

## Stability of Model Membranes in Extreme Environments

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**Abstract** The first forms of cellular life required a source of amphiphilic compounds capable of assembling into stable boundary structures. Membranes composed of fatty acids have been proposed as model systems of primitive membranes, but their bilayer structure is stable only within a narrow pH range and low ionic strength. They are particularly sensitive to aggregating effects of divalent cations ( $Mg^{+2}$ ,  $Ca^{+2}$ ,  $Fe^{+2}$ ) that would be present in Archaean sea water. Here we report that mixtures of alkyl amines and fatty acids form vesicles at strongly basic and acidic pH ranges which are resistant to the effects of divalent cations up to 0.1 M. Vesicles formed by mixtures of decylamine and decanoic acid (1:1 mole ratio) are relatively permeable to pyranine, a fluorescent anionic dye, but permeability could be reduced by adding 2 mol% of a polycyclic aromatic hydrocarbon such as pyrene. Permeability to the dye was also reduced by increasing the chain length of the amphiphiles. For instance, 1:1 mole ratio mixtures of dodecylamine and dodecanoic acid were able to retain pyranine dye during and following gel filtration. We conclude that primitive cell membranes were likely to be composed of mixtures of amphiphilic and hydrophobic molecules that manifested increased stability over pure fatty acid membranes.

**Keywords** Amphiphiles · Alkylamines · Early cellular life · Extreme environments · Fatty acids · Primitive membranes · Self-assembly

### Introduction

The membrane boundaries of contemporary cells are composed largely of phospholipids and, in the case of eukaryotes, phospholipids and cholesterol. However, it is unlikely that membranes of the earliest cellular forms of life incorporated such lipids, which require complex metabolic pathways for their synthesis. Instead it has been proposed that primitive membranes self-assembled from simpler amphiphilic molecules such as fatty acids (for review, see Deamer and

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Dworkin 2005). These are present in carbonaceous meteorites (Lawless and Yuen 1978; Shimoyama et al. 1989), and can be synthesized under geochemical conditions by Fischer-Tropsch type reactions (Nooner et al. 1976; McCollom et al. 1999; Rushdi and Simoneit 2001).

It is well-established that fatty acids can self-assemble into membranous compartments (Ekwall and Mandell 1969; Gebicki and Hicks 1973; Hargreaves and Deamer 1978). Saturated fatty acids having chain lengths in the range of 10–12 carbons form bilayer membranes and vesicular structures at room temperature when the pH of the solution is near the apparent  $pK_a$ , typically in the range of 7–8 (Apel and Deamer 2005; Morigaki and Walde 2007). Simoneit et al. (2007) reported that monoglycerides produced by condensation reactions under simulated prebiotic conditions can self-assemble into membranous structures. Longer chain saturated fatty acids also form bilayer membranes, but only if their hydrocarbon chains are maintained in a fluid state, either by increasing the temperature or by the presence of double bonds (Hargreaves and Deamer 1978). In addition to these structural properties, fatty acid vesicles are capable of growth (Berclaz et al. 2001; Cheng and Luisi 2003; Chen and Szostak 2004), self-reproduction (Luisi et al. 2004; Hanczyc et al. 2003), and a primitive form of competition for resources (Chen et al. 2004; Stano 2007).

The relevance of fatty acid vesicles to origin of life scenarios is recognized because they are chemically simple versions of amphiphiles, in contrast to “modern” phospholipids (Monnard and Deamer 2003; Hanczyc et al. 2003; Luisi et al. 2006; Thomas and Rana 2007). Fatty acids and other amphiphilic compounds present in carbonaceous meteorites can participate in membrane self-assembly processes (Deamer 1986; Deamer and Pashley 1989), as can the mixtures of fatty acids and fatty alcohols synthesized by Fischer-Tropsch type reactions under simulated prebiotic conditions (Monnard et al. 2002; Rushdi and Simoneit 2001). Furthermore, fatty acid vesicles can encapsulate functional enzymes such as polymerases (Walde et al. 1994; Oberholzer et al. 1995) and also act as catalytic templates for the polymerization of aniline (Namani and Walde 2005). However, the complex self-assembly behavior of fatty acids in aqueous solution depends strongly on ionization state, concentration, temperature and counter ions (Cistola et al. 1986, 1988), and their bilayer structure is disrupted by divalent cations (Monnard et al. 2002; Morigaki and Walde 2007).

For these reasons, it is desirable to explore mixtures of amphiphilic compounds that are expected to increase the range of stability of the resulting membranes by reducing their sensitivity to environmental parameters. For instance, it has previously been reported that the pH range of vesicle formation can be shifted to acidic pH by addition of surfactants such as sodium dodecylbenzene sulfonate (SDBS) to decanoic acid (Namani and Walde 2005) and to the alkaline pH by the addition of decanol to decanoic acid (Monnard et al. 2002).

