

# Fatty Acid/Phospholipid Blended Membranes: A Potential Intermediate State in Protocellular Evolution

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Prior to the evolution of membrane proteins, intrinsic membrane stability and permeability to polar solutes are essential features of a primitive cell membrane. These features are difficult to achieve simultaneously in model protocells made of either pure fatty acid or phospholipid membranes, raising the intriguing question of how the transition from fatty acid to phospholipid membranes might have occurred while continuously supporting encapsulated reactions required for genomic replication. Here, the properties of a blended membrane system composed of both oleic acid (OA), a monoacyl fatty acid, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), a diacyl phospholipid are described. This hybrid vesicle system exhibits high stability to divalent cations ( $Mg^{2+}$ ), while simultaneously maintaining its permeability to small charged molecules such as nucleotides and divalent ions such as  $Mg^{2+}$ . This combination of features facilitates key reactions expected to occur during a transition from primitive to modern cells, including nonenzymatic RNA replication, and is also compatible with highly evolved functions such as the ribosomal translation of a protein. The observations support the hypothesis that the early transition from fatty acid to phospholipid membranes could be accomplished through intermediate states in which membranes are composed of amphiphile mixtures, and do not require protein transporters.

## 1. Introduction

Understanding how membranes and genetic polymers could have coexisted and coevolved is critical to understanding the origins of primitive cells. Catalytic activities that would enable

RNA replication are fundamental to the emergence of Darwinian evolution. Yet, membrane stability and membrane permeability, two features that allow such reactions to occur within vesicles, are often difficult to achieve simultaneously. Monoacyl lipids confer essential properties to protocell membranes, including high permeability to ions and small charged molecules, as well as the dynamic exchange properties that enable multiple pathways for vesicle growth.<sup>[1,2]</sup> These characteristics make fatty acid membranes a promising candidate for the first primitive cells. On the other hand, the nonenzymatic replication of RNA, and the development of catalytic structures from RNA and peptides often benefit from chemical or physical features such as high concentrations of divalent cations or peptide sequences that interact with fatty acids, which can disrupt fatty acid membranes.<sup>[3,4]</sup>

How might a protocellular system evolve in response to strong selective pressures for enhanced membrane stability alongside membrane permeability? One

route to imparting stability to single chain lipid membranes is to improve fatty acid retention in membranes via high affinity interactions with other membrane amphiphiles. Mixtures of monoacyl amphiphiles, containing varied fatty acid chain lengths or head groups, have been shown to stabilize vesicles against certain environmental conditions like high salt,<sup>[4]</sup> pH,<sup>[5]</sup> temperature,<sup>[6]</sup> and low levels of divalent cations.<sup>[3,4]</sup> For example, mixtures of myristoleic acid and its glycerol monoester result in vesicles that can withstand concentrations of up to  $\approx 4 \times 10^{-3}$  M  $Mg^{2+}$  versus only  $\approx 1 \times 10^{-3}$  M  $Mg^{2+}$  for myristoleic acid alone.<sup>[3]</sup> However, RNA chemistry typically requires much higher levels of  $Mg^{2+}$ , on the order of  $50\text{--}100 \times 10^{-3}$  M, suggesting that it would be useful to explore the properties of membranes composed of mixtures of mono- and di-acyl lipids. The interactions of oleic acid with POPC membranes have been previously explored, primarily in the context of vesicle growth and stability,<sup>[7–9]</sup> but little is known of the permeability properties of such membranes, or their stability in the presence of divalent cations. The transition of primitive cells to modern ones is likely to have involved a shift in membrane composition from single chain lipids to phospholipids, making the latter lipid a natural starting point when investigating membrane stabilizing mechanisms. The presence of phospholipids can provide selective advantages to bilayer membranes, including promoting membrane growth. We previously showed that

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increasing phospholipid content in fatty acid vesicles reduced fatty acid desorption and led to the competitive growth of such vesicles over vesicles with less phospholipid.<sup>[1]</sup> In addition, phospholipid membranes are stable in the presence of proteins that are essential for modern cells but can bind and be inhibited by fatty acids.<sup>[10]</sup> However, pure phospholipid membranes are highly impermeable to divalent cations and small polar solutes. We wondered if intermediate states characterized by mixtures of the mono- and di-acyl amphiphiles might retain the advantages of both membrane compositions.

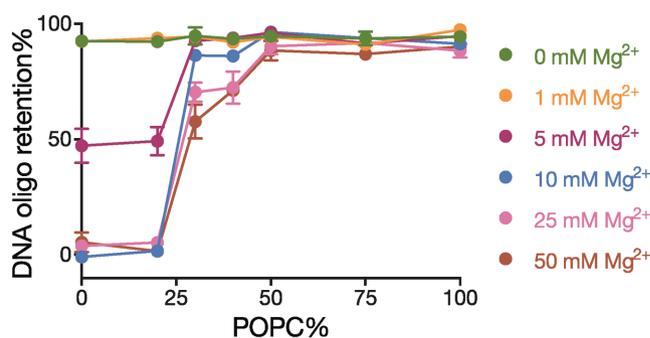
Here, we investigate whether a hybrid fatty acid/phospholipid system can address the need for both membrane stability and solute permeability in a model protocell system. Our observations expand upon previous results that showed increasing phospholipid content decreases membrane permeability.<sup>[1]</sup> Here, we show that phospholipids stabilize fatty acid vesicles to divalent cations while maintaining sufficient permeability to allow for the entry of essential solutes. We also show that the enhanced retention of fatty acids within membranes containing phospholipids allows a modern polymerase reaction, normally inhibited by fatty acids, to occur. The ability of a hybrid system to retain encapsulated polymers, permit solute entry, and retain membrane amphiphiles, points to a possible pathway by which primitive fatty acid based cell membranes would have been able to transition to a mixed fatty acid–phospholipid composition, while maintaining critical metabolic and replication reactions in the protocell interior.

