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LETTER TO THE EDITOR

Dynamics of the vesicles composed of fatty acids and other amphiphile mixtures: unveiling the role of fatty acids as a model protocell membrane

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Abstract

Fundamental research at the interface of chemistry and biology has the potential to shine light on the question of how living cells can be synthesized from inanimate matter thereby providing plausible pathways for the emergence of cellular life. Compartmentalization of different biochemical reactions within a membrane bound water environment is considered an essential first step in any origin of life pathway. It has been suggested that fatty acid-based vesicles can be considered a model protocell having the potential for change via Darwinian evolution. As such, protocell models have the potential to assist in furthering our understanding of the origin of life in the laboratory. Fatty acids, both by themselves and in mixtures with other amphiphiles, can form different self-assembled structures depending on their surroundings. Recent studies of fatty acid-based membranes have suggested likely pathways of protocell growth, division and membrane permeabilisation for the transport of different nutrients, such as nucleotides across the membrane. In this review, different dynamic processes related to the growth and division of the protocell membrane are discussed and possible pathways for transition of the protocell to the modern cell are explored. These areas of research may lead to a better understanding of the synthesis of artificial cell-like entities and thus herald the possibility of creating new form of life distinct from existing biology.

Keywords Vesicle · Fatty acids · Phospholipid · Ultrafast dynamics · Protocell

Introduction

The term ‘protocell’ refers to the hypothetical precursor of the first biological cell (Dzieciol and Mann 2012). Model protocells are created to study the early evaluation of life (Dzieciol and Mann 2012; Budin and Szostak 2010; Blain and Szostak 2014). The basic characteristics of any biological cell are compartmentalization, replication, transcription and metabolism (Budin and Szostak 2010). Even the simplest forms of life consist of a complex network of genes and proteins which are confined within a cell membrane. It is generally believed that protocells would have lacked the advanced machinery required for the complex biochemical reactions observed in modern cells. Therefore, it is reasonable to speculate that protocells were much simpler than any form of present-day living organisms. To try and understand the origin of life in a biophysical manner, two significant challenges must be overcome: (i) development of conceptual models able to span the transition of complexity in going from a protocell to a modern cell, and (ii) realization of these model transitions ex novo in the laboratory (Dzieciol and Mann 2012; Luisi 2006; Chiarabelli et al. 2013). The level of simplicity of our model of life depends on what we consider the momentous properties of life. A minimal cellular system should capable of self-replication, metabolism and Darwinian evolution. Self-replication is defined as continued growth and division which is dependent on the input of small molecules and energy only, and does not rely on the product of pre-existing living organisms (Chiarabelli et al. 2013). Darwinian evolution is dependent on the biological aspects of genetic variation and its phenotypic expression. The development of modern research into

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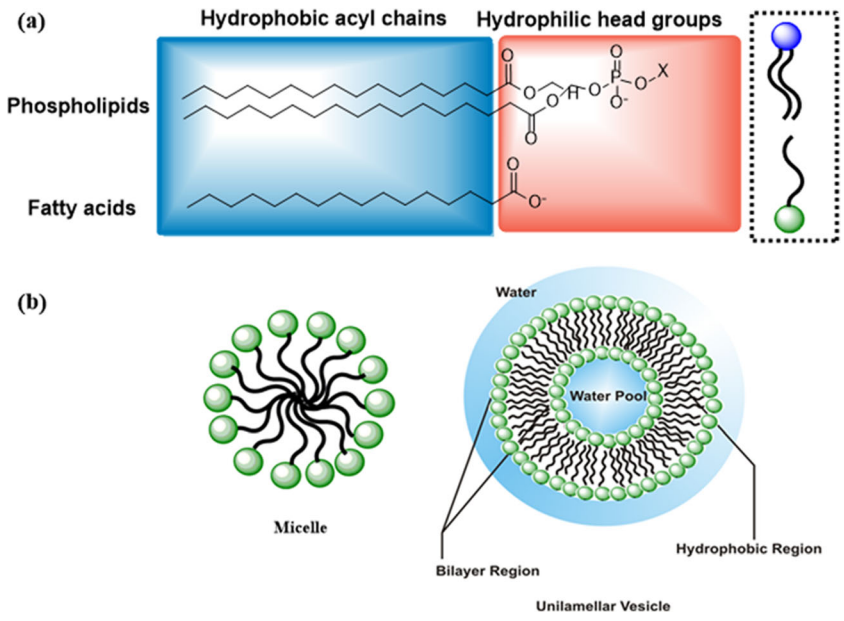
the origin of life is based on the elementary idea of molecular evolution, proposed by Oparin (Oparin 1938). According to Oparin, life is developed from the inanimate matters through a spontaneous and gradual build-up of molecular complexity. Oparin hypothesised that three essential properties, small molecule catalysis, molecular self- aggregation and amphiphilicity, are the critical factors for the creation of self-contained chemical reactions necessary for the development of self-replication, compartmentalization and coupled growth and division (Adamala and Szostak 2013a; Mansy et al. 2008; Zhu et al. 2012; Szostak et al. 2001). Developments in these topics are the subject of the present review. We specially focus on current research in which possible chemical and physical pathways are modelled with experimentally and physically (Adamala and Szostak 2013b; Mansy et al. 2008; Zhu et al. 2012; Szostak et al. 2001; Coveney et al. 2012; Mann 2013).

Construction of a model protocell in the laboratory requires an understanding of the self-assembly of different biological molecules of increasing complexity from simple structures likely to be present in the pre-biotic environment. Different biological components and other synthetic constituents are used to construct the model protocell to mimic different cellular functions under simplified conditions. All modern cells are bounded by a semi-permeable membrane which acts as a barrier between the inside and outside of the cell and regulates the flow of materials (Fraser et al. 1995). Therefore, it has been postulated that protocells were also membrane bound structures although the structure thought to be very different to that of the modern cell membrane (Chiarabelli et al. 2013). Modern cell membranes are nanometer-sized bilayer assemblies composed of mixtures of long chain amphiphilic molecules, such as phospholipids and glycolipids which consist of two hydrophobic acyl chains (Scheme 1) and one hydrophilic head group. The hydrophobic bilayer of the phospholipid membrane provides a selective barrier which is important for nutrient transport. Therefore, modern cells have complete control over the uptake of nutrients and release of waste side products through different channels, pore proteins embedded in the membrane. In water, phospholipids can self-assemble into vesicular structure with entropic hydrophobic force being principally responsible for the self-assembly (Chen and Walde 2010). The hydrophobic tail of the phospholipids disrupts hydrogen bonding networks of water molecules and lowers the free energy. Several drug molecules, including nucleic acids and proteins, can be encapsulated inside the vesicle (Tang et al. 2014). This facile entrapment of molecules by self-ordering amphiphiles at normal physiological condition has a radical impact on the modelling of protocell membrane (Tang et al. 2014). Shape changes in vesicles under the presence of different stimuli can be potentially correlated with different cellular functions such as membrane budding (Bhattacharya and Devaraj 2019). Different properties exhibited by vesicles might have been functional in the evolution of biological systems as they can be thought to be involved in some basic characteristics of life. Thus, it is tempting to suggest that today's complex cellular system is an upgraded version of primitive cell and that fatty acid-based vesicles were a possible component of these primitive cells.