The present study addresses three questions related to the stability of membrane vesicles in the prebiotic environment:

1. Under what conditions can membranes assembled from simple amphiphiles be made stable at extreme pH ranges and high divalent cation concentrations?
2. What is the minimal lipid concentration and chain length for the assembly of amphiphiles into stable membranes under these conditions?
3. What amphiphile compositions are sufficiently stable to provide a permeability barrier against the leakage of encapsulated solutes?

Backlund et al. (1997) investigated the phase behavior of various alkyl amines, carboxylic acid and water complexes by constructing a ternary phase diagram. The phase diagrams suggest that mixtures of alkyl amines and fatty acids might display unusual properties in regard to their ability to form lamellar aggregates. We decided to explore such binary mixtures in terms of their ability to assemble into vesicular structures at extreme pH

ranges and divalent cation concentrations. Because polycyclic aromatic hydrocarbons (PAHs) are also abundant organic components of carbonaceous meteorites (Stephan et al. 2003) and resemble cholesterol, we also decided to investigate admixtures of PAHs as potential stabilizing agents that could enhance the ability of such vesicles to maintain concentration gradients of ionic solutes.

## Materials and Methods

Decanoic acid (capric acid, 99%), dodecylamine (>95%), dodecanoic acid (>99%), pyrene (>99%), and decylamine (>95%) were purchased from Fluka (USA), pyranine (1-hydroxypyrene-3,6,8-trisulfonic acid, 98% pure) from Acros, sea salts from Sigma, and Sephadex G25 from Amersham Biosciences (Sweden).

### Preparation of Vesicles

In a typical preparation, appropriate amounts of decylamine and decanoic acid (1:1 mole ratio) were weighed into a glass test tube and mixed with 10% molar excess of NaOH in order to titrate the decanoic acid completely to sodium decanoate. The mixture was then suspended in a 0.1 M CHES buffer (2-(*N*-cyclohexylamino)ethane sulfonic acid) pH 11 to a final concentration of 0.1 M. The suspension pH was adjusted to pH 11 and the presence of vesicles was confirmed by phase microscopy.

To prepare vesicles at lower pH ranges, 1:1 mole ratios of decylamine and decanoic acid were dissolved in chloroform. This ratio was chosen because of the expectation that stabilizing hydrogen bonds or electrostatic bonds would form between equimolar concentrations of the two amphiphilic molecules. Other ratios (2:1 and 1:2) were also investigated in preliminary experiments, and the 1:1 ratio was found to be optimal. Aliquots were evaporated by a gentle stream of nitrogen gas, the resulting thin films were dispersed in 0.1 M sodium phosphate or borate and the pH was adjusted to the desired range with 1 N HCl.

In some cases 1 mol% pyrene was added to the lipid solutions in chloroform so that the resulting vesicles could be viewed by epifluorescence microscopy. Pyrene and benzylanthracene at 2 mole% were also prepared to investigate the effects of added PAH on membrane stability and permeability.

### Titration Experiments and Microscopy

A stock dispersion of 0.1 M decylamine and 0.1 M decanoic acid was prepared in 0.11 M NaOH. Aliquots of the stock (250  $\mu$ l) were placed in 25 small glass test tubes and titrated with 0.1 N HCl (from 25  $\mu$ l to 500  $\mu$ l). The final volumes were adjusted to 1 ml with Milli Q water so that each sample contained 25 mM of decylamine and decanoic acid. After one day equilibration the pH of the sample was measured and recorded. The samples were examined by phase microscopy at  $\times 40$  and  $\times 100$  magnification.

### Determining Critical Concentration of Vesicle Formation (CVC)

Decylamine/decanoic acid mixtures at different total concentrations were prepared and absorbance (turbidity) was measured at 350 nm in 0.1 M CHES buffer (pH 11) and also in 0.1 M borate (pH 3.0). The concentration at which the absorbance first began to increase was taken to be the critical concentration for vesicle formation (CVC).

## Stability of Decylamine and Decanoic Acid

The stability of vesicles was tested by measuring the turbidity as a function of time. Vesicles at pH 11 and pH 2 containing 1 mol% of pyrene were prepared as described in this section, followed by bath sonication for about 15 min. The turbidity of the resulting small vesicles was monitored at 500 nm as a function of time, and increased turbidity was taken to indicate physical changes in the vesicle dispersion, such as vesicle growth, fusion or aggregation.