## 2. Results

### 2.1. Stability and Permeability of Hybrid Vesicles in the Presence of $Mg^{2+}$

#### 2.1.1. Stability of Blended Membranes in the Presence $Mg^{2+}$

We began by asking whether phospholipids could enhance the stability of fatty acid based protocell membranes in the presence of  $Mg^{2+}$ . This is an important question because high concentrations of  $Mg^{2+}$  are necessary for both nonenzymatic and enzymatic RNA catalysis, but are known to disrupt fatty acid membranes. We prepared oleic acid (OA, C18:1) vesicles that contained increasing fractions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC, C16:0–18:1). To test the stability of vesicles as a function of their phospholipid content, we measured the release of a DNA oligomer (a 5'-Cy5- labeled 30mer) from vesicles at a total lipid concentration of  $7.5 \times 10^{-3}$  M, after a 12 h incubation with different concentrations of  $MgCl_2$ . The retention of DNA within the vesicles varied as a function of both magnesium concentration and the phospholipid content in the membrane (Figure 1). Pure fatty acid vesicles (100% OA) fully retained the DNA oligomer in  $\leq 1 \times 10^{-3}$  M  $Mg^{2+}$ , but in the presence of  $>5 \times 10^{-3}$  M  $Mg^{2+}$  the vesicles were unstable and released almost all of the encapsulated DNA. In contrast, vesicles that contained 50% or more phospholipid in their membranes retained more than 95% of the DNA oligomer over the entire range of  $Mg^{2+}$  concentrations tested ( $1$ – $50 \times 10^{-3}$  M). Vesicles with 20–40% of POPC in their membranes showed



**Figure 1.** Phospholipids stabilize fatty acid vesicles. Retention of a 30-mer DNA oligomer inside OA/POPC small unilamellar vesicles was monitored after an overnight incubation with different concentrations of  $Mg^{2+}$  in order to determine vesicle stability.  $n = 3$ ; error bars represent standard deviation (s.d.).

intermediate behavior, releasing progressively more encapsulated DNA at higher  $Mg^{2+}$  concentrations.

Similarly, we measured the retention of calcein, a small highly charged fluorescent molecule, after mixing purified vesicles containing encapsulated dye with various concentrations of  $Mg^{2+}$  (Figure S1, Supporting Information). In this case, we measured dye retention immediately after mixing, to distinguish between vesicle disruption and slow loss by permeation through the membrane. Pure OA vesicles and vesicles containing 10% POPC lost almost all of the encapsulated dye at  $Mg^{2+}$  concentrations of  $5 \times 10^{-3}$  M or above, while vesicles containing 50% or more POPC retained essentially all calcein over the range of tested  $Mg^{2+}$  concentrations.

#### 2.1.2. Permeability of Blended Membranes in the Presence of $Mg^{2+}$

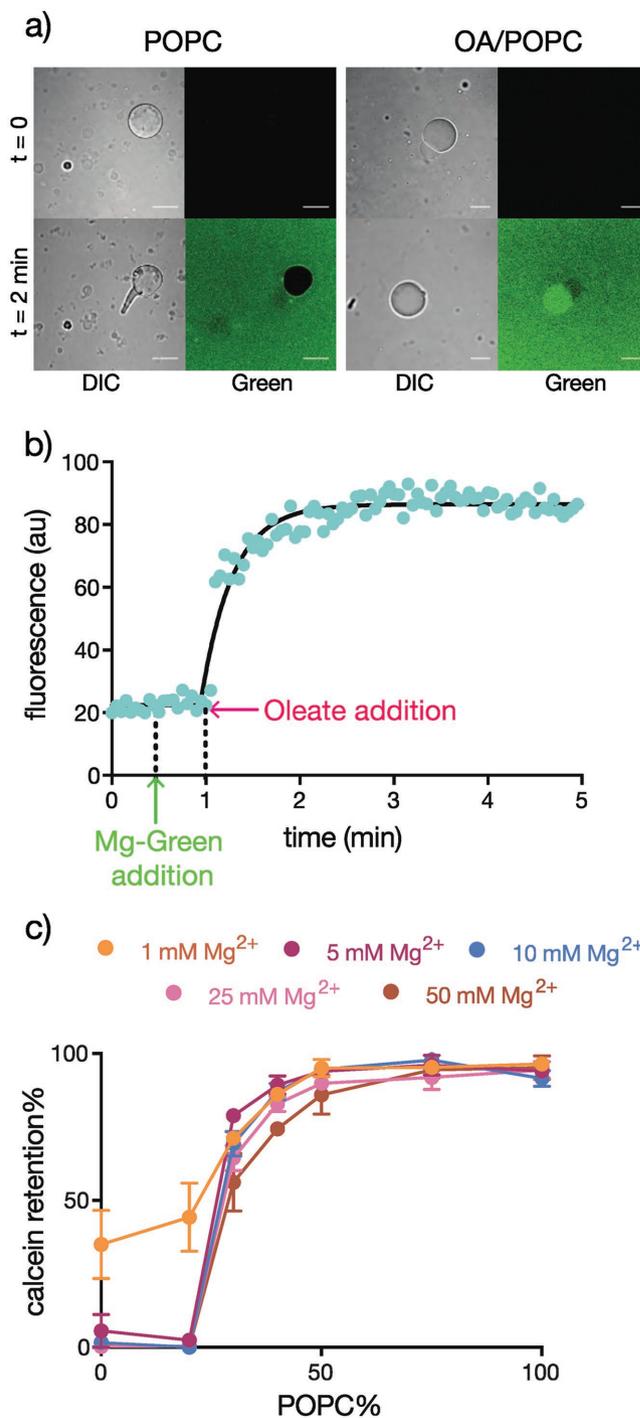
We next examined the permeability of mixed fatty acid–phospholipid membranes to various solutes in the presence of  $Mg^{2+}$ . While membranes composed entirely of single chain amphiphiles are highly permeable to ions and small molecules,<sup>[11]</sup> most pure phospholipid membranes are not. Thus, if primordial protocell membranes were composed of fatty acids and related single chain amphiphiles, the protocell would have access to ions and polar nutrients from the external environment. However, a gradual increase in phospholipid content during the evolutionary transition to more modern membrane compositions would likely cause a problem as decreasing permeability led to decreased access to ions and nutrients. Indeed, we have previously shown that increasing phospholipid content leads to a gradually decreasing permeability to the sugar ribose.<sup>[1]</sup> However, the permeability of mixed fatty acid–phospholipid membranes to essential ions such as  $Mg^{2+}$ , and nutrients such as nucleotides, has not been systematically examined. Here, we examine the effect of increasing phospholipid content on the spontaneous transmembrane transport of  $Mg^{2+}$  and activated ribonucleotides, which are thought to be crucial to prebiotic RNA chemistry.