Due to the low-permeability of phospholipid membranes, vesicles composed of them are implausible candidates for the model protocell membrane as various nutrients cannot permeate through the membrane, which is required for the basic functionalization of cell. Moreover, compared with other amphiphiles, phospholipids are structurally complex and in modern cells, several enzymes are required for their synthesis (Chiarabelli et al. 2013; Bhattacharya et al. 2019).



Scheme 1 **a** Chemical structure of phospholipids and fatty Acids. *X* denotes the polar groups, such as choline, glycerol or ethanolamine. This *X* is coupled with the negatively charged phosphate group. Fatty acids are the single-chain amphiphile in which carboxylate ion is the polar head group. The schematic presentation of amphiphiles is also shown. **b** Different self-assembled structures of fatty acid, such as micelle, vesicles, are formed at different pH of the medium

Therefore, it was postulated that simple amphiphiles, such as fatty acids, may be better suited as the principle component of protocell membranes. The bilayer membrane of fatty acid vesicles permits the passive diffusion of solutes in and out of the vesicle compartment. Furthermore, compared with the double chain phospholipids, the concentration of non-associated monomer of fatty acids in equilibrium with vesicles is much higher (Morigaki and Walde 2007). Thus, the flip-flop of fatty acid amphiphiles in vesicle membrane is expected to be much higher than that of phospholipid vesicles (Kamp et al. 1995). It has been hypothesised that important prebiotic molecules (including fatty acids) were formed in the hydrothermal vents (Schrum et al. 2010). From such vents, hydrogen and carbon monoxide are also released and with the help of catalysis, they were able to undergo stepwise formation of hydrocarbon tails of fatty acids, and fatty acids with different hydrocarbon chains were released into the water environment (Schrum et al. 2010). Interestingly, these single-chain fatty acids have been isolated from carbonaceous meteorites (Deamer 1985; Lawless and Yuen 1979) and synthesized under simulated pre-biotic conditions such as hydrothermal vents, interstellar ice environments (McCollom et al. 1999; Dworkin et al. 2001). Potential protocell model membranes aim to replicate a few of the characteristics of living cells, such as metabolism, replication, growth and division (Zhu et al. 2012; Zhu and Szostak 2009). In this manuscript, we first review the self-assembly behaviour and important properties of vesicles made of single-chain fatty acid amphiphile and their mixture with phospholipids or other amphiphiles. We then discuss membrane growth, division and membrane permeabilisation in the presence of different nutrients and their transition from vesicle to model protocell.

Fatty acid self-assembly

Depending on the pH of the medium, fatty acids form different self-aggregated structures. The stability of the aggregated structure is dependent on the ratio of the neutral and ionised form of carboxylic acid groups of the fatty acids. Amongst the different saturated and unsaturated fatty acids, the self-assembly of oleic acid has been the most heavily studied (Fameau et al. 2014; Janke et al. 2014; Arai et al. 2016; Luisi and Stano 2011; Cistola et al. 1988). Structure formation is dependent on the chain length and degree of unsaturation of the fatty acids. The critical bilayer concentration (CBC) of fatty acids for bilayer formation increases with a decrease in the chain length and degree of unsaturation. For example, the CBC of octanoic acid (C_8:0; the shortest fatty acid which can form a vesicle), decanoic acid (C_{10}:0) and oleic acid (C_{18}:1) at pH 7 are 145 mM, 10 mM and 0.19 mM respectively (Luisi and Stano 2011). If fatty acids were the primary components of a protocell membrane, then high concentrations of short-chain fatty acids in solution would be required to ensure vesicle formation. Short-chain fatty acids were likely to be present at low concentration and therefore to reach the CBC required for vesicle formation, an efficient concentration mechanism was needed. It is reasonable to hypothesise that vesicles with short-chain fatty acid could have existed in the transient pool and water evaporation could increase the concentration of fatty acids. This mechanism also increases the probability of encapsulation of catalytic species which were present in the environment into the fatty acid membranes (Monnard and Deamer 2002).

Oleic acid (OA) forms self-assembled structures, such as micelle, worm-like micelle, vesicle and oil droplets at different pH of the medium (Kanicky and Shah 2003; Theander and Pugh 2001). The self-assembly of OA is also dependent on the concentration of fatty acids in solution and temperature. The reported pKa value of OA is 8 (Kanicky and Shah 2003). Therefore, at pH > 8, the major component is oleate and at pH < 8, oleic acid is the principle component. The critical micellar concentration (CMC) of oleic acid at pH 12 is reported to be 0.1 mM (Theander and Pugh 2001). In general, OA exists at oleate micelles at higher pH (pH > 10). When the pH of the medium is rearranged in between 9 and 10, both micelle and vesicle coexist. However, cylindrical or worm-like micelle is also formed at this pH. OA exists as vesicle, when the pH is adjusted in between 9 and 7.5. The vesicles are fairly stable and can last for several days, although the monomers or the constituents interchange rapidly. Below pH 7.5, different meta-stable forms, such as dispersed cubic phase, are generated and it forms oil droplet type structures if the pH is further dropped (Fig. 1a) (Suga et al. 2016). Thus, the formation of OA/oleate vesicle is predominantly governed by hydrogen bonding interaction between the deprotonated and protonated form of OA. Due to the dynamic behaviour of fatty acids, they are constantly flipping between the inner and outer leaflet of the membrane. Fatty acid flipping may play an important role in the permeability for some small molecules, such as RNA nucleotides, to enter the vesicle. This high permeability of fatty acid vesicles was likely to be indispensable in the primitive cell before the advancement of membrane transport machinery.

