# Compositional heterogeneity of protocellular membranes: Implications for emergence of mixed vesicular systems

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Running title: Compositional heterogeneity and protomembrane landscape

**Abstract**

Protocell membranes are considered the boundaries of cellular life that first emerged on the prebiotic Earth. They are thought to be comprised of mixtures of single chain amphiphiles, such as fatty acids and their derivatives, moieties which would have been part of the complex prebiotic chemical landscape. In addition to their composition, the physico-chemical properties of these prebiological membranes would have been significantly affected, and regulated, by the physical environment that they were present in. In this study, the physico-chemical properties of two different chain length membrane systems, were systematically characterized, under prebiotically pertinent environmental conditions. The membrane systems have been designed to be composed of fatty acid and/or their alcohol and glycerol monoester derivatives, to make a range of vesicle combinations (e.g. binary and tertiary systems). Their properties were evaluated as a function of multiple factors including their composition, stability under varying pH, $Mg^{2+}$ ion concentration and dilution regimes, and permeability. These environmental constraints would have acted as important prebiotic selection pressures to shape the evolution of prebiological membranes. Our results indicate that complex membrane systems are more stable and robust to multiple selection pressures, thereby making them more suitable for supporting protocellular life. Furthermore, different fatty acid derivatives conferred varying degrees of stability when mixed with their respective fatty acid moiety. Importantly, the aforesaid depended on the chain length of the system, and the selection pressure that was applied. Significantly, when the systems were subjected to multiple selection pressures in a consecutive manner, only the heterogeneous membrane systems survived the race. These results highlight the requirement of compositional complexity, and underscore its implications for the emergence of mixed membrane systems during the dawn of life on Earth.

**Key words**

Protocells, Fatty acid membranes, Compositional heterogeneity, Selection pressure, Fitness landscape.

## 1. Introduction

The earliest forms of cellular life are considered to be entities that were comprised of dynamic chemical reactions, encapsulated in an amphiphilic compartment (1). In addition to fulfilling crucial physiological functions, compartmentalization helps in separating the internal components from the outside environment, a feature that has been argued to be crucial for Darwinian evolution of primitive cellular systems (1, 2). Unlike the contemporary biological membranes that potentially resulted over millions of years of evolution, prebiological membranes are thought to have been simple and composed of single chain amphiphiles (SCAs) (3). These SCAs could have come about on the early Earth either by endogenous synthesis, in the form of Fisher-Tropsch Type (FTT) reactions, or via exogenous delivery from extra-terrestrial space (4, 5). Amphiphiles such as short chain fatty acids, and their derivatives, including alcohol, amine, glycerol monoester and phosphate versions, fall within the description of prebiotically plausible SCAs. Nonetheless, only the longer chain fatty acids have been predominantly studied in the context of early compartments (3, 6). Compartmentalization is crucial for the encapsulation of chemical reactions or networks, while also maintaining the integrity of a protocellular entity. Lacking complex protein machinery, such entities must have relied on their surrounding physico-chemical environment in order to survive, grow, divide and undergo evolution. In this regard, their self-assembly property, chemical stability, and membrane integrity, against different environmental parameters, such as pH, ionic strength, temperature, dilution etc., need to be systematically explored. This is pertinent to gain a better understanding of how relevant environmental parameters would have constrained and shaped the functional landscape of prebiological membranes. Thus far, studies have predominantly focused on delineating one, or up to two of the aforesaid parameters at any given time (7-11). However, these conditions would have acted in concert as a combination of prebiotic selection pressures, which would have played a vital role in shaping the evolutionary landscape of prebiological membranes. Fatty-acid based membranes have large critical vesicular concentrations (CVC), and are sensitive to cation concentration and pH fluctuations (6, 11-12). Therefore, homogenous fatty acid membrane systems are less suitable as candidates that would have supported the emergence of cellular life. On the contrary, it is more rational to envisage heterogeneous membrane systems as these could have been readily available in an, arguably, large prebiotic chemical space. Growing literature also suggests that the lack of membrane stability can be counterbalanced by increasing the heterogeneity. For example, Namani and Deamer showed that membranes composed of decanoic acid (C10:0) and decylamine can form vesicles at very low as well as high pH, and are stable in the presence of 100 mM concentration of divalent cations (7). In 2002, Apel et al. showed that, when nonanol and decanol were mixed with their corresponding fatty acids, it resulted in a decrease in the CVC of the resultant binary systems. The acid-alcohol mixed systems were also able to form vesicles over a wide range of alkaline pH (8). In 2012, Rendo´n et al. showed on the same lines that the mixture of oleic acid and varying long chain alcohols, can assemble into vesicles over a wide alkaline pH

regime (9). Monnard et al. and Chen et al. demonstrated that binary membrane systems comprising of either C10:0 or C14:1 chain length fatty acids, and their respective glycerol monoester derivatives, are more resistant to soluble monovalent and divalent cations (10-11). However, the mechanism behind this increase in stability towards divalent cations, in the presence of derivatives, is not clearly understood. Although insightful, the aforementioned studies predominantly looked at binary membrane systems. Given the heterogeneous nature of prebiotic soup, and the niche parameters, it would be worthwhile to complexify the starting mix, to better understand how membrane related processes would have advent under 'prebiotically realistic' conditions. In this context, it is pertinent to highlight a study by Mansy et al. who looked at the thermostablity of a tertiary membrane system composed of decanoic acid, and its corresponding alcohol and glycerol monoester derivative (13). To our knowledge, this is the only tertiary system that has been explored thus far, and was done so in a very specific context. To gain a deeper understanding of how compositional heterogeneity would impinge on a membrane system's survivability, especially when subjected to multiple prebiotic selection pressures, we set out to characterize tertiary membrane systems of selected SCAs. In the present study, fatty acids of two different chain lengths, i.e. oleic acid (C18:1) and undecylenic acid (C11:1), were mixed with their corresponding alcohol and/or glycerol monoester derivatives, and used as a proxy for mixed membrane systems. Yet another important goal of this study pertains to discerning the role of the different head groups, on the assembly and stability process. A total of four systems, viz. the homogenous fatty acid, two binary systems containing fatty acid with either fatty alcohol or glycerol monoester derivative, and the tertiary system containing all the three components, were explored for each chain length. The ratio of fatty acid to its derivative was maintained at 2:1. To our knowledge, our study might be the first report detailing the self-assembly and stability of mixed undecylenic acid membrane systems under different prebiotic conditions. The physical parameters that were characterized include, formation of protocellular membranes at alkaline pH, their CVC, ionic stability and the permeability of the said systems. Our results indicate that the heterogeneous membrane systems are indeed more stable, and robust under different environmental conditions. Therefore, these would have been more suitable to support protocellular life forms. The systems comprised of only the fatty acid moieties, were found to be the most sensitive ones among the lot. Our results also illustrate that the head groups of the different amphiphiles used in this study, play an important role in stabilizing primitive membranes under specific selection conditions. Significantly, when above a certain concentration, they tend to destabilize the mixed membrane system rather than stabilizing it. Given this interesting finding, we also attempted to delineate the contribution of individual membrane components, and the plausible mechanism that might be involved, in stabilizing the protomembranes that were evaluated. Systems containing different derivatives, i.e. either the fatty alcohol or the glycerol monoester moiety, possess different survival rates when subjected to specific selection pressures. Therefore, the evolution of protomembranes could have been shaped, both, by their compositional heterogeneity, and the niche parameters (selection conditions) that they are subjected to. The possibility of being affected by multiple selection pressures is a relevant prebiotically "realistic" scenario, which is

what this study has aims to characterize. The overall outcome of this study clearly shows that the tertiary systems possess the best chance at survival when subjected to multiple selection pressures. This work, thus, has implications for discerning the emergence of mixed membrane systems, and highlights the need to factor prebiotically realistic conditions, to better understand how they would have impinged on the prebiotic molecular landscape.