*Permeability of Blended Membranes to  $Mg^{2+}$ :* For an initial qualitative investigation of the permeability of blended fatty acid/phospholipid membranes to magnesium, we employed Magnesium Green, a cell-impermeable dye that fluoresces in the

presence of  $Mg^{2+}$  ( $K_d \approx 1 \times 10^{-3}$  M). Having determined that a phospholipid content of 50% is sufficient to confer membrane stability to  $Mg^{2+}$ , we prepared giant unilamellar vesicles (GUVs) from either pure POPC (100%) or 50% OA:50% POPC blended lipids in buffer that contained  $2 \times 10^{-6}$  M Magnesium Green. The vesicles were diluted into an isotonic solution (see the Supporting Information for details) that also contained  $2 \times 10^{-6}$  M Magnesium Green. We then added  $25 \times 10^{-3}$  M  $MgCl_2$  to the external solution and monitored the permeation of  $Mg^{2+}$  across the membrane by visualizing the resulting change in internal Magnesium Green fluorescence. While the interior of pure POPC vesicles remained dark (nonfluorescent) over a 10 min observation period, hybrid vesicles containing 50% OA allowed  $Mg^{2+}$  equilibration across the membrane, leading to a rapid and strong increase in fluorescence in the vesicle interior (Figure 2a).

In order to obtain more quantitative data on the permeability of  $Mg^{2+}$  to mixed phospholipid–fatty acid membranes, we used a fluorometer to monitor the efflux of  $Mg^{2+}$  in real time from purified vesicles containing encapsulated  $Mg^{2+}$ . Because pure POPC vesicles are impermeable to  $Mg^{2+}$ , we first prepared monodisperse, unilamellar 100 nm 100% POPC vesicles containing  $50 \times 10^{-3}$  M  $Mg^{2+}$ , and purified these vesicles to remove unencapsulated  $Mg^{2+}$  (Materials and Methods, Supporting Information). We then added Magnesium Green to the outside of the vesicles and monitored  $Mg^{2+}$  efflux through the vesicle membranes. In the absence of any fatty acid, pure POPC vesicles remained impermeable to  $Mg^{2+}$  (Figure 2b, and Figure S2, Supporting Information). In order to monitor the effect of fatty acids on  $Mg^{2+}$  permeability, we added oleate micelles to the POPC vesicles, which is known to result in the rapid incorporation of fatty acids into preexisting phospholipid membranes.<sup>[12]</sup> Upon the addition of oleate at a 1:4 OA:POPC ratio, we observed rapid and complete  $Mg^{2+}$  equilibration across the membrane in less than 2 min, indicating that the presence of oleate permits  $Mg^{2+}$  transit across a phospholipid membrane. Since this assay convolves the time required for oleate incorporation into the phospholipid membrane with the time required for  $Mg^{2+}$  permeation, the observed rate of roughly  $3 \text{ min}^{-1}$  must be considered a lower limit, and the real rate could be significantly faster.

*Permeability of Blended Membranes to Calcein:* We measured calcein leakage over a 12 h period to examine the permeability of blended membranes to small charged molecules. For membranes composed of 50% or more phospholipids, less than 10% calcein leakage was detected during a 12 h incubation in the absence of  $Mg^{2+}$ ; while for pure oleic acid vesicles, more than 30% of calcein leaked out even in the absence of  $Mg^{2+}$ , indicating, as expected, a higher membrane permeability of oleate versus POPC membranes (Figure 2c). In order to study the effect of  $Mg^{2+}$  on hybrid membrane permeability change, we plotted calcein release in the presence of increasing  $Mg^{2+}$  concentrations ( $1$ – $50 \times 10^{-3}$  M) for a series of membrane compositions ranging from 100% OA to 100% POPC. In the presence of  $1 \times 10^{-3}$  M  $Mg^{2+}$ , where all vesicle compositions were stable, we observed that pure OA vesicles and 20% POPC vesicles released significantly more of the encapsulated calcein than in the absence of  $Mg^{2+}$ , indicating a  $Mg^{2+}$ -induced increase in permeability. In the presence of higher concentrations of  $Mg^{2+}$ ,



**Figure 2.** Fatty acids enhance the permeability of phospholipid vesicles. a) Microscopy of POPC and 50% OA giant vesicles upon addition of  $Mg^{2+}$  shows that the presence of oleate in membranes allows  $Mg^{2+}$  entry into vesicles. The presence of  $Mg^{2+}$  is indicated by increases in Magnesium Green fluorescence (green). Scale bar for POPC vesicles is  $20 \mu\text{m}$ . Scale bar for OA/POPC vesicles is  $10 \mu\text{m}$ . b) A fluorometer study shows the addition of oleate to pure phospholipid vesicles containing  $Mg^{2+}$  results in  $Mg^{2+}$  exit from vesicle interior, and can be monitored through an increase in Magnesium Green fluorescence in solution. Data are fitted to one phase exponential decay model with  $k = 3.0 \text{ min}^{-1}$  and  $t_{1/2} = 0.23 \text{ min}$ . c) Vesicle permeability to the small molecule dye calcein is represented by calcein retention after 12 h.  $n = 3$ ; error bars represent s.d.

pure OA and 20% POPC vesicles are destabilized and release all calcein. For all the  $Mg^{2+}$  concentrations tested ( $0\text{--}50 \times 10^{-3}$  M), pure POPC and 75% POPC vesicles had no observable calcein leakage increase, while 50% POPC vesicles showed a small increase in calcein leakage in  $50 \times 10^{-3}$  M  $Mg^{2+}$ .