## Dye Encapsulation

The 1:1 mole ratio mixtures of the dodecylamine and dodecanoic acid were dissolved in 1 ml of chloroform, and the chloroform was evaporated by nitrogen gas. To the resulting thin film, 2 ml of 0.1 M CHES buffer was added containing 1.0 mM pyranine. The suspension was then vortexed and heated in order to produce efficient mixing of the components. The pH of the suspension was adjusted to 11.5 by addition of NaOH, followed by extrusion through 200 nm Nucleopore polycarbonate membrane filters using a Liposfast extruding device (Avanti Polar Lipids). An aliquot of the extruded suspension (0.5 ml) was applied to a Sephadex G25 column (1 cm diameter x 12 cm length) and eluted with the same buffer. If dye was encapsulated under these conditions, it was present as a separate peak and the vesicles were collected in the excluded volume of the eluant.

Similarly, a thin film of decylamine and decanoic acid (1:1 molar ratio) was prepared with 2 mol% pyrene. The thin film was dispersed in 0.2 M CHES buffer at pH 11 containing 1 mM pyranine. The suspension was vortexed and sonicated for 10 min to produce smaller vesicles, as described above. Aliquots of the preparation (0.5 ml) were then applied to a Sephadex G25 column (1 cm diameter × 10 cm length) and eluted with 0.2 M CHES buffer.

## Results

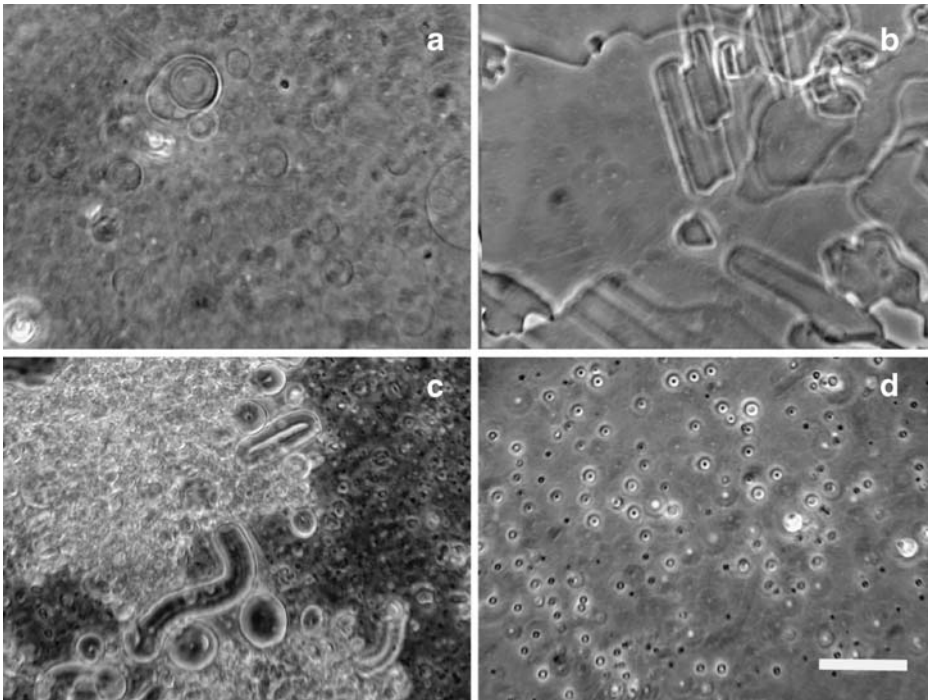
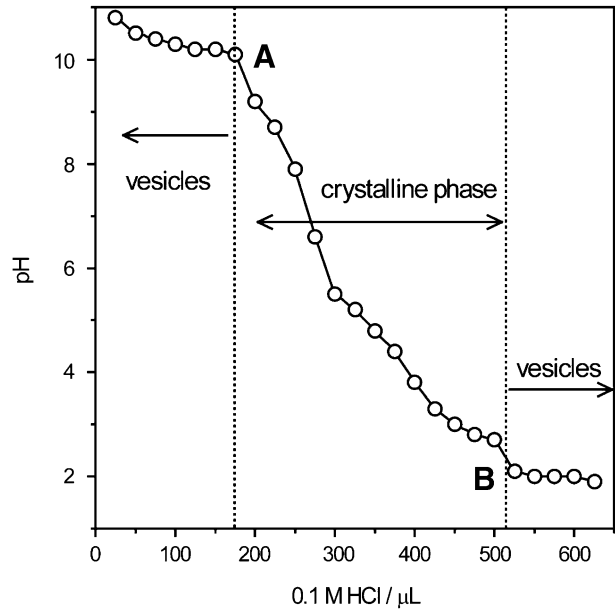
### Titration of Decylamine/Decanoic Acid Mixtures