The different phase behaviour of fatty acid assemblies has been well characterised using different techniques, such as Raman spectroscopy and fluorescence (Suga et al. 2016). The microscopic properties of the membrane can be determined using different fluorescent probes, such as Laurdan (6-lauroyl-2-dimethylaminonaphthalene) and DPH(1,6-diphenyl-1,3,5-hexatriene). Membrane polarity is determined using the fluorescence property of Laurdan and DPH is used to determine the micro-viscosity of the membrane. Suga et al. (2016) first suggested the formation of a cubosome type of structure at pH 7.5. At this pH, the membrane surface is more hydrophobic and more viscous compared with the other

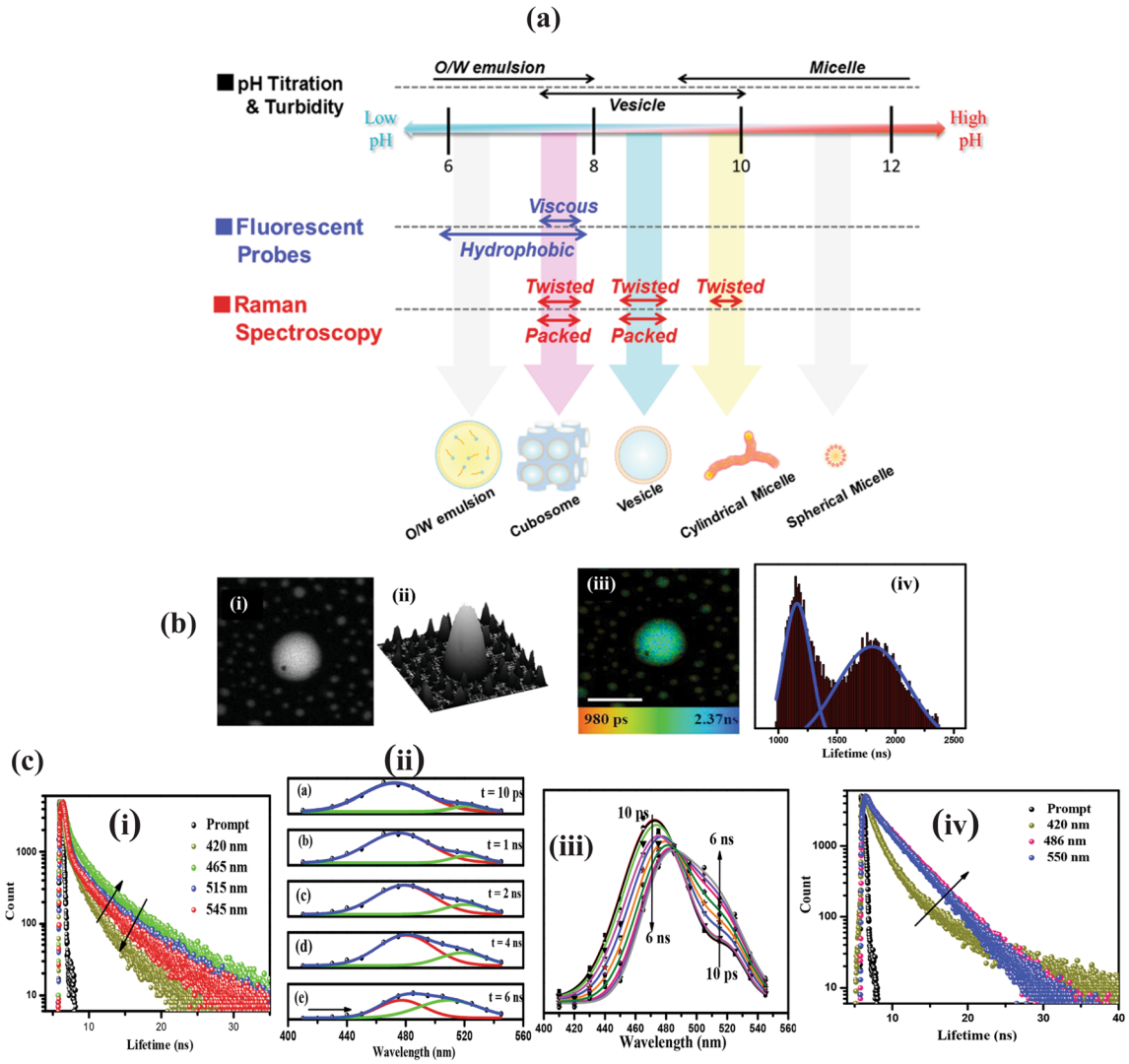


Fig. 1 **a** Schematic representation of the phase behaviour of OA/oleate assemblies determined by fluorescent probes and Raman Spectroscopy. **b** Fluorescence lifetime imaging microscopy (FLIM) images of OA/oleate vesicles using $BP(OH)_2$ as a fluorescent molecule. (i) Intensity image, (ii) 3D distribution of the vesicles obtained from the intensity image, (iii) FLIM image and (iv) lifetime distribution of the fluorophore obtained from the FLIM image; the broad distribution in lifetime signifies the heterogenetic distribution of fluorophore in the vesicle. **c** Fluorescence lifetime decays of $BP(OH)_2$ in different OA/oleate assemblies, (i) lifetime decays of $BP(OH)_2$ in OA micelle collected in different emission wavelengths, (ii) time-resolved emission spectra (TRES) obtained from the lifetime decays in oleate micelle. The overall spectrum is signified by the blue curve and they are deconvoluted into two spectra, (iii) time-resolved area-normalized emission spectra (TRANES) obtained from TRES in OA micelle. Here the iso-emissive point signifies the presence of multiple protropic forms of $BP(OH)_2$ in the excited state in oleate micelle, (iv) lifetime decays of $BP(OH)_2$ in OA/oleate vesicles. **a** is reprinted with the permission from Suga et al. 2016 (copyright 2016, American Chemical Society) and **b**, **c** are reprinted with the permission from Kundu et al. 2016 (copyright 2016, American Chemical Society)

structural assemblies composed of OA. The cubosomes are found to occupy a small area in the phase diagram and it also requires longer incubation time to form the structure. The chain torsion and packing of the fatty acid assemblies can be understood by Raman spectroscopy. The formation of both the dispersed cubic phase and cylindrical micelle is synonymous with a high value of chain torsion and dense chain packing of fatty acid assemblies. The different phase behaviours of OA at different pH have also been studied by molecular dynamics simulation (Janke et al. 2014; Arai et al. 2016).

In order to understand the kinetic and thermodynamic aspects of the different self-assembled structures of fatty acids, it is important to study the dynamics of the micelle to vesicle transition. The rotational dynamics of different non-polar fluorophore molecules has been studied for non-polar region of micelles and vesicles composed of sodium decanoate/decanoic acid (Stevenson and Blanchard 2006) and it was observed that the rotational relaxation time of fluorophore in micellar media is higher compared with that in vesicle. Therefore, it was suggested

that the micellar environment is more viscous compared with the vesicular media. Furthermore, Kundu et al. (2016) have also studied the pH dependent–excited state proton transfer dynamics of $BP(OH)_2$ (2,2′-Bipyridine-3,3′-diol) during the transformation of oleate micelle into OA/oleate vesicle.