## 2. Results

### 2.1 Influence of compositional heterogeneity on self-assembly of primitive membrane systems

The CVC of an amphiphile is a narrow regime of concentration where monomers come together and assemble into a higher ordered structure i.e. a vesicle (14). Higher CVC of fatty acids poses significant obstacle towards their self- assembly in prebioitic scenarios, wherein meeting this high concentration prerequisite would have been difficult (2, 8). In order to understand the self-assembly process and determine the CVC of the systems mentioned in the previous section, 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a bilayer reporter fluorescent probe (15) (SI, Method 2.3). DPH fluorescence was plotted as a function of lipid concentration to determine the CVC of the membrane system. The concentration of lipid, where a sudden increase in fluorescence was observed (the inflection point), represents the CVC of the membrane system. Turbidometric assay and microscopy were also used to confirm the CVC results (SI, Figure S2, S3, and S4) (16). The fluorescence assay revealed that the CVC of the homogenous undecylenic acid system (C11_UDA) was near 35 mM. Upon mixing undecylenic acid (UDA) with glyceryl 1-undecylenate (UDG) in 2:1 ratio, as mentioned earlier (C11_2:1_UDG), the CVC drastically decreased to 2 mM. For the tertiary system (C11_4:1:1_T), and the binary acid-alcohol mixed system (C11_2:1_UDOH), the CVC was found to be around 2 and 3 mM, respectively. Microscopic analysis of all the systems confirmed the presence of vesicles, as shown in Figure 2. Table S1, (SI) summarizes all the results. In the C18:1-based systems, for the homogenous oleic acid (C18_OA) system, the CVC from fluorescence and turbidity assays was found to be approximately 0.09 mM. Upon adding either the glycerol monoester (C18_2:1_GMO), or the alcohol derivative (C18_2:1_OOH), the CVC decreased to about 0.02 and 0.06 mM, respectively (Figure S1). For the tertiary system (C18_4:1:1_T), CVC was found to be approximately 0.02 mM. Interestingly vesicles were observed under the microscope at 40X magnification at slightly higher concentration than what was expected from the fluorescence assay as microscopy is diffraction limited (SI, Table S1, Figure S4).

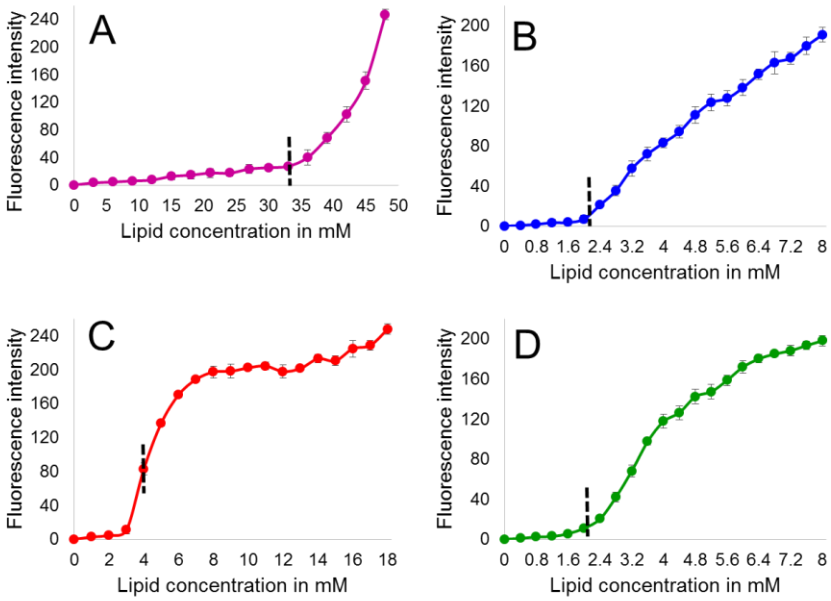


**Figure 1**: CVC estimation of different membrane systems by fluorescence assay. The increase in fluorescence is plotted as a function of lipid concentration. Panel A to D represents the different C11:1 membrane systems, (A) homogenous undecylenic acid system (C11_UDA), (B) undecylenic acid:glyceryl 1-undecylenate mixed system (C11_2:1_UDG). (C) and (D) represents the undecylenic acid and undecylenyl alcohol containing binary system (C11_2:1_UDOH), and the tertiary system comprising all three moieties (C11_4:1:1_T), respectively. The inflection point is represented with the black dash line. n = 3; error bars represent standard deviation (s.d.).

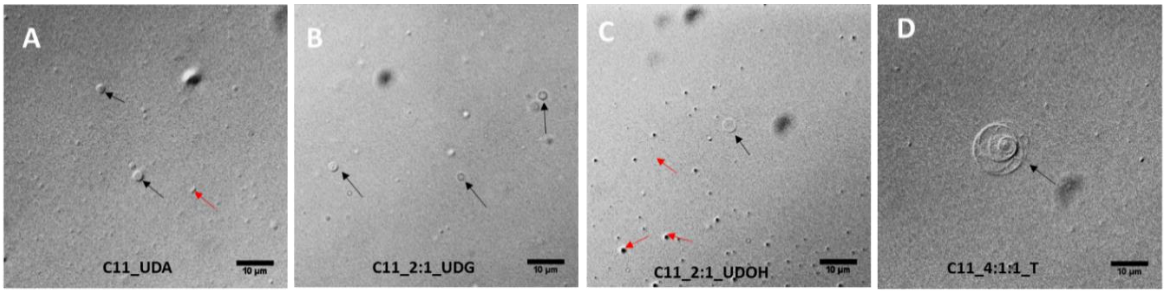


**Figure 2:** CVC estimation using microscopic analysis. The black and red arrows indicate vesicles and aggregates, respectively. Panel A to D shows the different C11:1 membrane systems at their CVC, (A) 40 mM homogenous undecylenic acid system (C11_UDA), (B) 2 mM of undecylenic acid- gyceryl 1-undecylenate mixed system (C11_2:1_UDG), (C) and (D) represent undecylenic acid-undecylenyl alcohol mixed binary system (C11_2:1_UDOH) and the tertiary system (C11_4:1:1_T), respectively at 40X magnification. Scale bar in all the images are 10 microns.