Our first goal was to determine the pH ranges at which vesicles produced by 1:1 mixtures of decylamine and decanoic acid are stable. To address this question, we titrated the vesicle preparations between pH 11 and 2 as described in “Materials and methods”, but without CHES buffer, and used phase contrast microscopy to visualize the physical state of the components. The titration curve (Fig. 1) shows plateaus at high pH (>10; above point B in Fig. 1) and at low pH (<3; below point A in Fig. 1), with little buffer capacity at neutral pH ranges. At both the high and low pH range, vesicular structures were apparent, with several micrometers in diameter (see Fig. 2a and d). Remarkably, at intermediate pH ranges only crystalline aggregates were present (Fig. 2b and c), but the crystalline phase could be reversibly transform into vesicles either by raising or lowering the pH. Figure 3 shows a photograph of the samples from the titration curve, and the physical changes are clearly apparent to the eye as the mixture goes from vesicular to crystalline to vesicular phase during the titration from pH 2 to pH 11.

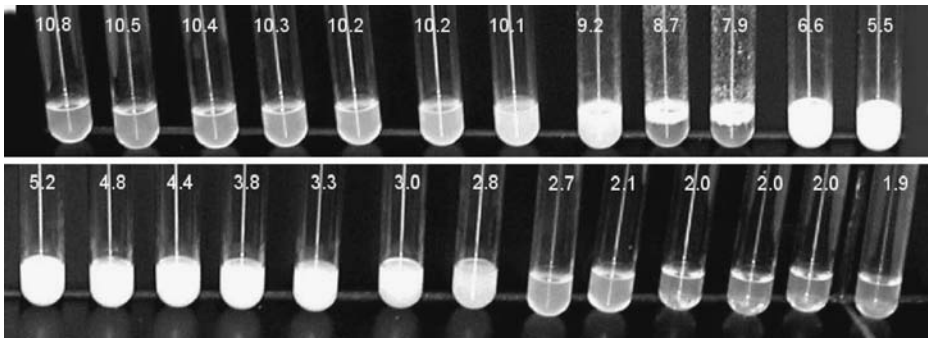
### Critical Concentration for Vesicle Formation (CVC)

In general, pure fatty acid vesicles form at relatively high concentrations of fatty acid (above CVC), and vesicles disperse into free fatty acid monomers if the concentration falls

**Fig. 1** Titration curve of decylamine and decanoic acid mixtures (25 mM each) at room temperature. The vesicle regions are shown separated by dotted lines drawn at *A* and *B*. A crystalline phase was present in the region between *A* and *B*



**Fig. 2** Phase contrast light micrographs of the titration curve samples of decylamine and decanoic acid mixtures (25 mM each) in aqueous solutions at **a** pH 10.8, **b** pH 6.6, **c** pH 4.4 and **d** pH 2.0



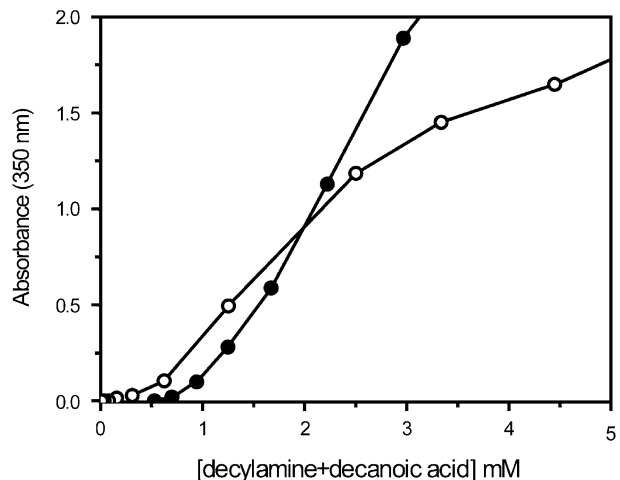
**Fig. 3** Samples from the titration curve of Fig. 1 are shown with the pH of each sample noted on each test tube

below CVC. We tested the CVC of decylamine/decanoic acid mixtures at pH 11 and pH 3 by turbidity measurements, in which turbidity is monitored at 350 nm as a function of total amphiphile concentration. As clearly seen in Fig. 4, an increase in absorbance is observed in both cases at specific amphiphile concentrations. The break points (ca. 0.5 and 1 mM, respectively) are estimates of the CVC of decylamine and decanoic acid vesicles at both pH's 11 and 3. Significantly, these values are about 10 times lower than the pure decanoic acid vesicles (Namani and Walde 2005).