**Permeability of Blended Membranes to Activated Nucleotides:** We next investigated the permeability of blended membranes to activated nucleotides, which are the substrates for non-enzymatic RNA replication. We have previously shown that such nucleotides can enter fatty acid vesicles, but not phospholipid vesicles.<sup>[13,14]</sup> We measured nucleotide permeability by encapsulating a radiolabeled imidazole-activated nucleotide monophosphate, 2-MeImpA (adenosine 5'-phosphoro-2-methylimidazolide) within vesicles. We then used size exclusion chromatography to separate the retained and leaked nucleotides over a series of time points. We observed that the rate at which 2-MeImpA permeated across vesicle membranes decreased as phospholipid content increased (Figure S4, Supporting Information), while the presence of  $Mg^{2+}$  enhanced nucleotide permeability.

The much greater permeability of membranes to the activated nucleotide 2-MeImpA compared to calcein presumably reflects its much lower charge density of either  $-1$  for the unprotonated form or  $0$  for the protonated zwitterionic form.<sup>[15]</sup> The increased permeability seen in the presence of  $Mg^{2+}$  may be due to binding of the divalent cation to oleate molecules in the membrane, creating transient defect structures that allow solutes to transit the membrane.<sup>[16]</sup> Our results indicate that although membrane permeability is reduced by the incorporation of phospholipids, hybrid membranes containing up to 75% POPC are still highly permeable to  $Mg^{2+}$ , and a POPC content of 25% in the presence of  $5 \times 10^{-3}$  M  $Mg^{2+}$  allows nucleotide permeation at a rate comparable to that seen with pure oleate membranes. At 50% POPC, membrane permeability to 2-MeImpA is strongly reduced even in the presence of  $50 \times 10^{-3}$  M  $Mg^{2+}$ .

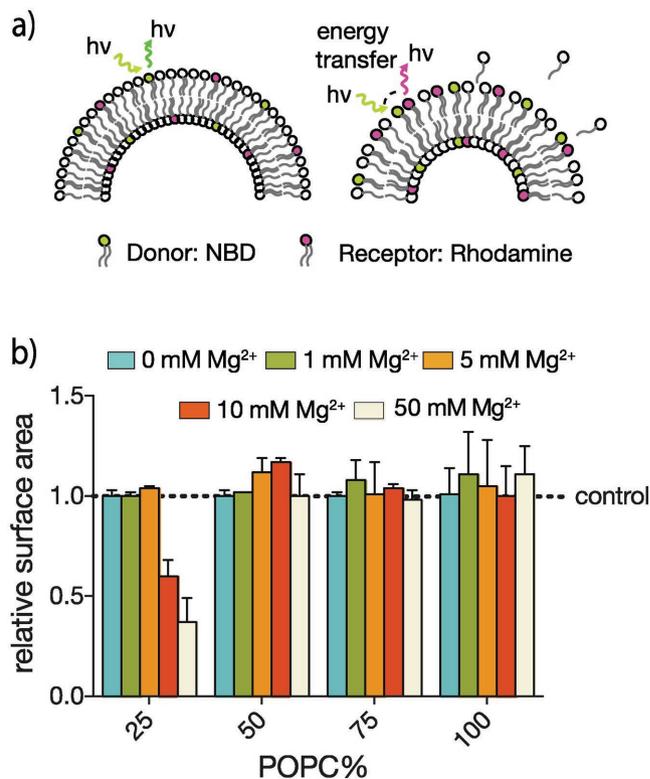
## 2.2. Mechanism by Which POPC Stabilizes Oleate Vesicles to the Presence of $Mg^{2+}$

### 2.2.1. Phospholipids Favor the Partitioning of Oleate into the Membrane

We first investigated the mechanism by which phospholipids enhance the stability of hybrid membranes by examining the partitioning of fatty acids between the membrane and solution. Oleate exists in a dynamic equilibrium between the bilayer membrane of vesicles and micelles and free oleate molecules in the surrounding solution.<sup>[17]</sup> We have previously shown that phospholipids decrease the rate of dissociation of fatty acids from mixed membranes.<sup>[1]</sup> We hypothesized that this decreased dissociation rate would result in a lower concentration of free fatty acid in solution, which in turn could decrease or abolish the ability of  $Mg^{2+}$  to aggregate and precipitate the fatty acid. To investigate the extent to which POPC in membranes increased oleate retention in the membrane phase, we measured the free fatty acid concentration in the solution surrounding vesicles using an enzyme-based assay for fatty acid quantification. We

first collected a small fraction of the solution surrounding vesicles by centrifuging vesicles through a 20 kDa molecular weight membrane, thus separating the vesicles from the surrounding solution containing free fatty acid and micelles. For a fatty acid vesicle preparation containing  $20 \times 10^{-3}$  M pure oleate vesicles, we found that the oleate concentration in the surrounding solution was  $20 \times 10^{-6}$  M (Figure S5, Supporting Information). As we increased the phospholipid fraction in the membrane from 0 to 50%, the concentration of free oleate in solution steadily decreased to  $3.5 \times 10^{-6}$  M, indicating increased membrane retention of the fatty acid. These observations confirm that the presence of phospholipids in fatty acid membranes increases the partitioning of fatty acids into the membrane from solution, a feature that correlates with and could help to explain the stabilization of vesicles in the presence of  $Mg^{2+}$ .