### 2.2 Effect of compositional heterogeneity on the formation of protocellular membranes under alkaline pH regimes

It has been hypothesized that the pH of certain early Earth terrestrial hydrothermal pools would have been neutral to alkaline (17). These have been proposed as one of the primary niches that would have supported the emergence of life. Recent studies

have also shown that alkaline pH can drive prebiotically pertinent reactions, including formose reaction (18), polymerization of non-activated amino acids (19), and non-canonical nucleoside or nucleotide formation (20). Given the sensitivity of fatty acid membranes to changes in pH, the encapsulation of the aforementioned reactions or networks, and their sustenance, in these membranes would have been extremely challenging. Therefore, we investigated whether increasing the compositional heterogeneity of the membrane, could facilitate vesicle formation and confer stability at alkaline pH regimes (SI, Method 2.4). It was observed that both the alcohol and glycerol monoester derivatives could indeed stabilize the fatty acid vesicles over a wide range of pH. Turbidity estimation indicated that C11_UDA system could form vesicles from pH 7.5 to 8, which was also confirmed using microscopy (Figure 3). Nonetheless, above pH 8, it predictably was assembling into micelles. The C11_2:1_UDG system showed vesicle formation from pH 7 to 9, above which micelle formation resulted in a decrease in the turbidity values (SI, Figure S7). On the other hand, C11_2:1_UDOH and the C11_4:1:1_T systems were able to form vesicles, all the way from pH 7.5 to 11 as shown in Figure 3. In the case of the C18 systems, the C18_OA system formed vesicles from pH 8 to 9; below pH 8 and above 9, disordered aggregates and micelles, respectively, were noted. The C18_2:1_OOH system could form vesicles from pH 8.5 to 11. However, below 8.5 it assembled into disordered aggregates, which was further confirmed by microscopy (SI, Figure S5). In the case of C18_2:1_GMO and the C18_4:1:1_T systems, vesicles were observed over a wide range of pH from 7.5 to 11, as summarized in Figure 4B; below pH 7.5, both the systems only formed aggregates.

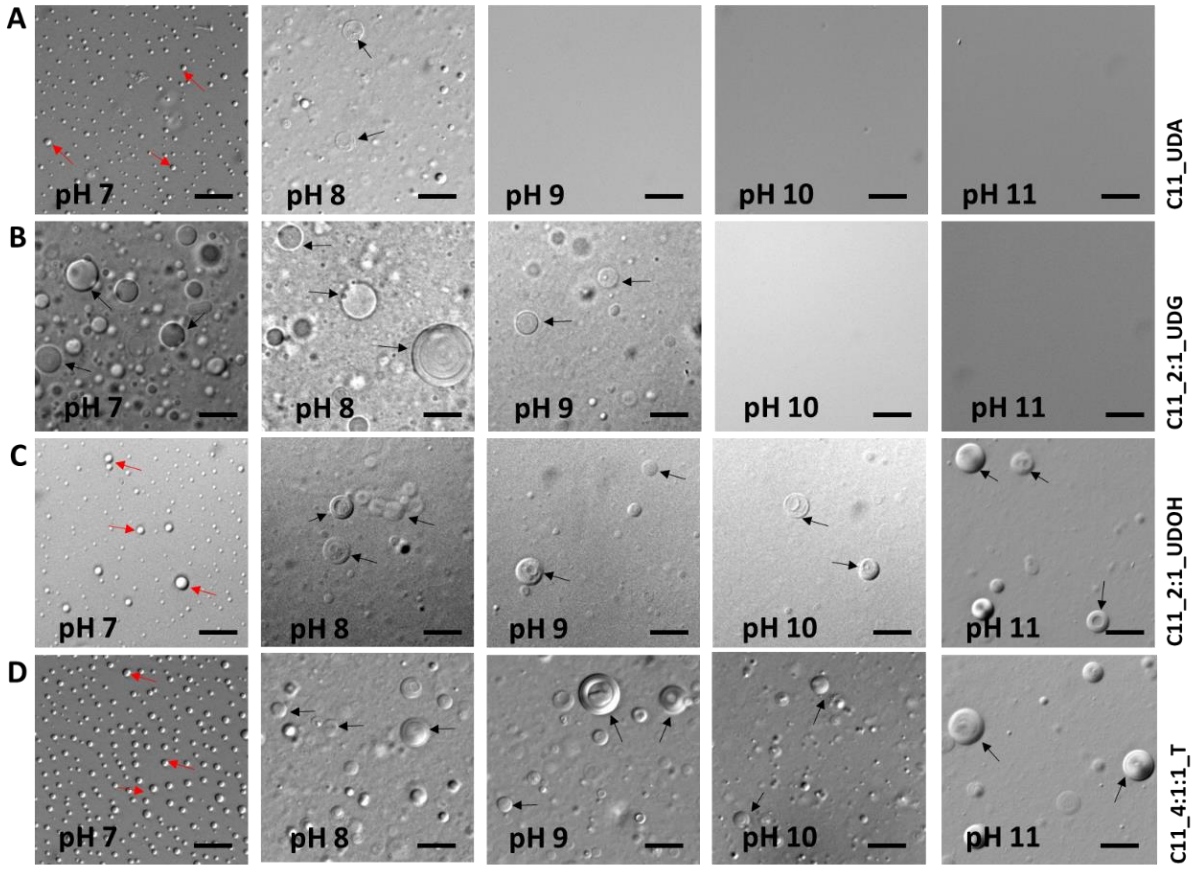

**Figure 3:** Microscopic analysis of C11:1 systems. These images demonstrate the formation of different higher order assemblies (e.g. vesicles, oil droplets etc.), depending on the pH of the surrounding environment. Panels A to D show four different C11-based systems. A) homogenous acid (C11_UDA) system, B) the binary system of acid-glycerol monoester (C11_2:1_UDG), C) the binary system of acid-alcohol (C11_2:1_UDOH) and D) the tertiary system (C11_4:1:1_T). The black and red arrows indicate vesicles and aggregates, respectively. The scale bar in all the images is 10 microns.

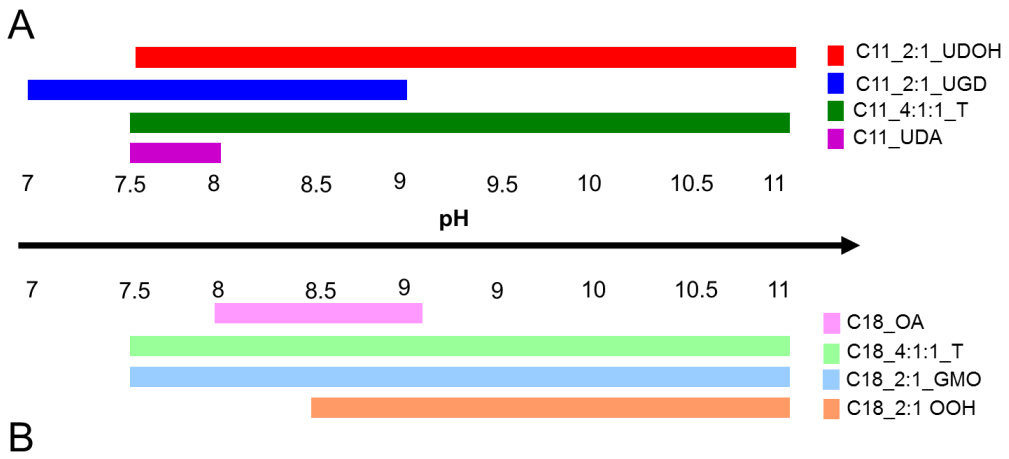


**Figure 4:** Ability of the different fatty acid-based systems to form vesicles over varying pH regimes has been illustrated. The ability of a system to form vesicles over a range of pH is represented by the differently colored horizontal bars. The length of the bar is directly proportional to the range of pH at which the system can assemble into vesicles. Panels A and B represent the C11:1 and C18:1 systems, respectively. C11_UDA, C11_2:1_UDG, C11_2:1_UDOH and C11_4:1:1_T represent, homogenous undecylenic acid system, undecylenic acid- glyceryl 1-undecylenate mixed binary system, the undecylenic acid-undecylenyl alcohol mixed binary system, and the tertiary C11:1 system, respectively. In case of panel B, C18_OA, C18_2:1_GMO, C18_2:1 OOH and C18_4:1:1_T represent homogenous oleic acid, oleic acid-glycerol 1-monooleate mixed binary system, oleic acid-oleyl alcohol mixed binary system, and the tertiary C18:1 system, respectively.