Similar to earlier experiments, the micro-viscosity experienced by $BP(OH)_2$ in micellar media is greater than that in vesicle. The formation of different prototropic forms of $BP(OH)_2$ in different fatty acid assemblies was demonstrated using time-resolved emission spectra (TRES) and fluorescence anisotropy measurements (Fig. 1c). Furthermore, multi-wavelength fluorescence lifetime imaging microscopy (MW-FLIM) study was performed to understand the excited state lifetime distribution of $BP(OH)_2$ in a single vesicle, and the results were compared between multiple fatty acid vesicles, obtained from the lifetime measurements performed in bulk solution and single fatty acid vesicle, obtained from FLIM study (Fig. 1b). The kinetics of micelle to vesicle transition for fatty acid has been studied by several groups (Chen et al. 2004). The nucleation and growth kinetics of fatty acid micelles are quite fast occurring on time-scale of microseconds to milliseconds. However, monomer exchange by water molecules occurs on a time-scale of the order of microseconds to nanoseconds. Vesicle formation from micelle has two relaxation times; a rapid mixing of micelles, accompanied by moderate growth and closure of the structure of vesicle. The aggregation of micelle occurs very quickly (< 12 ms). However, growth and division of the vesicle take seconds to minutes. The detailed growth and division mechanism of fatty acid vesicle are discussed in the next section. The kinetics of formation of fatty acid vesicles is very important in terms of considering such type of origin of life questions as whether the growth reaction can be autocatalytic, which means that the formation of vesicle can accelerate the formation of other vesicles. Besides, vesicle formation can also be catalysed by other molecules, such as mononitrile, which also catalyses the RNA polymerization from activated monomers (Hanczyc et al. 2003).

Growth and division mechanism of fatty acid vesicles: autocatalysis

Fatty acid vesicles can be grown through two different pathways; growth through the inclusion of fatty acids from added micelles and growth through the interchange of the fatty acids amongst themselves in the vesicles (Schrum et al. 2010). Luisi and co-workers first established that vesicle growth can be driven by the addition of alkaline fatty acid micellar solution to the suspension of preformed vesicle (Berclaz et al. 2001; Stano and Luisi 2010). When the micellar solution is introduced into a lower pH environment, they become thermodynamically destabilised. Different quantitative techniques, such as light scattering and Förster resonance energy transfer (FRET), have been employed to understand the growth and division process of fatty acid vesicles (Zhu and Szostak 2009; Blochliger et al. 1998). Initially, spherical vesicles are reshaped into long thread-like vesicles, when multilamellar vesicles were nourished with micellar solution (Fig. 2). This process is driven by transient disproportion between surface area and volume enlargement. Thereafter, thread-like vesicles are divided into small unilamellar vesicles without any loss of internal content under mild shear forces. These studies were initially performed by light scattering and cryo-TEM measurements (Berclaz et al. 2001; Blochliger et al. 1998). Use of light scattering makes it difficult to interpret samples with heterogenous size distribution whereas cryo-TEM measurements does not allow for the monitoring of growth of the vesicle in real-time. On the other hand, by using FRET, it is possible to measure the area of the vesicles in real-time. The efficiency of FRET is dependent on the donor to acceptor separation distance (R_{DA}). When the vesicle grows in size after the addition of fatty acid micelles, the value of R_{DA} increases and a decrease in the FRET efficiency was observed. The real-time FRET assay indicated a kinetic segmentation in the growth of vesicle and reveals a complex series of actions after addition of micelles. Vesicle's growth occurs in two stages; fast and slow. Chen and Szostak (2004b) have proposed that the fast phase (up to 40% growth) is stoichiometrically restricted by the preformed vesicles which is due to the formation of a fatty acid shell around the vesicle and they rapidly transfer into the preformed vesicle. The slower phase rises due to the assimilation of amphiphiles which have been stuck in an intermediate state. This kinetically stable intermediate state is the consequence of micelle-micelle interaction. Zhu and Szostak (2009) further showed that encapsulated RNA in the parent vesicle (which signifies a primitive genome) were dispersed into multiple daughter vesicles. These simple and robust pathways suggest that analogous processes might have happened under the prebiotic condition of early earth. The mechanistic detail of the mode of division is remained unclear. Some microscopic studies suggest that long thread membranes are subjected to 'pearling instability' and they minimize their surface energy by transforming into a string of beads morphology (Bar-Ziv and Moses 1994). Thus, the tether joining spherical beads may be the feeble point which can be easily destroyed by the shear forces.

Hentrich and Szostak (2014) have further demonstrated the controlled growth of the filamentous fatty acid vesicles under flow (Fig. 2e). For vesicle growth, vesicles were immobilised on the modified glass surface which was then monitored for membrane growth under different flow rates. The initial growth velocity of the vesicle was dependent on the initial vesicle size and it was therefore, suggested that the added fatty acid molecules were located into the membrane over the