To further investigate the ability of phospholipid to retain fatty acids in mixed composition membranes, we measured the change in surface area of vesicles as a function of initial phospholipid content in the membrane, following the addition of  $Mg^{2+}$  to the external solution. We used a Forster resonance energy transfer (FRET) assay to measure changes in membrane area by monitoring changes in FRET efficiency between membrane-labeled fluorophores (Figure 3a). We reasoned that if fatty acids in solution were being removed by the precipitation of insoluble complexes with  $Mg^{2+}$ , they would be removed from membranes over time resulting in a decrease in total membrane lipid and a corresponding loss of vesicle surface



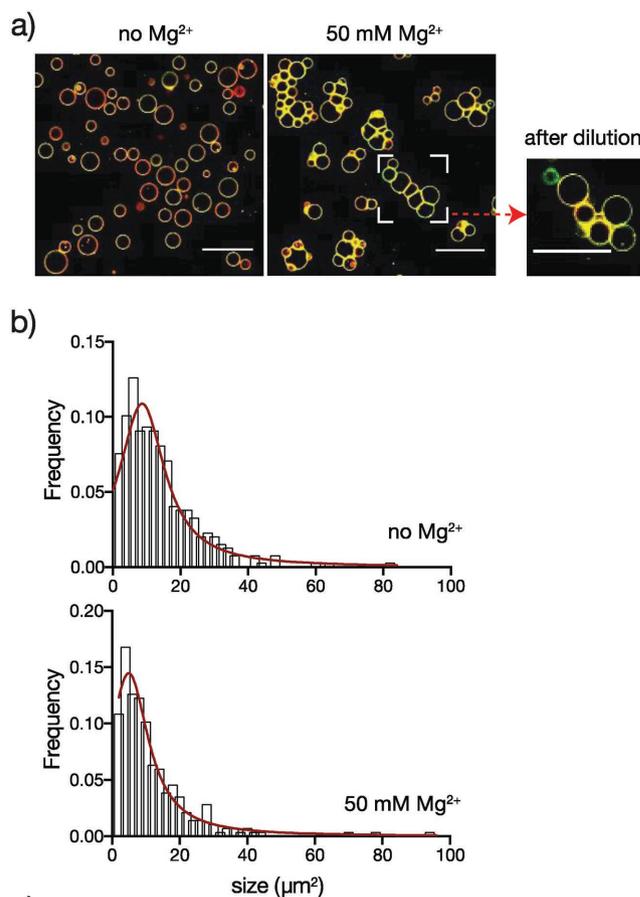
**Figure 3.** Membrane surface area analysis. a) Scheme for membrane surface area analysis based on a FRET assay. b) Fatty acid loss upon addition of  $Mg^{2+}$  was detected by measuring changes in membrane surface area in FRET-labeled OA/POPC small unilamellar vesicles.  $n = 3$ ; error bars represent s.d.

area. In membranes containing a mixture of oleic acid and POPC, the POPC is expected to occupy more membrane area than the oleate,<sup>[18]</sup> however, we still expect to detect a change in surface area if significant levels of oleate were removed. We observed that the surface area of vesicles with membranes containing 50 or 100% phospholipid remained constant following the addition of  $Mg^{2+}$  over the entire range of concentrations tested (Figure 3b). At a phospholipid content of 25%, however, the vesicles lost progressively more surface area in the presence of higher concentrations of  $Mg^{2+}$ , which we attribute to irreversible oleate desorption and a corresponding vesicle area shrinkage. As expected, pure fatty acid membranes were completely destroyed upon the addition of  $\geq 5 \times 10^{-3}$  M  $Mg^{2+}$ . In this case, the total lipid surface area greatly increased, presumably as a result of the dissolution of membranes (Figure S6, Supporting Information). Taken together, our free fatty acid assay and surface area studies suggest that a critical concentration of phospholipid is required in a fatty acid membrane to prevent fatty acid loss to  $Mg^{2+}$ , and that below 50% phospholipid content, membranes lose fatty acid in a way that leads to membrane instability.

### 2.2.2. Morphological Changes of Hybrid Vesicles in the Presence of $Mg^{2+}$

To further understand the physical properties of hybrid vesicles and their response to external  $Mg^{2+}$ , we used confocal microscopy to directly visualize 50% POPC:50% OA hybrid GUVs following the addition of  $Mg^{2+}$ . We prepared the GUVs with encapsulated sucrose, so that they would sink to the bottom of the observation chamber where they could be readily observed. As the added  $Mg^{2+}$  diffused to the bottom of the chamber, the vesicles began to aggregate and assemble into clusters with a honeycomb morphology (Figure 4a, middle, Movie 1). As the concentration of  $Mg^{2+}$  increased, the proportion of vesicles in aggregates increased and the planar surface contact area between neighboring vesicles increased. We quantified the average vesicle cross-sectional area in the absence of  $Mg^{2+}$  and then after the addition of  $50 \times 10^{-3}$  M  $Mg^{2+}$  by using the first and last frames in Movies 1 and 2; images were processed using ImageJ (Figure S7, Supporting Information). We found that cross-sectional area of hybrid vesicles in the no  $Mg^{2+}$  environment fitted well to a Cauchy distribution with an average size of  $8.6 \mu m^2$  ( $N = 397$ ), which decreased to  $5.0 \mu m^2$  ( $N = 286$ ) in  $50 \times 10^{-3}$  M  $Mg^{2+}$  (Figure 4b). Both the increased planar surface contact area and the shrinkage of vesicle size imply a decrease in vesicle volume.