### 2.3 Effect of membrane heterogeneity on its stability, in the presence of $Mg^{2+}$ ion

In order to replicate and carry out catalytic functions, RNA molecules, which are thought to be the first biomolecules to have emerged, require high amounts of divalent cations (50 to 200 mM of $Mg^{2+}$ ion) (21-22). These high concentrations have been shown to not be compatible with homogenous fatty acid membranes (10, 23). Moreover, recent studies have demonstrated the reconstitution of certain primitive metabolic pathways in the presence of high concentration of mono and divalent cations (24-25). This poses an imminent question of how such metabolic networks and RNA replicators, would have coexisted along with fatty acid membranes. Previous studies has demonstrated that presence of chelating agents such as citrate can stabilize oleic acid membrane in presence of high concentration of $Mg^{2+}$ ion

without affecting the function of $Mg^{2+}$ ion dependent ribozyme (23). In this present study systematic evaluation of the influence of alcohol and glycerol monoester head groups, on the ionic stability of fatty acid membranes was carried out. $Mg^{2+}$ ion was used as a representative divalent cation for its prebiotic relevance (21-23). Optical microscopy and dynamic light scattering spectroscopy (DLS), was used to check the stability of the vesicles in the presence of $Mg^{2+}$ ions (SI, Method 2.5,). The concentration of $Mg^{2+}$ ion at which the average size of the vesicle population increases, in comparison to the initial size (when no $Mg^{2+}$ is added), was considered to be the aggregation-inducing concentration ($Mg^{2+}_{AIC}$) (26). At this concentration, both, vesicles and $Mg^{2+}$ induced aggregates can be present simultaneously in the solution (Figure 5). In case of C11:1 systems, the C11_2:1_UDG system was found to be the most stable one, with an $Mg^{2+}_{AIC}$ of 16 mM, followed by the C11_4:1:1_T and the C11_2:1_UDOH systems, with an $Mg^{2+}_{AIC}$ of 12 and 8 mM, respectively. The C11_UDA system was found to be extremely labile to $Mg^{2+}$ ions, where aggregation started even at concentrations as low as 3 mM of $Mg^{2+}$ ion as shown in Figure 6. On microscopic analysis, large fatty acid crystals and aggregates were found at 4 mM $Mg^{2+}$ ion concentration in the C11_UDA system, and beyond 8 mM, no vesicles were observed in the solution (Figure 6, panel A). However, in the case of the C11_2:1_UDG system, lipid aggregates and crystals started appearing at 12 mM $Mg^{2+}$ ion concentration, and vesicles persisted even in solutions with 24 mM of $Mg^{2+}$ ions, as shown in Figure 6, panel B. Between the C11_2:1_UDOH and the C11_4:1:1_T systems, the latter seemed more stable, with vesicles being observed along with some aggregates in the presence of 16 mM $Mg^{2+}$ ion. However, only crystals and droplets were found in the C11_2:1_UDOH system at 16 mM $Mg^{2+}$ ion (Figure 6, panel C). While in the case of the C18:1 systems, as expected, C18_OA system was found to be the most sensitive, with an $Mg^{2+}_{AIC}$ of 3.5 mM (SI, Figure S8). Unlike the C11:1-based system, the acid-glycerol monoester mixed system (C18_2:1_GMO), was found to be the second most sensitive towards $Mg^{2+}$ ions, with an $Mg^{2+}_{AIC}$ of 5 mM. Both the tertiary C18_4:1:1_T and the binary C18_2:1_OOH systems showed an $Mg^{2+}_{AIC}$ of 6 mM. Similar observations were confirmed using microscopy (SI, Figure S9)

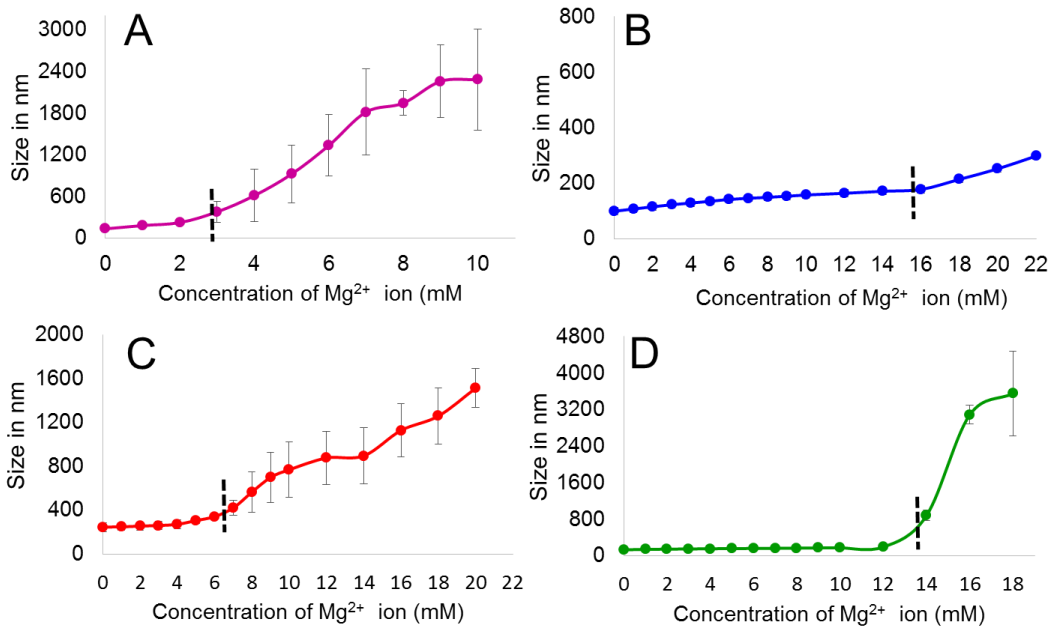

**Figure 5:** DLS measurements to determine $Mg^{2+}$ ion induced aggregate formation. The particle diameter (in nm) is plotted as a function of the $Mg^{2+}$ ion concentration. Panels A to D represents four different C11:1 systems. A) homogenous acid (C11_UDA) system, B) the binary acid-glycerol monoester system (C11_2:1_UDG), C) the binary acid-alcohol system (C11_2:1_UDOH) and D) the tertiary system (C11_4:1:1_T). The vertical black dashed line indicates the $Mg^{2+}$ ion induced aggregation formation concentration ($Mg^{2+}_{AIC}$). n = 3; error bars represent standard deviation (s.d.).