#### Membrane Stability in the Presence of Divalent Cations

The formation of membranes on the early Earth is generally assumed to have occurred in marine environments. It has been demonstrated that fatty acid vesicles precipitate in the presence of low concentrations of divalent cations such as  $Mg^{+2}$ ,  $Ca^{+2}$  and  $Fe^{+2}$  when the cations form insoluble soaps with the anionic fatty acids (Monnard et al. 2002; Chen et al. 2005). The chemical incompatibility between pure fatty acid vesicles and divalent cations represents a significant question when we consider the origin of the first cellular compartments.

**Fig. 4** Critical vesicle concentration (CVC) of decylamine and decanoic acid mixtures (1:1 mole ratio) was determined by measuring turbidity at 350 nm as a function of concentration. The *open circles* correspond to vesicles were prepared in 0.1 M borate at pH 3 and the *closed circles* correspond to vesicles prepared in 0.1 M CHES buffer at pH 11.5



We tested the stability of decylamine and decanoic acid vesicles (at pH 2) in the presence of divalent cations such as  $Mg^{+2}$ ,  $Ca^{+2}$ , and also in sea water. As shown in Fig. 5, light microscopy reveals that decylamine/decanoic acid vesicles are stable in the presence of 0.1 M  $MgCl_2$  (Fig. 5a), 0.1 M  $CaCl_2$  (Fig. 5b) and 40 mg/ml sea salts (Fig. 5c). We account for the stability of vesicles to the fact that only neutral (protonated) decanoic acid is present at low pH, and therefore cannot undergo electrostatic association with cations. In contrast, at high pH, when decanoic acid is in its anionic form, the presence of cations even at low concentrations ( $<0.1$  mM  $Mg^{+2}$ ) strongly affects the stability of vesicles.

### Encapsulation of Pyranine Dye

The membranes of primitive cells formed by amphiphilic compounds in the early Earth environment must have been sufficiently stable to provide a diffusion barrier to ionic solutes, as phospholipids do in contemporary cells. However, it is also true that extremely low permeability would hinder the development of protocells, since selective material exchange with the environment favors the maintenance of protocells. Therefore the permeability must be fixed at some intermediate range, mediated either by passive diffusion through the bilayer or by incorporation of some form of channel.

We tested the permeability of the alkyl amine and fatty acid mixtures by capturing pyranine, a strongly fluorescent anionic dye that can be used as a marker for encapsulated volume. We found that these vesicles are relatively permeable to pyranine, and the entrapped dye cannot be recovered after elution through a gel permeation column. In other words, pyranine was released from the internal aqueous volume of vesicles as soon as a concentration gradient was established during gel filtration.

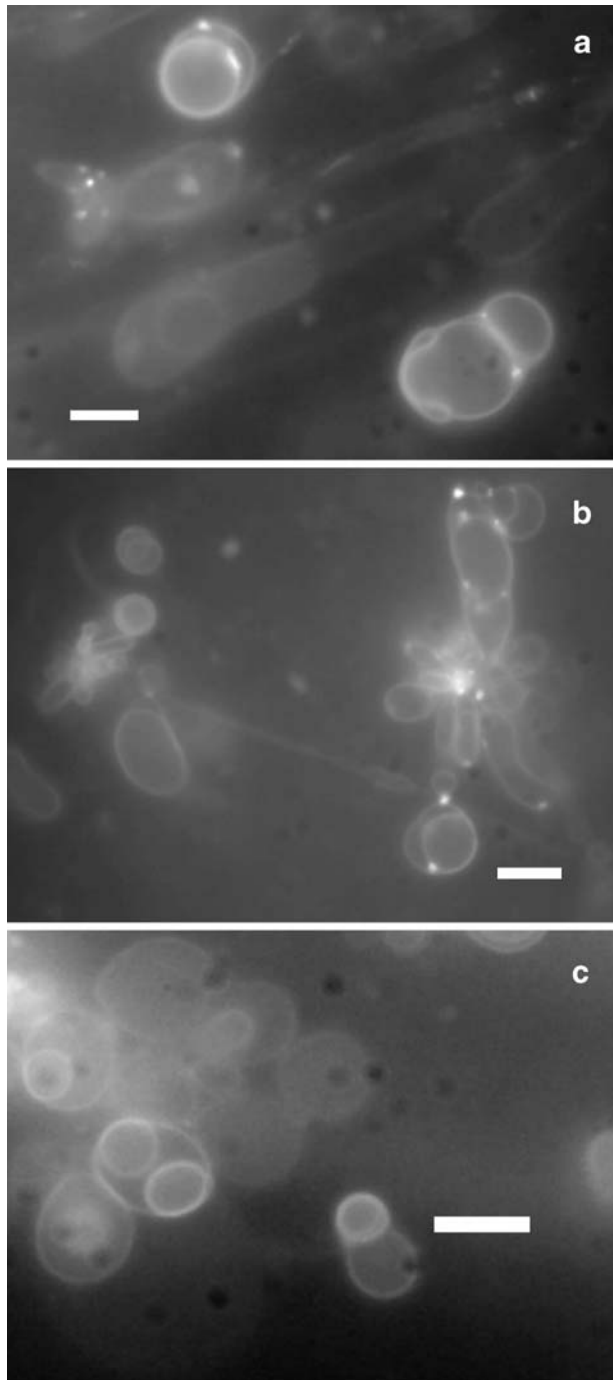
We therefore investigated the possibility that the permeability could be decreased by the addition of simple non-polar molecules, with preference for prebiotically plausible molecules. We found that a significant decrease of permeability can be attained by the addition of small amount of aromatic hydrocarbon molecules such as pyrene and benzantracene, which presumably act to stabilize the membranes by a mechanism analogous to the role of cholesterol in biological membranes (Szabo 1974). The elution profile of pyranine (2 mol%)-loaded decylamine/decanoic acid vesicles through a Sephadex G25 column is shown in Fig. 6, left panel. There is a clear separation of vesicles containing pyranine (fraction A) and free dye (fraction B).