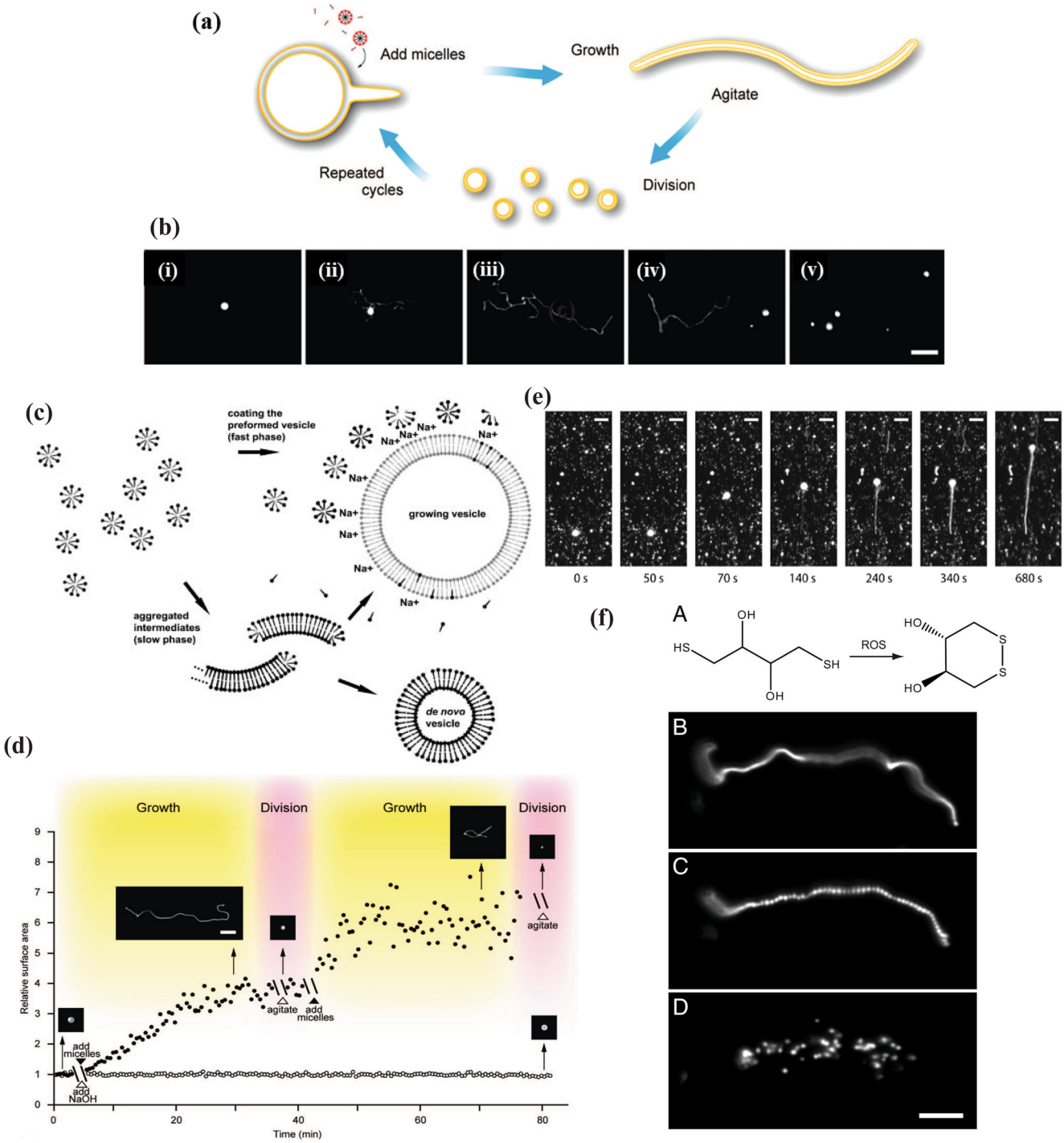


Fig. 2 **a** Schematic presentation of cyclic multilamellar growth and division of fatty acid vesicle; **b** growth and division of single multilamellar vesicle after adding of 5 equivalent of oleate micelle, i–iii demonstrate the growth profile after 3 min, 10 min and 25 min addition of micelle, iv and v demonstrate the division of vesicle after mild agitation. Vesicles were labelled with encapsulated fluorescent dye and they remain entrapped throughout the cycle; **c** different dynamic processes occurring during micelle addition into preformed vesicle; **d** determination of relative surface area of vesicle with respect to time after two cycle of addition of 5 equivalent of oleate micelle, followed by agitation; **e** filamentous growth of OA/oleate vesicle at high flow rate (100 μl/min); **f** pearling and division of OA/oleate vesicles, (A) radical facilitated oxidation reaction of dithiothreitol, (B–D) under intense illumination vesicle in presence of OA micelle is transferred into thread-like vesicle followed by small daughter vesicles. **a**, **b** and **d** are reproduced with the permission from Zhu and Szostak (2009) (copyright 2009, American Chemical Society); **c** is reprinted with the permission from Chen and Szostak (2004a) (copyright 2004, Biophysical Journal); **e** and **f** are reprinted with the permission from Hentrich and Szostak (2014) (copyright 2014, American Chemical Society) and Zhu et al. 2012 (copyright 2012, National Academy of Science) respectively

complete surface. Filamentous growth and vesicle shape were dependent on several factors, such as, flow rate, amphiphile concentration and salt concentration. Molecular dynamics simulation studies also confirmed that the vesicle reproduction process involving disproportion of material across both leaflets with a sluggish flip-flop rate were key features (Markvoort et al. 2010). Beside this pathway, Zhu et al. (2012) have demonstrated another vesicle division pathway which is not dependent of any external mechanical forces. Vesicles, which contain hydroxypyrene in the membrane, induced pearling and division after illumination in the presence of thiols (Fig. 2f). The fluorescent dye, hydroxypyrene generated reactive oxygen species (ROS) after illumination and it oxidised thiols to disulphide-containing compounds. The disulphide-membrane interaction induced a change in the surface tension and caused pearling and subsequent division. This provides an alternative route for the self-reproduction of the protocells in the thiol rich environments as the thiols were most likely to be found in the prebiotic hydrothermal systems, and different UV absorbing polycyclic aromatic hydrocarbons (PAH) could have facilitated the division of the membrane.

Another route for vesicle growth involves the exchange of fatty acid molecules between vesicles (Chen et al. 2004). Such exchange does not significantly alter the size of the vesicles for osmotically relaxed vesicles. However, when osmotically swelled vesicles are mixed with the vesicles in isotonic solution, rapid exchange between fatty acids occurs and it results in the growth of swelled vesicles with consequent shrinkage of the relaxed vesicles. Therefore, this mechanism permits the growth of vesicles which contain genetic materials, such as RNA at the disbursement of empty vesicles. Faster replication increases the internal concentration of nucleic acid. Thus, this pathway provides a direct connection between the rate of the growth of protocell and rate of replication of encapsulated genetic materials. Therefore, this process could lead to the emergence of Darwinian evolution at cellular level.

Luisi et al. also demonstrated the autopoietic self-reproduction of fatty acid vesicles (Stano and Luisi 2010; Walde et al. 1994) finding significant relevance to the field of the origin of life and synthetic biology. Autopoiesis identifies the self-maintenance of a cell/organisms own structure and dynamic organization. The basics of autopoiesis are described in Fig. 3a, b. In this system, the external component (indicated by P) is transformed into an element (indicated by S) of the autopoiesis system. This transformation occurs within the self-generating boundary which separates the system from the external environment. In self-organising system, the precursor of S is P. The autopoietic mechanism of vesicle reproduction is much more complex compared with that of either a reverse micelle or micelle as it involves more complicated routes which depend on the critical stage (Bachmann et al. 1990). Therefore, thermodynamics and kinetics both play a major role to determine the pathway. The self-reproduction of conventional fatty acid vesicles, mixed vesicles of fatty acid and phospholipids, is well documented in the literature (Blochliger et al. 1998; Luisi et al. 1993; Lonchin et al. 1999; Rogerson et al. 2006). To investigate the autopoietic self-reproduction of fatty acid vesicles, different water-insoluble precursors, such as fatty acid anhydrides, fatty acid ethyl and methyl esters were successfully employed (Walde et al. 1994). Anhydride molecules were transferred to the droplet to the bilayer of the vesicle through the collision process in which bilayers were in transient contact with the droplet.