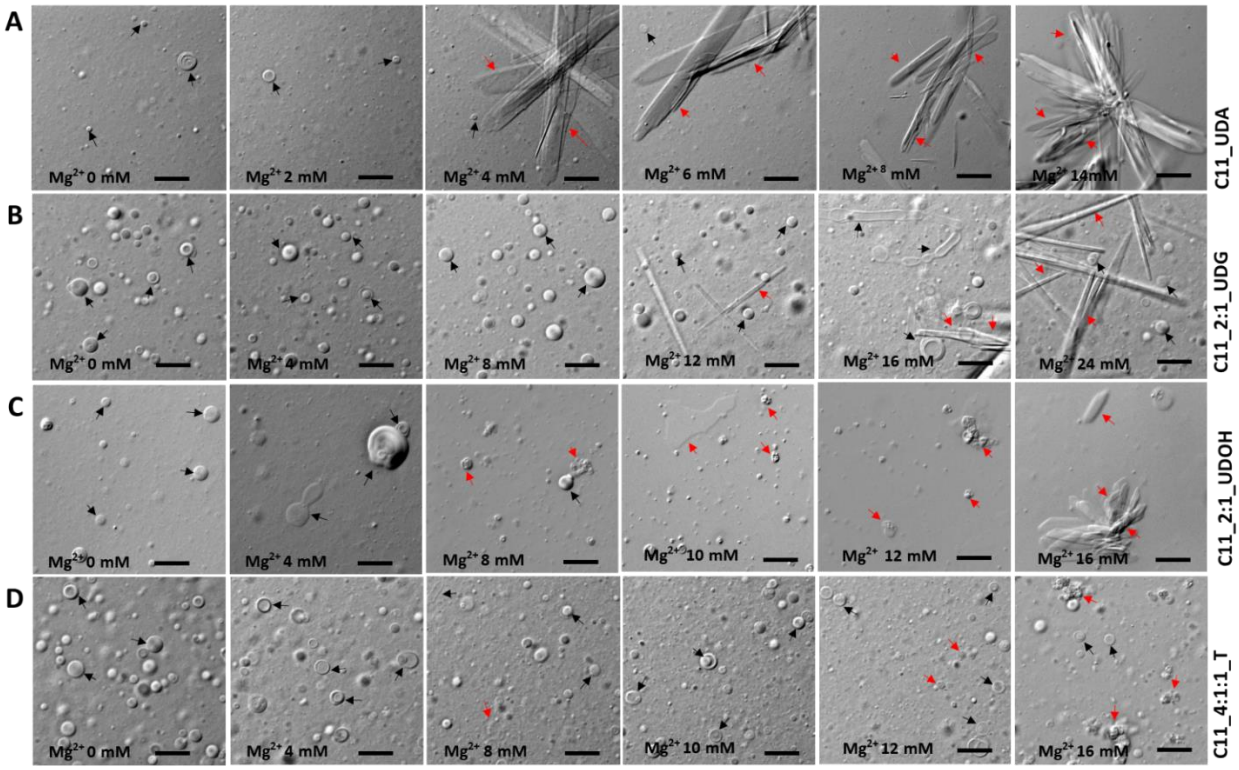


**Figure 6:** $Mg^{2+}$ ion induced vesicle and aggregate forming properties of all the four C11 systems. Panels A to D show four different C11:1 based systems. A) homogenous acid (C11_UDA) system, B) the binary system comprising of acid and glycerol monoester (C11_2:1_UDG), C) the binary system comprising of acid and alcohol (C11_2:1_UDOH), and D) the tertiary system that is composed of all the three amphiphiles (C18_4:1:1_T). From left to right, $Mg^{2+}$ ions concentration was increased gradually by keeping the lipid concentration constant. In terms of cation sensitivity among the four systems, the following order is seen: C11_UDA> C11_2:1_UDOH> C11_4:1:1_T> C11_2:1_UDG. The black and red arrows indicate vesicles and aggregates (fatty acid crystals and droplets), respectively. The scale bar in all images is 10 microns.

### 2.4 Influence of compositional heterogeneity on the permeability of protocell membrane

Being dynamic in nature, fatty acid membranes facilitate the permeation of polar molecules. This is an important feature for the primitive cell to possess as the membrane dynamics is only way by which this cell can exchange matter with its

environment (27). In order to understand the effect that each head group might have on the permeability of model protocell membranes, calcein leakage assay was carried out using the four different C11:1 systems (SI, Method 2.6). Calcein, a small charged molecule, was encapsulated in the different vesicular systems, at a concentration above its self-quenching concentration, and its release was measured over time (26). It was observed that when gyceryl 1-undecylenate (UDG) was mixed with undecylenic acid (UDA), the permeability of the system (C11_2:1_UDG) for calcein increased in comparison to the homogenous C11_UDA membrane system. Over a period of 180 min, 80 percent of the encapsulated calcein was released. Whereas in the case of the C11_UDA system, only 22 percent of the encapsulated calcein was released. Interestingly, the C11_2:1_UDOH system was found to be impermeable, wherein none of the encapsulated calcein was released even after a period of 180 min, as shown in Figure 7**.** The C11_4:1:1_T system was found to possess moderate permeability to calcein, i.e. it was less permeable than the binary system of C11_2:1_UDG, and the C11_UDA system, but more permeable than the binary system of C11_2:1_UDOH system, releasing only 14 percent of encapsulated calcein over 180 mins.

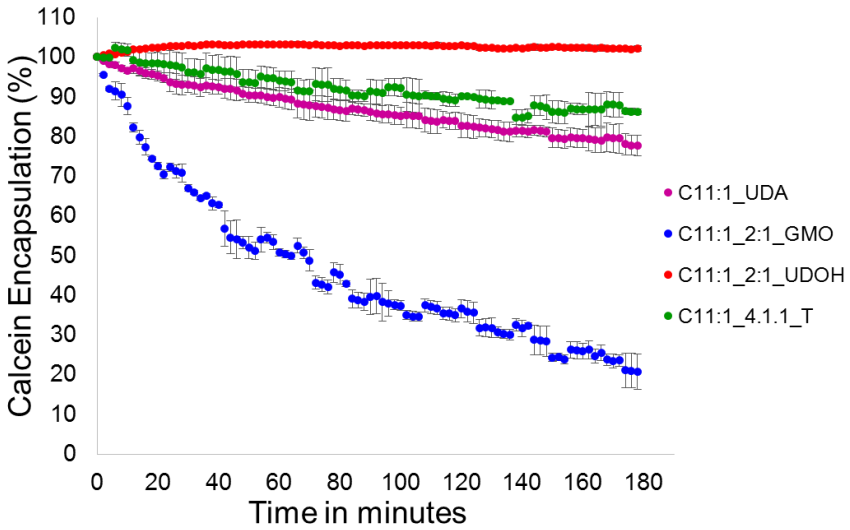


**Figure 7:** Calcein leakage assay to evaluate the permeability of the four different C11:1 system towards calcein. Percentage of calcein encapsulation was plotted against time (in minutes). Decrease in the % encapsulation, indicates increased permeability. C11_UDA, C11_2:1_UDG, C11_2:1_UDOH and C11_4:1:1_T represents, homogenous undecylenic acid system, undecylenic acid- glyceryl 1-undecylenate mixed binary system, the undecylenic acid**-** undecylenyl alcohol mixed binary system, and the tertiary C11:1 system, respectively. n = 4; error bars represent standard deviation (s.d.).