Encapsulation of water soluble dyes can occur when the chain length of the amphiphile is increased by two carbon atoms. The entrapment of pyranine inside dodecylamine/dodecanoic acid vesicles (Fig. 6, right panel) at pH 11 is similar to that of decylamine/decanoic acid/pyrene vesicles (49/49/2 mol%). The absorbance of each fraction was recorded at a wavelength of 450 nm and the amount of dye entrapped was determined by the molar extinction coefficient of pyranine. The entrapment of pyranine was also tested with dodecylamine/dodecanoic acid vesicles at pH 2, using 1 mM pyranine as a marker for internal volume. The vesicles eluted without trapped dye, suggesting that the vesicles at low pH are significantly more permeable than at high pH ranges (results not shown).

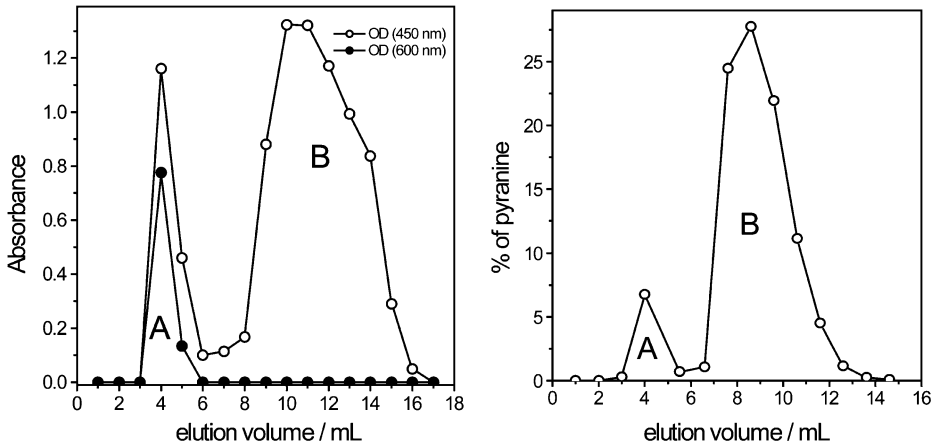
### Stability of Decylamine/Decanoic Acid Vesicles

The general stability of vesicle preparations was monitored by measuring the turbidity of sonicated vesicles as a function of time (Fig. 7). The turbidity increased over time at both pH 2 and pH 11 in vesicles prepared with 1 mol% pyrene, indicating that the vesicles were either aggregating or fusing to produce larger structures. The effect is more pronounced at

**Fig. 5** Epifluorescence micrograph of decylamine/decanoic acid (25 mM each) vesicles in presence of **a** 0.1 M  $\text{MgCl}_2$ , **b** 0.1 M  $\text{CaCl}_2$  and **c** sea salts 40 g/l. All the vesicles were prepared in 0.1 M borate at pH 2. The scale bars indicate 5  $\mu\text{m}$