At pH 8.5, in the presence of OA/oleate vesicle, oleic anhydride (P) was hydrolysed into oleic acid (S). Due to their minimal water solubility, oleic anhydride was placed at the bilayer region and the hydrolysis reaction occurred at the interface between the membrane and water. The initial concentration of released OA/Oleate was reported to be very slow. However, afterwards, the presence of vesicle rapidly increased the reaction rate. The catalytic process was characterised by turbidity measurements, chemical analysis, TEM and light microscopy measurements. The process was examined in different temperatures and it was observed that with increasing temperature, the time profile of vesicle generation became steeper and initial slow phase became considerably shortened. The growth and division of vesicles were directly monitored by light microscopy measurements also (Fig. 3c) (Wick et al. 1995). The hydrolysis of the anhydrides probably led to the swelling of the bilayer. Budding of the vesicle was also observed and it also led to increase the number of vesicles. The autopoietic self-reproduction was also investigated with chiral fatty acid vesicle formed by (S)- and (R)-2-methyldodecanoic acid to relate the autocatalytic efficiency with enantioselectivity (Morigaki et al. 1997). Similar studies are also in progress in semi-synthetic minimal cells which are artificial cell-like compartments and they are constructed by filling with liposomes with different biomolecules, such as DNA, protein and enzyme (Mann 2013). Therefore, this budding process, growth and division mechanism might provide the answer about the role of vesicle in the transition from inanimate to animate state of matter (Svetina 2009).

Fatty acid modified membrane

One of the drawbacks of pure fatty acid membranes as potential protocells is that they are extremely permeable to protons and therefore, incompetent to sustain a pH gradient across the bilayer (Chen et al. 2004). In the presence of alkali metal ions, pH gradient decays rapidly. In phospholipid vesicles, the prestabilised pH gradient dissipates immediately after the addition of fatty acids. Therefore, the usefulness of pure fatty acid-based vesicles is dependent on the pH of the medium, temperature and

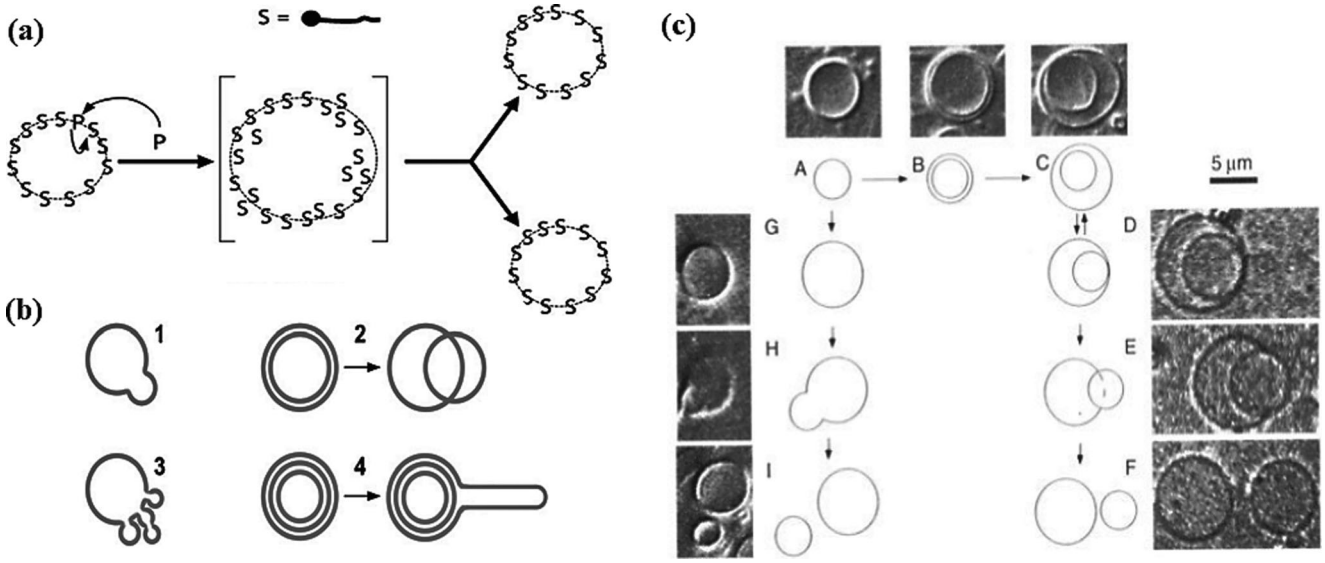


Fig. 3 **a** General mechanism for the self-reproduction of surfactant systems. A suitable precursor, P, is added to the self-assembled system and it can passively diffuse into the boundary of the system. Furthermore, it can be transformed into the boundary forming compound, S. Therefore, the size of the aggregate is increased and it may bring about destabilisation and consequent division into two or more new aggregates; **b** several intermediates which have been observed during the transformation of OA/oleate vesicles in presence of oleate micelle: (1) budding mechanism, (2) translocation, (3) evagination, (4) tubular growth and division; (c) two self-reproduction pathways for OA/oleate vesicles after addition of oleate anhydride, (A–C) formation of 'inclusion' vesicles, (D–F) vesicle 'birthing', (G–I) vesicle 'budding'. **a** and **b** are reproduced with the permission from Stano and Luisi (2010) (copyright 2010, Royal Society of Chemistry) and **c** is reproduced with the permission from Wick et al. (1995) (copyright 1995, American Chemical Society)

concentration of amphiphiles. This stability can be improved to a wider range of pH by mixing the fatty acids with fatty alcohol, fatty acid glycerol ester and other amphiphiles (Budin et al. 2014; Kundu et al. 2018).