### 2.5 Mechanism by which compositional heterogeneity might confer stability on fatty acid membranes in the presence of $Mg^{2+}$

In order to understand the mechanism behind the increased stability of fatty acid membranes in the presence of their derivatives, the retention of the fatty acid moieties in the membrane was first investigated as a function of their composition.

Fatty acid molecules stay in dynamic equilibrium and can therefore readily exchange between the membrane phase and the free monomers present in the solution (28). The free monomers possess a negative charge and can interact with the free $Mg^{2+}$ ions, potentially forming crystals and unordered aggregates. Therefore, more the amount of free fatty acids in the solution, greater will be the aggregate formation. It is known from literature that phospholipids can increase the retention of fatty acid molecules in the membrane, resulting in a decrease in the free fatty acids, thus stabilizing the membrane in the presence of $Mg^{2+}$ ions (29-30). We started by investigating to understand whether the fatty acid derivatives might stabilize the membranes in a manner similar to how the phospholipids do. The free fatty acid monomers present in in the solution was estimated for all the eight membrane systems using liquid chromatography coupled with mass spectrometry (SI, Method 2.8). It was observed that the amount of free fatty acid present in the C11_UDA system was higher compared to the other C11-based membrane systems as shown in Figure 8A. Presence of both the derivatives showed a decrease in the dissociation of free UDA molecules into the solution, thereby stabilizing the membrane. In case of the C18 membrane systems, it was observed that both the derivatives decrease the dissociation of OA into the solution which is similar with the C11:1 based systems (SI, Figure S13). The amount of free fatty acids present in the solution for each system corroborates with their CVC (Figure 1; SI, Figure S1). Higher CVC indicates higher amount of monomers present in the solution. However, this did not explain the ionic stability difference between the systems, C11_2:1_UDG and C11_2:1_UDOH, as the C11_2:1_UDG system was found to be more stable than the C11_2:1_UDOH system, in the presence of $Mg^{2+}$ ion (Figure 6). In order to understand the aforesaid further, the zeta potential (ζ) of all the systems was measured. This was to get an idea of the surface charge density associated with the different vesicle systems. The free $Mg^{2+}$ ions present in the solution tend to interact with the negatively charged fatty acid vesicles. Therefore, a decrease in the net negative charge on the vesicle surface would lead to a weakening in the interaction with the $Mg^{2+}$ ions. It has been previously shown that, addition of phospholipids to oleic acid membranes can decrease the zeta potential of the membranes, which in turn stabilizes the fatty acid membranes in the presence of $Mg^{2+}$ ions (26). The zeta potential measurements (SI, method 2.7) revealed that addition of UDG to the UDA membrane, decreased the net negative charge density, as shown in Figure 8B. The net negative charge of the C11_2:1_UDOH binary system was found to be equal to that of the C11_UDA system, while the negative charge on the tertiary C11_4:1:1_T system was found to be somewhere in between, i.e it was higher than the C11_2:1_UDG system but lower than the C11_UDA system. This observed change in the negative charge density can be explained by considering the size of the different head groups. Because of the bulky head group of the UDG moiety, less number of deprotonated fatty acid molecules would be present in a unit surface area. This is in contrast to the number of fatty acid molecules that could be present in the same unit surface area of the C11_UDA and C11_2:1_UDOH membrane systems. This observation can be further justified by taking into account the permeability of the membrane systems as shown in Figure 7. The presence of bigger head group leads to less efficient packing, thus increasing the overall permeability of the said system. The same pattern of zeta potential values were also observed for the C18:1-based systems (SI, Figure S12). The C18_2:1_GMO binary system was found to possess the lowest negative charge. Therefore both of the components, i.e. retention of fatty acid

molecules in the membrane, and changes in the zeta potential on adding derivatives, could contribute towards the increased stability of the mixed membrane systems.

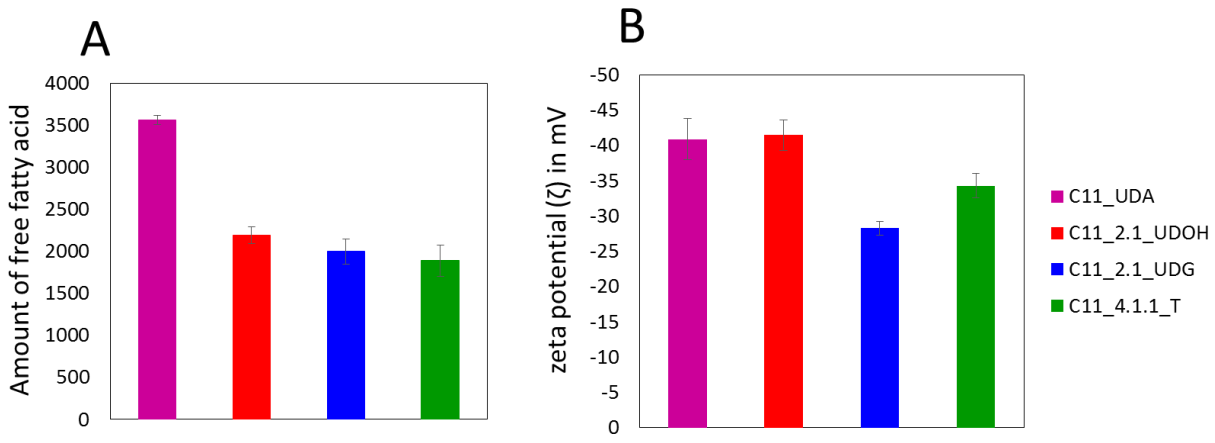


**Figure 8:** 8A shows the relative amount of free fatty acids in the solution as a function of the membrane composition. C11_UDA, C11_2:1_UDG, C11_2:1_UDOH and C11_4:1:1_T represents, homogenous undecylenic acid system, undecylenic acid- glyceryl 1-undecylenate mixed binary system, the undecylenic acid-undecylenyl alcohol mixed binary system, and the tertiary C11:1 system, respectively. Presence of the derivatives UDG and UDOH increases the retention of UDA in the membrane. n = 6; error bars represent standard deviation (s.d.). The difference between the mean values for homogenous C11_UDA with other three heterogeneous systems is significant based on student t test with *p*-value < 0.05 by a one-tailed test; 8B indicates the zeta potential measurements of the various C11:1 systems as a function of their composition. n = 5; error bars represent standard deviation (s.d.). The difference between the mean values for C11_UDA and C11_2:1_UDG is significant based on student t test: *p*-value < 0.00001. The difference between the means for C11_UDA and C11_4:1:1_T is significant based on student t test: *p*-value < 0.00083 by a one-tailed test.