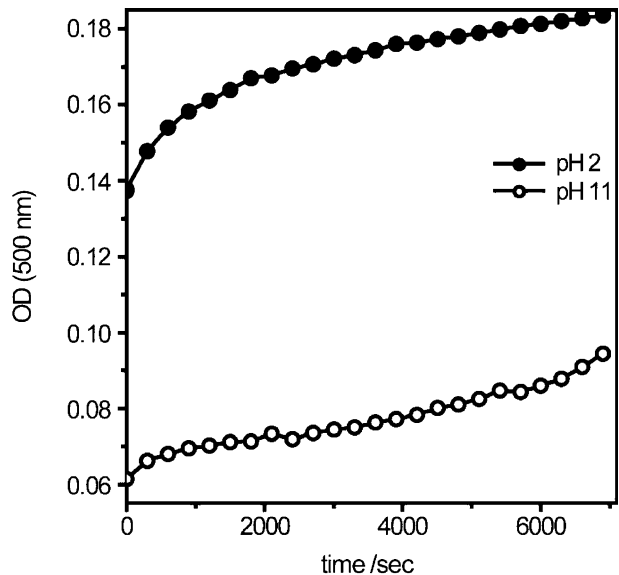




**Fig. 6** *Left panel*, vesicles were prepared from decylamine and decanoic acid (50 mM each) in 0.2 M CHES buffer at pH 11.5, with 2 mM pyranine added. The vesicle suspension was sonicated for 10 min to reduce the number of larger vesicles, and 0.5 ml of the sonicated sample was applied to a Sephadex G25 column (12 × 1 cm). The absorbance of the dye at 450 nm (*open circles*) and turbidity at 600 nm (*filled circles*) were recorded during elution from the column as a function of eluted volume. *Right panel*, dodecylamine/dodecanoic acid (25 mM each) vesicles suspension was prepared in presence of pyranine (1 mM) in 0.1 M CHES buffer at pH 11 and the suspension was extruded through 200 nm polycarbonate membranes. 0.5 ml of the extruded suspension was applied to a Sephadex G25 column (15 × 1 cm). The percent of eluted pyranine was plotted as function of elution volume. The first fraction peak in the elution profile corresponds to pyranine-containing vesicles and later fractions correspond to non-entrapped pyranine

pH 2 vesicles compared to pH 11. This result is consistent with the entrapment experiment described in the previous section, in which it was shown that decylamine/decanoic acid vesicles at pH 2 were unable to retain pyranine during gel filtration, while vesicles at pH 11 could. A more extensive study will be required to elucidate the relationship between turbidity increments and possible structural effects.

**Fig. 7** The stability of decylamine (10 mM) and decanoic (10 mM) acid vesicles at pH 11 and pH 3 was monitored as turbidity changes over time, using absorbance at 500 nm to measure turbidity. Open circles correspond to vesicles at pH 11 and the closed circles correspond to pH 2. The vesicles were sonicated for 15 min in a bath sonicator before measuring the absorbance



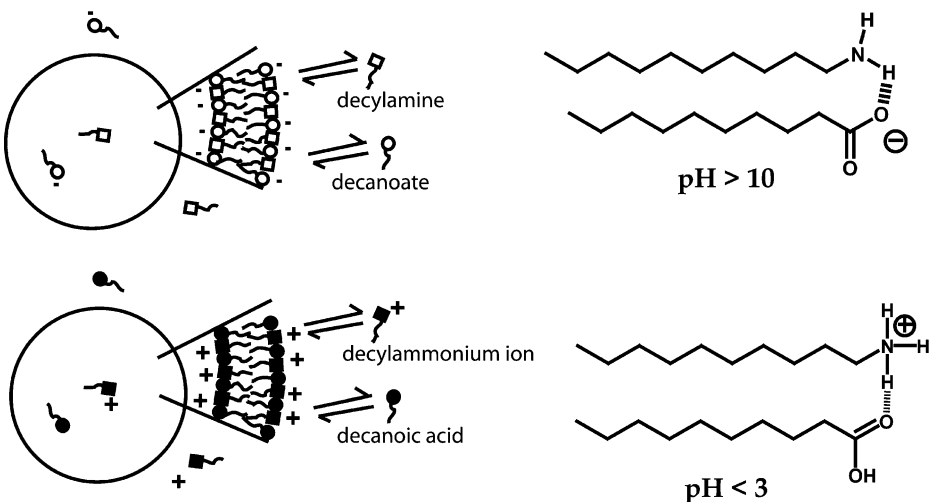
## Discussion and Conclusion

A wide range of possible environments have been proposed for the origin of life, including hydrothermal vents (Baross and Hoffman 1983) mineral surfaces such as pyrite or clay (Wachtershauser 1988; Ferris 2002) evaporating lagoons (Levy and Miller 1999) and global ice melted by giant impacts (Bada et al. 1994). In general, little attention has been paid to the ionic composition of the aqueous phase, and there has been a tacit assumption that life began in a marine environment. However, at some point of this process life became cellular, so that amphiphilic compounds able to form stable membranous boundary structures would be required. Fatty acids have been proposed as a possible primordial amphiphile (Hargreaves and Deamer 1978), but Monnard et al. (2002) pointed out that such membranes are very sensitive to pH and divalent ion composition, and argued that fresh water environments should be considered as an alternative site for life's origin.