Fatty acid and phospholipid mixture

Similar to fatty acid vesicles, addition of fatty acid micellar solution into the preformed lipid vesicle results in vesicle self-replication and the lipid vesicles show 'matrix effect' on the newly formed vesicles (the size of the newly formed vesicle by growth and division mimics the size of preformed vesicles) (Blochliger et al. 1998; Lonchin et al. 1999). Souza et al. (2015) have studied the self-reproduction process using Cryo-TEM and free-flow electrophoresis (FFE) measurements.[56] FFE technique allows separation of the vesicle according to their charges. In the process of self-reproduction, this technique becomes convenient when the negatively charged fatty acid micelle is added to the zwitterionic lipid POPC (1-palmitoyl 2-oleoyl-sn-glycero-3-phosphocholine), as the charge density of the lipid is depended on the extent of micelle uptake and the result showed that the uptake of micelle by lipid vesicle shows different pathways which depend on the micelle-lipid ratio. At low OA mole fraction, oleate molecules are incorporated into the parent lipid vesicles. However, at high mole fraction, daughter vesicles with different OA populations are produced from the parent POPC vesicle. These pathways are also supported by Cryo-TEM measurements of ferritin-labelled vesicles. Different compositions of lipid and fatty acid exhibit complex morphological changes such as growth, fusion, fission and budding (Peterlina et al. 2009; Herrero et al. 2019; Ediss et al. 2014; Mally et al. 2013; Piedrafita et al. 2017). In this regard, Mondal et al. (2019) have recently demonstrated the role of unsaturated fatty acid in phospholipid membrane structure using fluorescence microscopy and time-resolved fluorescence spectroscopy (Fig. 4) measurements. For this, vesicle dispersions were prepared using mixtures of saturated lipid (DMPC, 1,2-Dimyristoyl-racglycero-3-phosphocholine) and unsaturated lipid (LAPC, L-α-phosphatidylcholine) molecules. LAPC lipid contains mixture of saturated and unsaturated fatty acids in their hydrophobic tail, and vesicles composed of LAPC and DMPC exhibit a variety of structural arrangements (Fig. 4a). To understand the role of fatty acid geometry on lipid membrane structure, the authors further elucidated the individual role of unsaturated fatty acids (oleic acid and linoleic acid) on saturated lipid (DMPC) vesicle. Oleic acid and linoleic acid are mono-unsaturated and poly-unsaturated respectively. The different geometric arrangement of the lipids in the vesicle was understood by the FLIM measurements (Fig. 4b). Furthermore, the solvation dynamics of a dye molecule distributed in a different region of the vesicle was measured to understand the arrangement of water molecules around the lipid surface containing unsaturated fatty acids (Fig. 4c). Solvation dynamics of water is related to the water reorganisation process due to rapid changes in the

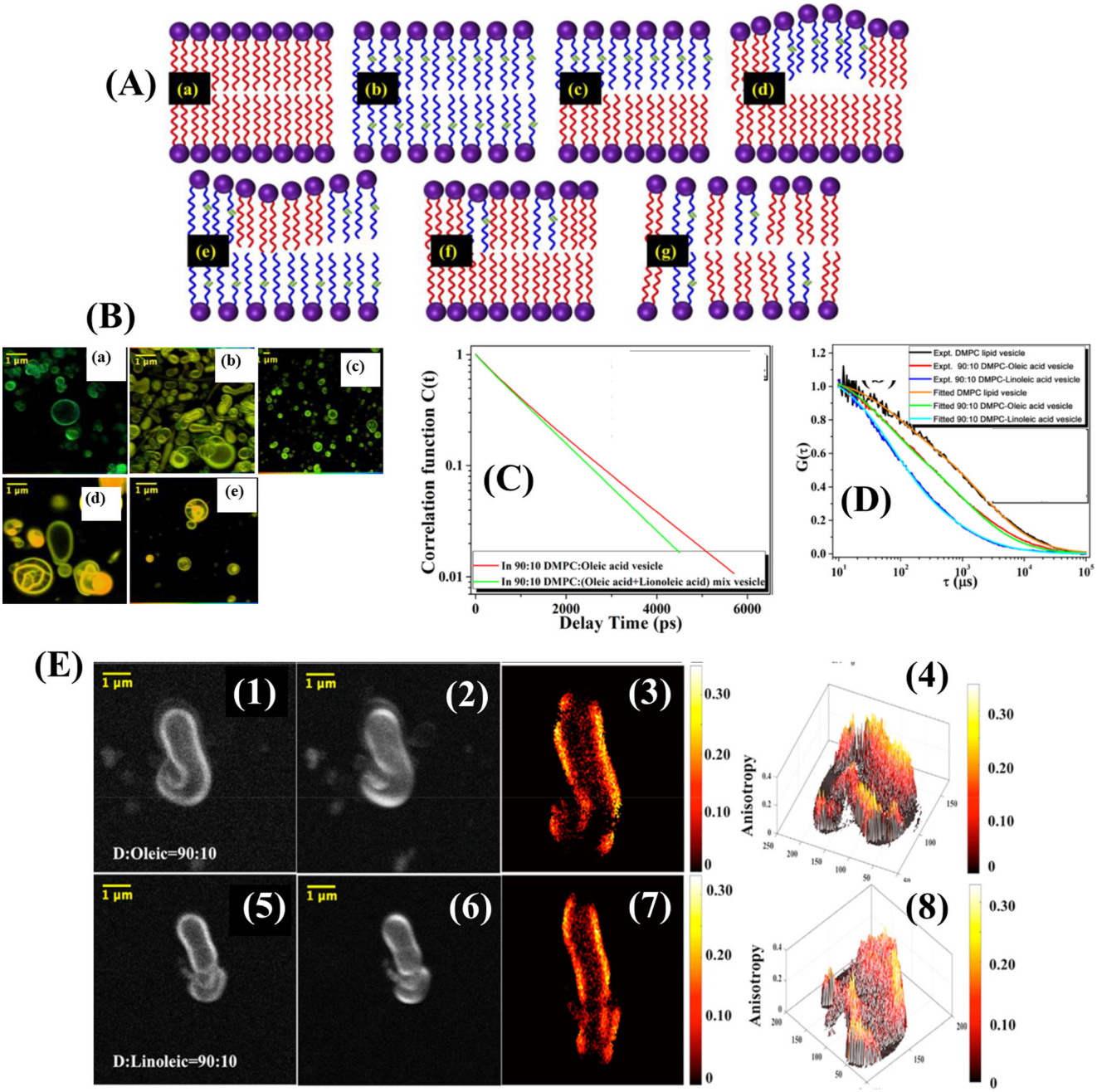


Fig. 4 **a** Probable orientation of lipids in bilayer composed of DMPC and LAPC. Bilayer packing of (a) DMPC lipid and (b) unsaturated LAPC lipid, (c) symmetric packing of DMPC and LAPC lipid, (d) asymmetric inclusion of LAPC lipid into DMPC lipid, (d) asymmetric inclusion of DMPC into LAPC lipid, (f) LAPC only present at the outer leaflet of bilayer and (g) most asymmetric bilayer formation of DMPC and LAPC mixture; **b** FLIM images of (a) DMPC vesicle, (b) 90:10 DMPC: LAPC vesicle, (c) 60:40 DMPC:LAPC vesicle, (d) 90:10 DMPC: oleic acid vesicle, (e) 90: 10 DMPC: linoleic acid vesicle; **c** decay of solvent correlation function, C(t) of coumarin-153 in 90:10 DMPC: oleic acid vesicle (red line) and 90:10 DMPC: linoleic acid (green line); **d** FCS traces of Rh-6G in DMPC lipid vesicle (black line), 90:10 DMPC: oleic acid vesicle (red line) and 90:10 DMPC: linoleic acid vesicle (blue line); **e** FAIM images of 90:10 DMPC: oleic acid vesicle (1-4) and 90:10 DMPC: linoleic acid vesicle (5-8). Images (1, 3) and (2, 6) are collected at the perpendicular and parallel w.r.t the linear polarization of the excited light, (3, 6) are calculated fluorescence anisotropy images and (4, 8) are the 3D surface image. All the figures are reprinted with the permission from Mondal et al. (2019) (copyright 2019, American Chemical Society)