### 2.6 Multiple selection pressures (MSPs) and the fitness landscape of protocell membranes as a function of their composition

As the stabilizing effect of different derivatives seem to be context dependent and vary on the selection pressure and the environment, the stability of different membrane systems under multiple selection pressures was investigated using the C11:1 systems. The three selection pressures discussed above i.e. CVC, formation of vesicles in alkaline pH regimes and stability in the presence of $Mg^{2+}$ ion, were applied in a sequential manner. Furthermore, to understand if there was any influence coming from the order of the applied selection pressures, a total of six different combinations were tried by varying their sequence. After applying each selection pressure, the solution was observed under microscope to look for the presence of vesicles (SI, Method 2.9). The MSP experiment revealed that even though the binary mixed systems (C11_2:1_UDOH and C11_2:1_UDG) are more stable under a given selection pressure, when all the three selection pressures were applied, the tertiary system (C11_4:1:1_T) stood a better chance at survival. As shown in Figure 9, all the four C11:1 membrane systems formed vesicles at a concentration of 60 mM at pH 8. When all the four systems were diluted to a final lipid concentration of 20 mM using 200 mM bicine buffer of pH 8, the C11_UDA

system failed to form vesicles because of its high CVC, while the other three mixed systems continued to show vesicles. Next, out of the three mixed systems, only lipid crystals and aggregates were observed in C11_2:1_UDOH system when $Mg^{2+}$ ions were added to the solution at a concentration of 14 mM. Among the other two mixed systems, C11_2:1_UDG and C11_4:1:1_T, being less sensitive to $Mg^{2+}$ ion concentration (Figure 6), continued to show vesicle formation even at 14 mM concentration of $Mg^{2+}$ ions. Finally, when the pH of the solution was adjusted to 10, the C11_2:1_UDG system failed to assemble into vesicles. Consequently, only the tertiary C11_4:1:1_T system was able to assemble into vesicles when all the selection pressures were applied back to back. Significantly, this observation was independent of the sequence of the selection pressures applied, as shown in Figure S14 to S18 (SI).

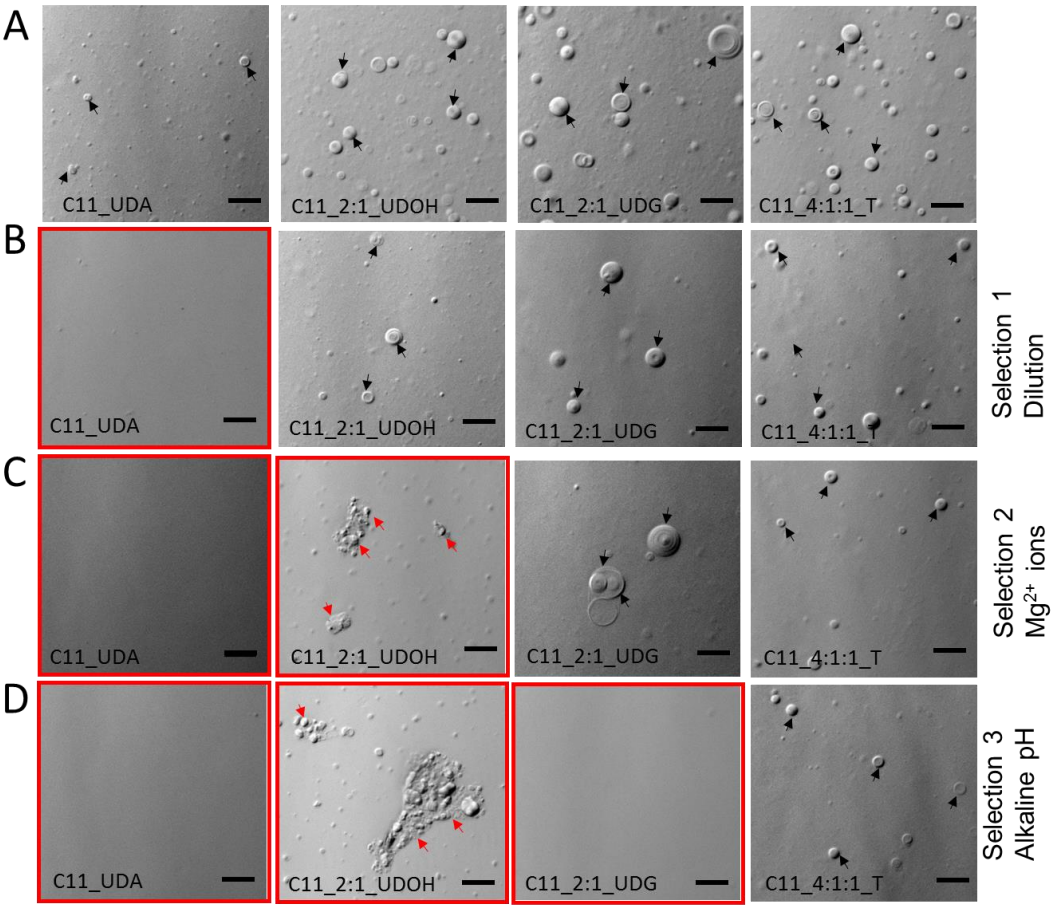


**Figure 9:** Vesicle stability as a function of their composition under multiple selection pressures applied consecutively. C11_UDA, C11_2:1_UDG, C11_2:1_UDOH and C11_4:1:1_T represent, homogenous undecylenic acid system, undecylenic acid-glyceryl 1-undecylenate mixed binary system, the undecylenic acid- undecylenyl alcohol mixed binary system, and the tertiary C11:1 system, respectively. Panels A to D represent different pebiotically relevant selection conditions. A) 60 mM total lipid concentration at pH 8. B) Dilution of the total lipid concentration to 20 mM at pH 8. C) 20 mM total lipid concentration at pH 8, in the presence of 14 mM $Mg^{2+}$. D) 20 mM total lipid concentration in presence of 14 mM $Mg^{2+}$ at pH 10. The red enclosures indicate absence of vesicles. The black and red arrows indicate vesicles and

aggregates (fatty acid crystals and droplets), respectively. The scale bar in all images are 10 microns.