Contemporary cellular life has evolved enzymatic pathways to synthesize a variety of complex amphiphilic lipids, and has adapted the lipid composition to virtually all the liquid water niches available in the biosphere, including temperature extremes from melting ice to hydrothermal water at 121 degrees C, from pH 1 to pH 11, and fresh water to saturated salt solutions. The single characteristic of all biological membranes is that they are not composed of a pure lipid, but always lipid mixtures, suggesting that there is a property of lipid mixtures that increases the robustness of bilayer membranes, while vesicles composed of pure lipids will be highly sensitive to environmental variables.

We do not claim that the amphiphilic mixture described in this report represents a plausible composition of primitive membrane, because as yet there are no reports indicating that alkylamines would be available in the prebiotic environment. However, we do suggest that it is worth investigating the properties of simple amphiphilic mixtures, and that the alkylamine–alkanoic acid system offers a convenient model for experimental tests.

The current study focused on finding conditions in which stable amphiphilic vesicles can form at extremes of pH and salt concentrations. We have shown that at low pH decylamine/



**Fig. 8** Schematic representation of possible structural associations of between decylamine and decanoic molecules at low pH (<3) and at high pH (>10). Square symbols represent amine head group and circles represent the carboxylate head groups. Filled and open symbols are protonated and deprotonated forms of decanoic acid (R–COOH and R–COO<sup>−</sup>) and decylamine (R–NH<sub>3</sub><sup>+</sup> and R–NH<sub>2</sub>)

decanoic acid vesicles form spontaneously, and that they are not destroyed by the presence of  $Mg^{+2}$  and  $Ca^{+2}$  ions at high concentrations. However, negatively charged vesicles at high pH ranges do tend to precipitate in the presence of divalent ions. We have also observed that these vesicles undergo fusion faster than pure decanoic acid vesicles (Namani and Walde 2005) and that their size can be reduced by sonication or extrusion.

The stability of pure fatty acid vesicles has been explained in terms of the presence of intermolecular H-bonds between the protonated and the ionic forms (Apel et al. 2002), so that pure fatty acid vesicles are stable only in the pH range where both forms coexist (generally between pH 7 and 9). In the case of decylamine/decanoic acid vesicles at high pH, we can postulate a similar behavior, in which the decylamine ( $R-NH_2$ ) acts as a hydrogen bond donor and the decanoate ( $R-COO^-$ ) as a hydrogen bond acceptor. In contrast, at low pH the decylammonium ions ( $R-NH_3^+$ ) form vesicles by hydrogen bonding with decanoic acid ( $R-COOH$ ) as tentatively depicted in Fig. 8.

The presence of vesicular structures was confirmed by epifluorescence microscopy using 1 mol% pyrene as a fluorescent label, as shown in Fig. 5. It has been problematic to demonstrate the existence of aqueous interior volumes of the decylamine/decanoic acid vesicles because these amphiphiles do not provide a sufficient permeability barrier. We were able to demonstrate internal volumes by adding 2 mol% pyrene to the decylamine/decanoic acid. The pyrene apparently acted as a stabilizing molecule, similar to the role of cholesterol in contemporary cell membranes. Stable membranes could also be produced by increasing the chain length by two carbon atoms in the dodecylamine/dodecanoic acid system, which was able to capture pyranine dye. However, vesicles composed of either system at low pH were unable to contain encapsulated dye, and microscopic examination showed that they tended to fuse to form larger vesicles (results not shown). Figure 7 also shows increased turbidity at pH 2 compared to pH 11 due to fusion of the vesicles, suggesting a relative instability of the bilayers at low pH ranges.

Finally, we observed that admixtures of small amounts of two PAH species—pyrene and benzantracene—also had a stabilizing effect on bilayer permeability of decanoic acid membranes. We propose that PAH species, which were likely to be abundant in the prebiotic environment, could have played a role similar to that of cholesterol in contemporary membranes (Szabo 1974). In fact, earlier studies of the vesicular membranes produced by amphiphilic components of the Murchison meteorite showed that the membranes were highly fluorescent, a clear indication of the presence of PAH derivatives in the bilayers (Deamer and Pashley 1989). It seems clear that further investigations of mixed amphiphilic components will be very fruitful in advancing our understanding of primitive membrane structure.

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