiotically plausible reagent[11,35], it has been produced from the prebiotically available trimetaphosphate[36] by reaction with ammonia[37,38], at relatively high pH. Because the results demonstrated in this work proceed at lower pH, an investigation of additional, prebiotically plausible and alternative pathways at milder pH, such as corrosion of phosphide minerals (schreibersite)[39,40], towards the formation of DAP would be desirable. In that context, the detection of PN-containing compounds in interstellar media[41,42] and star-forming regions[43] suggests that nitrogenous versions (or forerunners) of phosphate may not be unreasonable to contemplate[32,44]. The phosphorylation of small molecules by DAP and its analogues may also prove to be useful in synthetic chemistry[32].

Methods

Full details are provided in the Supplementary Information.

General phosphorylation protocols

DAP in water. DAP, with or without imidazole (and/or zinc chloride and/or magnesium chloride), was added to the substrates (nucleosides, nucleotides, oligonucleotides, glycerol, nonanoic acid or amino acids) in water. The pH was adjusted and maintained between 5.5 and 8 by the addition of 4 M HCl (aq) and agitated at room temperature. Additional DAP was added based on the progress of the reaction and its consumption, as monitored by NMR analyses.

DAP in the 'paste reaction'. The substrates (nucleosides, nucleotides, oligonucleotides and glycerol) and DAP, with or without imidazole, were ground together with a few drops of water. Additional DAP was added based on the progress of the reaction and its consumption as monitored by NMR analyses.

Phosphorylation reaction analyses. The progress of the reactions was monitored by the following techniques: ^{1}H, ^{13}C and ^{31}P NMR spectroscopy (nucleosides, nucleotides, glycerol, fatty acids and amino acids), fast protein liquid chromatography (FPLC; oligonucleotides), liquid chromatography–mass spectrometry (LC–MS; glycerol and amino acids) and matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF-MS; oligouridylate, oligonucleotides and amino acids).

Vesicle preparation. Fatty acid (1 equiv.), glycerol (1 equiv.), DAP (5 equiv.) and imidazole (5 equiv.) were mixed together with a few drops of water and left at room temperature. An aliquot of this crude reaction mixture was mixed with water, vortexed (with and without sonication and filtration) to allow for the formation of micelles/vesicles.

Vesicle characterization. The structures obtained from the vesicle preparation described above (and also from synthetic phospholipid **26**) were characterized by dynamic light scattering and visualized by TEM with negative staining and by confocal laser scanning microscopy with dye incorporation.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

R.K. conceived the project. R.K., C.G., S.B., M.K. and E.-K.K. designed the experiments. C.G. and S.B. performed the nucleoside/nucleotide/oligonucleotide phosphorylation experiments. M.K., S.B. and C.G. performed the amino acid phosphorylation experiments. M.K. performed the liposome studies. E.-K.K. and S.B. made the initial observations of DAP-mediated phosphorylation. R.K. wrote the paper with input from C.G., S.B., M.K. and E.-K.K. All authors discussed the results and commented on the manuscript. C.G., S.B. and M.K. contributed equally to this work.

Additional information

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Competing financial interests

The authors declare no competing financial interests.