## 3 Discussion

It is reasonable to assume that the environments of the early Earth would have been heterogeneous and replete with different kinds of amphiphiles that could readily assemble into membrane structures. The physico-chemical properties of these primitive compartments would have been largely affected by their environmental conditions such as pH, temperature, cation concentration, dilution events etc. Therefore, evaluating the formation and fitness of such primitive membranes against different environmental conditions, is a crucial step towards understanding the properties of such membrane systems. In the present study, the formation and stability of model proto-membrane systems, and the influence of membrane compositional heterogeneity on their fitness, was systematically evaluated under different environmental selection pressures. When the fitness of different membrane systems was tested against dilution regimes, it was observed that the incorporation of both of the derivatives, i.e. fatty alcohol and glycerol monoester versions, decreases the CVC of the system across varying chain lengths. In case of the C11:1 systems, the C11_2:1_UDG and the C11_4:1:1_T system were found to possess the lowest CVC, which is around 2 mM. The CVC of C11_2:1_UDOH system was found be much lower than that of the C11_UDA system, which is about 35 mm, but is higher than the C11_2:1_UDG system (Figure 1). Among the C18:1 systems, the C18_2:1_GMO system was found to have the lowest CVC of about 0.01 mM, followed by the C18_4:1:1_T system, then the C18_2:1_OOH system (SI, FigureS1). Therefore, the influence of the glycerol monoester on lowering the CVC is greater than the fatty alcohol. A crucial aspect to highlight is that three different methods were employed to estimate the CVC as any of the individual techniques is not sufficient in isolation to understand the complete picture. DPH can bind to both the higher order amphiphilic structures, i.e the ordered vesicles, and the disordered random aggregates (15). As for CVC estimation by turbidity measurement, both, vesicles and aggregates can contribute to the resultant turbidity of a solution, giving false positive values. Additionally, recent studies have highlighted that turbidity is a combined function of particle size, lamellarity and the structure of the particles (31), which makes the read out non-trivial to interpret. On the other hand, microscopic analysis tends to miss out on smaller sized vesicles due to them being diffraction limited. Given the aforesaid limitations of the techniques, three independent techniques were combined to narrow down the CVC to a confident range. This is pertinent as several previous studies have predominantly used turbidity measurements to report CVC values, which tend to be misleading due to the above detailed issues. Subsequently, the effect of alkaline pH on the vesicle forming capability was evaluated. In alkaline pH regimes, homogenous fatty acid systems were found to be most sensitive. The C11_2:1_UDG system failed to form vesicles above a pH of 9. Whereas, the C11_2:1_UDOH and the C11_4:1:1_T system continue to assemble into vesicles even at pH 11. In case of C18:1, all three mixed membrane systems, namely, the C18_2:1_GMO, C18_2:1_OOH and the C18_4:1:1_T systems, were found to form vesicles even at pH 11. These observations can be explained by factoring in the protonation status of the different species. As the pH increases, the fatty acid species get deprotonated, allowing it to

hydrogen bond with the hydroxyl group of the alcohol or the glycerol head group, resulting in membrane assembly (3, 6). In the C11_2:1_UDG system vesicles were not observed above pH 9 (Figure 4). Hence, the stability of the C11_4:1:1_T membrane systems predominantly seem to stem from the alcohol moiety above pH 9. While investigating the C18_2:1_GMO system, vesicles were observed even at pH 10 to 11 in the 2:1 ratio combination. Given this, the ratio of oleic acid to its glycerol monoester was decreased to 4:1 to see what this would do to the self-assembly capability. Upon decreasing the monoester moiety, vesicles were not observed above pH 10 (SI, Figure S6). This suggests that, at very alkaline pH, the glycerol monooleate starts to get deprotonated, thus failing to be a good hydrogen bonding partner. The C18_2:1_OOH system does not form vesicles at pH 8, while the C18_OA system can. This might potentially come from the predominance of the protonated species at this pH, which hampers efficient hydrogen bonding. This indicates to a pH range within which the derivative in the membrane could positively influence membrane stability. The ability of the C18_2:1_GMO and C11_2:1_UDG systems, to assemble into vesicles at lower pH, while the corresponding homogenous fatty acid systems cannot, indicates that at the lower pH regimes, the stability of the mixed membrane is predominantly conferred by the glycerol monoester moiety. When the stability of the membrane systems in question was evaluated against $Mg^{2+}$ ion concentration, the C11_2:1_UDG system was found to be the most resilient among the C11:1 systems, followed by the C11_4:1:1_T system. The C11_2:1_UDOH system was found to be the second most sensitive system, after the C11_UDA system. This result corroborates with a previous study where Monnard et al. demosntrated that the glycerol monoester derivatives possess greater stabilizing activity than that of the alcohol derivative, towards $Mg^{2+}$ ions using C10:0 fatty acid system (10). In the C18:1 systems, the C18_2:1_OOH and the C18_4:1:1_T system were found to be equally stable towards higher $Mg^{2+}$ ion concentrations. In this chain length system, the C18_2:1_GMO system was found to be the second most sensitive system, after the C18_OA system; a pattern that was different from what we saw in the C11:1 systems. This could potentially be attributed to the increase in the chain length. As the chain length increases, parameters like membrane thickness, membrane packing, CVC etc. also change. Previous studies carried out on C10:0 systems, emphasized on monoacyl glycerol as the most stabilizing in the presence to $Mg^{2+}$ ions, and hence, was argued as being a more suitable derivative to incorporate into fatty acid membranes (10-11). However, our results demonstrate that when moving to longer chain length system like the C18:1 systems, the fatty alcohol derivative seems to be more stabilizing against higher $Mg^{2+}$ ions, thereby highlighting the importance of long chain fatty alcohols. Protocells would have lacked membrane embedded complex transport machinery. Therefore, they must have depended on the membrane dynamics intrinsic to the system, for the transport of nutrients and wastes across the boundary (26-27). Compartments with very high or very low permeability are not suitable to support protocellular life forms. Rather, compartments possessing moderate permeability, which will allow small molecules to go in without letting the internal components to permeate out, would be more suitable to support protocellular life. Fatty acid membranes are dynamic, thermodynamically stable structures, therefore suitable for supporting primitive life forms (27). Recent studies suggest potential pathways for the transition from fatty acid to phospholipid rich membranes (26, 30). However, incorporation of phospholipid into fatty acid membranes decreases their permeability by decreasing the membrane dynamics (26-27, 30), which in turn will hinder the

transport of matter. Our results show that, incorporation of UDG into UDA membrane in 2:1 ratio increase the membrane permeability when compared to what is seen in the homogenous UDA membrane system. On the other hand, the addition of fatty alcohol, UDOH, to UDA, decreases the permeability of the system. The tertiary C11_4:1:1_T system possesses moderate permeability, a resultant of contributions coming from both of the derivatives. These observations can be explained by considering how different the head groups of the two derivatives are. Being a bulky head group, glycerol monoester hinders efficient membrane packing, thus leading to an increase in the permeability of the system. Fatty alcohols, on the other hand, possess a small head group, which can potentially increase membrane packing, resulting in a decrease in the permeability. A recent study from Rendón at al showed that in the presence of fatty alcohol, the vibrational freedom of the carboxyl group of the fatty acid decreases, as a result of increased packing, further supporting our hypothesis (9). In conclusion, our study highlights that the stability of the model protomembranes is not a linear property of its compositional heterogeneity. This is because, it is governed differently by different prebiotically relevant parameters. The effect of each head group upon the stability of the membrane, is context-dependent. It depends on the environment and the selection pressure that the system is being subjected to. For example, at alkaline pH of 10 or above, the UDOH stabilizes the mixed membranes, while UDG cannot, whereas in the presence of $Mg^{2+}$ ions, the UDG system is more stabilizing than the UDOH system. Overall, the tertiary system was found to be more stable in the presence of all the three selection conditions, when applied consecutively. Different selection pressures can shape the evolution of protomembranes in terms of its composition. Under multiple selection conditions, the more complex a membrane system is in terms of its composition, the more versatile it is, and possesses a better chance at survival. Thus, with time, membranes with greater compositional heterogeneity, would have gotten selected and become enriched for when subjected to varying selection pressures. Therefore, this study provides an insight towards understanding the molecular evolution of prebiological membranes and how the emergence of complex mixed membrane systems would have come about on the early Earth.

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## Conflicts of interest

The authors declare that they have no conflict of interest.

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