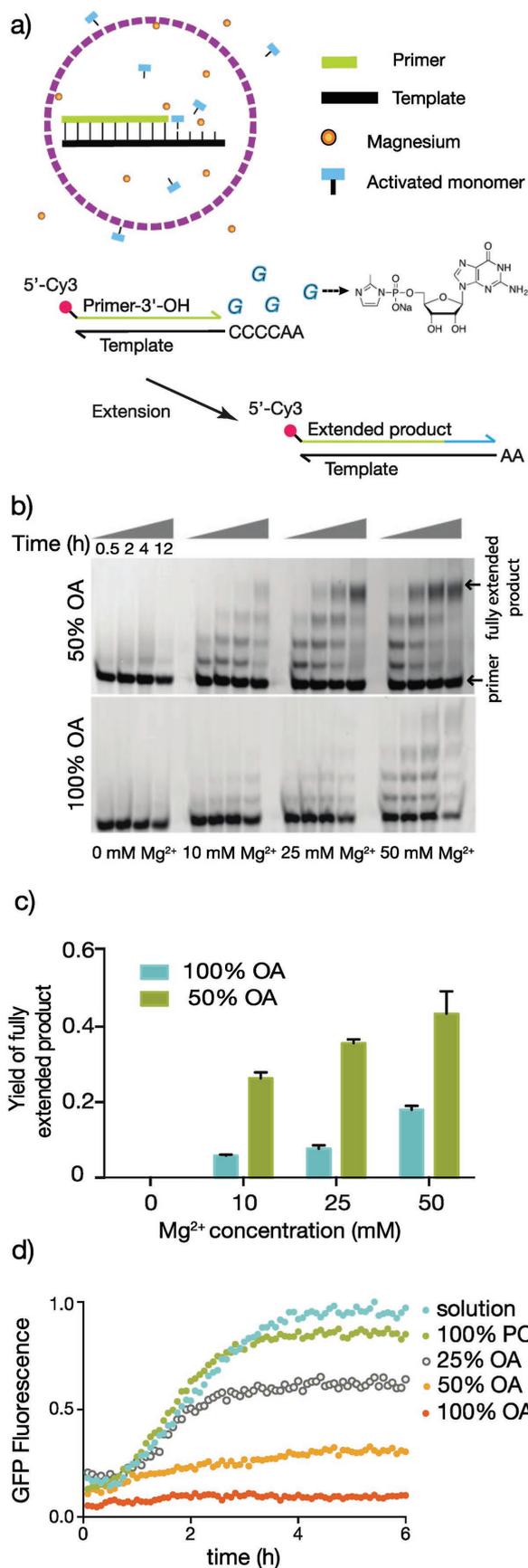
We hypothesized that this volume loss might result from water efflux from the vesicles due to the increased ionic strength of the medium following the addition of  $Mg^{2+}$ . To test this, we prepared pure POPC or 50% POPC hybrid vesicles that contained  $10 \times 10^{-3}$  M partially self-quenched calcein. We then increased the external ionic strength by adding  $Mg^{2+}$  and used the change in fluorescence intensity to calculate the vesicle volume change. We found that an increase of  $150 \times 10^{-3}$  M in ionic strength due to added  $MgCl_2$  caused a 50% volume decrease in POPC vesicles, while 50% POPC hybrid vesicles shrank only 20% presumably because of much faster



**Figure 4.** Hybrid vesicle morphological and size change. a) Microscopic images of 50% OA:50% POPC giant vesicles (labeled with red Rodamine-PE and green BODYPI-C12) in the absence of  $Mg^{2+}$  (left), after the slow addition of  $Mg^{2+}$  to a final concentration of  $50 \times 10^{-3}$  M (middle), and then after repeated partial dilution with water to  $\approx 6 \times 10^{-3}$  M  $Mg^{2+}$  (right), scale bar  $50 \mu m$ . b) Size distribution of vesicle population before (top) and after (bottom) addition of  $Mg^{2+}$ .

$Mg^{2+}$  permeation (Figure S8, Supporting Information). Taken together, the above observations suggest that when hybrid vesicles are exposed to added  $Mg^{2+}$ , the  $Mg^{2+}$  binds to the fatty acids to neutralize surface charge and reduce repulsion, with charge bridging ultimately leading to vesicle adhesion and aggregation. Following water efflux to equilibrate internal and external osmolarity, the resulting volume decrease would allow increased contact area between neighboring vesicles, leading to the observed honeycomb morphology within vesicle clusters.

We then asked whether the  $Mg^{2+}$  induced vesicle aggregation and enlarged surface contact area could be reversed by gradual dilution of the external  $Mg^{2+}$ . During careful and repeated gradual dilution with isotonic media, we found that the surface contact area between neighboring hybrid vesicles gradually decreased, and vesicles gradually regained their spherical morphology (Figure 4a, right, Movie 3). At the end of a 15 min dilution process, at which the overall  $Mg^{2+}$  concentration was  $\approx 6 \times 10^{-3}$  M, vesicle aggregates were largely but not completely dissociated, and over 93% of vesicles in the field had a circularity index ( $4\pi A/L^2$ ,  $A$ : area,  $L$ : perimeter) above 0.9 compared with less than 30% before dilution of the  $Mg^{2+}$ .



### 2.3. Primer Extension inside Vesicles

Ultimately, shifts in the composition of primitive membranes would be expected to affect protocellular fitness as a whole. A critical feature of the fitness of a protocell is the ability to retain and replicate its RNA genome. Our results so far show that increasing levels of phospholipid stabilize vesicles and increase the retention of oligonucleotides, even in the presence of otherwise disruptive concentrations of Mg<sup>2+</sup>. Furthermore, the permeability of the membrane to the essential catalytic divalent cation Mg<sup>2+</sup> remains high up to at least 50% POPC in the membrane. Finally, the permeability of substrate molecules such as activated nucleotides remains good at 25% POPC but is significantly decreased at 50% POPC. We therefore examined how blended membranes affected two RNA-dependent reactions: nonenzymatic template-directed primer extension and the ribosomal translation of green fluorescent protein (GFP).

#### 2.3.1. Nonenzymatic RNA Copying inside Blended Membranes

We began by assessing how nonenzymatic RNA copying would proceed inside hybrid vesicles. This reaction benefits from high membrane permeability in that both reactive nucleotides and divalent cations must cross the vesicle bilayer to react with and extend an encapsulated primer/template complex (Figure 5a). Divalent cations such as Mg<sup>2+</sup> must be chelated to ensure fatty acid vesicle stability,<sup>[13]</sup> but this leads to a decrease in the efficiency of the primer extension reaction. We therefore wondered if the ability to use higher concentrations of unchelated Mg<sup>2+</sup> with blended membranes would offset the reduction in nucleotide permeability, and ultimately enhance the efficiency of the RNA copying reaction inside vesicles. We encapsulated a primer/template complex within 100% oleic acid or 50% OA:50% POPC vesicles, removed unencapsulated RNA, and then added 50 × 10<sup>-3</sup> M activated nucleotide, 2-MeImpG, to the external solution. This nucleotide should gradually cross the vesicle membrane and, in the presence of Mg<sup>2+</sup>, copy the encapsulated template. To initiate the reaction, we then added citrate-chelated Mg<sup>2+</sup> to oleic acid vesicles and an equivalent amount of unchelated MgCl<sub>2</sub> to hybrid vesicles and allowed the reaction to proceed for 12 h.

**Figure 5.** Hybrid membranes promote nonenzymatic and enzymatic catalysis within vesicles. a) Scheme of nonenzymatic primer extension inside vesicle. b) PAGE analysis of a nonenzymatic primer extension reaction conducted in the presence of either 50% OA:50% POPC membranes (top) or 100% OA membranes (bottom). Reactions in the presence of the pure fatty acid membrane utilized magnesium chelated to citric acid (at a 1:4 Mg<sup>2+</sup>:citric acid ratio) while reactions with 50% OA used unchelated magnesium. c) Fraction of fully extended product in pure OA and 50% OA vesicles with different Mg<sup>2+</sup> concentration. d) In vitro translation of GFP monitored by GFP fluorescence of a cell free transcription/translation system incubated with vesicles of different compositions. GFP fluorescence is reported normalized to the GFP signal from the reaction conducted in solution (no vesicles). Increasing the content of oleic acid in the vesicle membranes leads to a reduction in translation of GFP, with negligible protein production measured in the presence of 100% OA membranes. All studies were conducted with small unilamellar vesicles.

Analysis of the reaction products showed that the RNA copying reaction was most efficient, and yielded more of the fully extended product, within the blended membrane system for all  $Mg^{2+}$  concentrations tested (Figure 5b,c). The benefit of using unchelated  $Mg^{2+}$  was most apparent at low concentrations ( $10 \times 10^{-3} M$ ) of the cation, where there was a fivefold increase in the fraction of fully copied template in blended membranes compared to pure OA vesicles that utilized chelated  $Mg^{2+}$ . On a primitive earth where low levels of divalent ions may have been present at any given time, the ability of a protocellular system to produce RNA products with greater efficiency than competing vesicles would be a selective advantage. On the other hand, the larger amount of unreacted primer observed within the hybrid vesicles likely reflects the variability in the permeability of the hybrid membrane to nucleotides; vesicles positioned in the middle of a honeycomb cluster in the  $Mg^{2+}$  solution would not be well-exposed to the surrounding media. Nevertheless, the benefit of hybrid membranes for nonenzymatic RNA copying is clear, because they allow both the use of free  $Mg^{2+}$  and the transit of  $Mg^{2+}$  and activated nucleotides into vesicles.

### 2.3.2. Transcription–Translation in the Presence of Blended Membranes

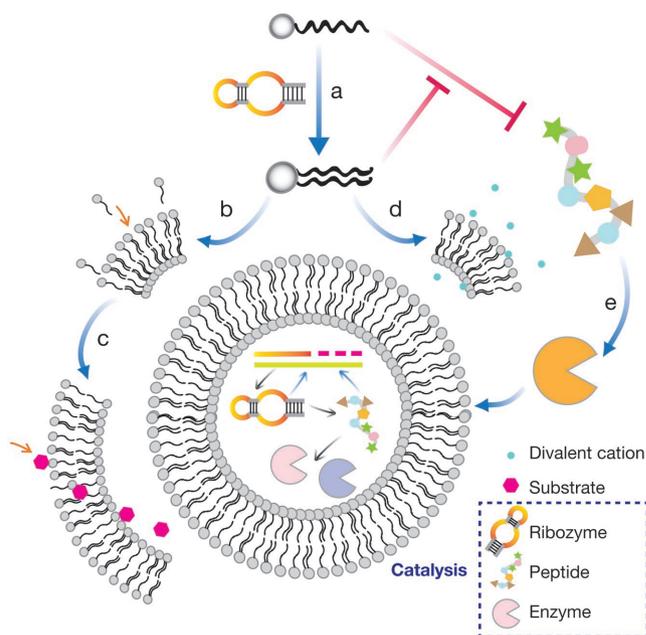
We next turned our attention to a multienzyme reaction that is a central aspect of modern cellular function: the transcription/translation system. All modern cells utilize proteins to maintain cellular growth, maintenance, and division. DNA and RNA polymerases are key enzymes in this process as they generate the genetic sequences that will be translated into specific proteins. Yet, many fatty acids strongly inhibit polymerase activity,<sup>[19]</sup> posing the question of how this enzymatic activity might have emerged in primitive membranes containing fatty acids. We wondered if phospholipids, which we previously showed improve fatty acid retention in membranes, might enable transcription and translation in the presence of fatty acid membranes by similarly preventing inhibitory fatty acid–polymerase interactions.

We utilized a cell-free *in vitro* transcription–translation system (PURExpress) and DNA encoding the protein GFP to assess gene transcription and translation. This reaction mixture was incubated with vesicles of varying compositions of fatty acid and phospholipid. We observed that in the presence of pure oleic acid vesicles, the appearance of GFP fluorescence was strongly inhibited (Figure 3d). As the vesicle composition increased in phospholipid content, GFP production increased. Pure phospholipid vesicles displayed similar levels of GFP production relative to samples that contained no vesicles (solution). Free fatty acids have long been known to inhibit a variety of enzymes. The mechanism of inhibition is thought to be due to hydrophobic interactions of the fatty acid with the substrate binding pocket of the enzyme and/or a detergent-mediated alteration to protein structure.<sup>[20,21]</sup> This inhibition would certainly create difficulties for emerging catalytic polymers that had an affinity for fatty acids. Our results suggest that phospholipids, by competitively binding fatty acids, may have allowed for the emergence of enzymatic activity in primitive cells.

## 3. Discussion

We have shown that phospholipids can stabilize model protocell vesicles made of oleic acid, allowing the vesicles to tolerate the presence of the divalent cation  $Mg^{2+}$ , which is essential for many catalytic processes. The presence of fatty acids, in turn, allows for small molecule permeability, including the permeation of divalent cations and nucleotides, both essential components of a primitive metabolic system. These complementary physical attributes of stability and permeability ultimately enable the encapsulated reactions that would have been critical in an evolving protocellular system. The fact that these two classes of amphiphiles would be expected to bracket the transition of protocell membrane composition from fatty acids to phospholipids, suggests that their coexistence, prior to the development of transporter molecules, could have facilitated the initial stages of the evolutionary transition in membrane composition.

Our study has shed further light on the selective pressures that may have prompted cell membranes to evolve toward phospholipids. In (Figure 6), we illustrate the various ways in which phospholipids could enhance the fitness of a protocellular system. As we have previously suggested, di-acyl phospholipids could have initially emerged in primitive cells upon the development of acyl-transferase catalysts, such as ribozymes, that were able to link two monoacyl chains together. Once present, even a small fraction of phospholipid in a fatty acid membrane will drive vesicle growth at the expense of nearby vesicles with less phospholipid.<sup>[1]</sup> Because of their strong growth advantage,



**Figure 6.** Scheme of lipid and catalysis co-evolution. a) Phospholipid synthesis from fatty acids by ribozyme catalysis; b) Competitive growth of blended membranes promoted by the presence of phospholipids; c) Fatty acid presence in blended membrane promotes permeability to small metabolic building blocks; d) Phospholipid presence in hybrid membranes promotes stability to divalent cations and e) protein-based enzymes. Evolution of enzymes is possible in the presence of phospholipids.

protocells able to synthesize phospholipids would be expected to take over the population, and subsequent competition would create a selective pressure for the evolution of increasing levels of phospholipids in protocell membranes. Our results show that as long as the membranes retain a substantial proportion of fatty acids, vesicle membranes would retain good permeability to  $Mg^{2+}$  and significant permeability to nutrients such as nucleotides. Interestingly, the presence of phospholipids in protocell membranes would confer another potentially very important selective advantage, namely, the ability to survive in environments containing high levels of  $Mg^{2+}$ . Because  $Mg^{2+}$  is an important catalyst of a variety of both nonenzymatic and enzymatic reactions, the ability to tolerate high levels of environmental  $Mg^{2+}$  would allow protocells to colonize new environments. Finally, we have shown that phospholipids help to retain fatty acids in membranes, thereby decreasing the concentration of free fatty acids in solution and consequently decreasing fatty acid inhibition of protein catalysts such as RNA polymerase. Fatty acid retention has been an overlooked issue in terms of vesicle-confined reactions. While fatty acids are compatible with many RNA-based catalysts,<sup>[22,23]</sup> fatty acids are known to inhibit a variety of protein-based enzymes. For example, linoleic acid (C18) was reported to result in 50% inhibition of DNA polymerase  $\alpha$  activity at a concentration of  $35 \times 10^{-6}$  M and complete inhibition at  $60 \times 10^{-6}$  M.<sup>[19]</sup> The confinement of fatty acids to membranes could therefore provide opportunities for the emergence of catalytic peptides with novel activities, including transporter capabilities that would further reduce the need for fatty acids to provide membrane permeability.

As we continue to gain understanding of how an adaptive protocellular system might have developed on the early earth, we increasingly find that heterogeneity imbues model prebiotic systems with unexpected and advantageous characteristics.<sup>[24]</sup> Mixtures of amphiphiles can lead to improved vesicle stability or membrane formation at lower amphiphile concentrations than the constituent parts allow.<sup>[25]</sup> In addition, mixtures of amphiphiles can influence features of new vesicle formation.<sup>[7]</sup> Heterogeneity in the structure of RNA, including variation in backbone linkages, oligomer length, and sequence, may also provide advantages, for example, by decreasing the melting temperatures of RNA duplexes compared to more homogeneous molecules.<sup>[26,27]</sup> Along these lines, we find that mixtures of membrane components enable the coexistence of increased permeability and stability, which would be expected to be advantageous to early protocells. While our study investigated a simple mixture of oleic acid and POPC, these amphiphiles represent simple models of a primitive monoacyl, and modern diacyl amphiphile. It is likely that other complex membrane compositions, not yet thoroughly studied, might allow for even greater stability alongside greater membrane permeability than our study reports. The work presented here, however, expands our understanding of how diacyl lipids affect the physical properties of monoacyl systems, and sets the stage for future investigations of more complex systems. Our results, along with evidence that the physical properties of the membrane can influence proximal catalytic events,<sup>[28,29]</sup> provide a further connection between vesicle membranes and their encapsulated genomic material and the reasons for their initial association.

## 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

artificial cells, fatty acids, membranes, origin of life, RNA

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