charge distribution of a dye molecule after electronic excitation (Nandi et al. 2000). In the case of vesicles, three different types of water, i.e., 'bound' water, 'exchange' water and 'free' water are reported by several groups (Pal et al. 2000; Roy et al. 2016). The relaxation time of bound water is very slow as it involves strong hydrogen bonding interaction between water molecules and the head groups of charged lipids. In the case of 'exchange' water, it continuously exchanges hydrogen bonds

between bound water and free water inside the vesicle. Thus, the exchange rate is dependent on the surrounding free and bound water inside the vesicle. The free water relaxation time is very fast compared with that of exchange water and bound water. However, the relaxation time is much slower compared with that of bulk water due to the confinements. The bulk water solvation time is less than 50 fs as reported by Jimenez et al. (1994) although the free water solvation time inside vesicle is reported to be around 1.5 ps (Sen et al. 2006). Unsaturation disrupts the lipid bilayer packing and it reduces the rigidity of the bilayer, which fundamentally controls the water dynamics. Average solvation times became significantly faster in the presence unsaturated fatty acids in DMPC vesicles. For saturated lipid vesicles, the hydrogen-bonded water molecules are oriented in an ordered fashion which extends multiple layers beyond the first hydration shell of the hydrophilic head groups, making a rigid hydrogen-bonded structure. However, after the insertion of unsaturated fatty acids, the first hydration shell turns into a fragile structure and the water molecules become free to move faster which is reflected by a faster solvation time. The effect of unsaturated fatty acids on bilayer of DMPC vesicles was also determined by FLIM measurements. The monounsaturated oleic acid disrupted the lipid packing, and therefore, the lifetime of dye molecules decreased in DMPC/oleic acid mixed vesicles compared with pure DMPC vesicle. The decrease was more prominent for DMPC/linoleic acid mixed vesicles because of the polyunsaturation in linoleic acid.

Asymmetrical packing in the bilayer induces transformation of the shape of the vesicle as evidenced in Fig. 4. The growth and shape transformation of other lipid vesicles in the presence of fatty acids have been reported by several other groups also (Peterlina et al. 2009; Herrero et al. 2019). The change in the fluidity of the membrane was determined by fluorescence anisotropy imaging microscopy (FAIM) (Mondal et al. 2019). As membrane fluidity depends on the hydrogen bond interactions between the charged lipid head groups and interfacial water molecules, the increase in the fluidity of the membrane can be correlated to the increase in the interspacing between the head groups. Vesicles made solely of saturated lipid molecules show high anisotropy values which indicate that the interspacing between charged head groups is much less and the lipid packing is highly ordered (Fig. 4e). For mixed fatty acid/lipid vesicles, the anisotropy values decreased significantly which directly indicates an increase in the fluidity of the membrane due to the loose arrangement of lipid head groups. As the unsaturation in the fatty acid structure is increased, the elasticity of the membrane is also increased. Similar to the FAIM measurements, the behaviour of the vesicles associated water can also be measured by fluorescence correlation spectroscopy (FCS) measurements using hydrophilic dye molecules (Fig. 4d). Due to the polar behaviour, hydrophilic dyes, such as Rhodamine-6G, are located within the water pool of the vesicle. Single-molecular level FCS measurements can explain the changes in the intravesicular environments due to the frictional forces arising from the water molecules inside vesicle. In presence of fatty acids, the diffusion of the dye molecules becomes significantly faster compared with the vesicles made solely of lipids. Furthermore, addition of fatty acids on the cell membrane significantly increases the membrane permeability due to the unsaturation. The transport mechanism of fatty acids across the cell membrane remains unsolved, and several theoretical and experimental studies have been performed to answer this question. It was concluded that non-ionised fatty acids flip-flop rapidly ($t_{\frac{1}{2}} \leq 2$sec) whereas ionised fatty acids flip-flop slowly ($t_{\frac{1}{2}}$of minutes) (Wei and Pohorille 2014; Kampf et al. 2006). During the process, oleic acid molecules move towards the centre of the lipid membrane bilayer, where they experience correlated translational and rotational motion. Theoretical calculations suggest that the flip-flop barrier at the centre of the bilayer is ~ 4.2 kcal/mol (Wei and Pohorille 2014) and the fast flip-flop rate proposed to result from the permeation of fatty acids membrane without any assistance of transport machinery.

Growth of the protocell membrane can also be driven by surprisingly low levels of phospholipids (Budin and Szostak 2011). As mentioned earlier, protocells were assumed to be comprised of single-chain fatty acids and modern cell membranes are comprised of diacyl or dialkyl phospholipids. Therefore, the question arises how the primitive cell membranes were transformed into the modern membranes, especially during the early stages which were characterised by low levels of phospholipids. To answer this, Budin and Szostak (2011) hypothesised that low concentration of phospholipids, synthesized from ribozymes, could drive competitive growth and thus, offer a selective pressure for the evolution of modern membrane transport machinery to overcome the reduced permeability of the lipid membranes. To address the hypothesis, FRET was used as a tool to measure the change in the surface area of the OA/oleate vesicle in the presence of 10% DOPA (di-oleoyl-phosphatidic acid) lipid. The membrane growth resulted from decreases fatty acid efflux with increasing phospholipid contents. Phospholipid-driven growth can also facilitate vesicle division. Rapid growth in a ratio solution composed of 90:10 oleate: DOPA vesicle was observed when they were mixed with 100-fold excess of oleate vesicle and the spherical vesicles were transformed into long filamenteous vesicle (Fig. 5a). The lag between surface area growth and volume growth was the reason for shape change, and under mild shear force, the filamenteous vesicles subsequently divided into small vesicles. Thus, the ability to synthesize the phospholipid from a single-chain amphiphile is highly advantageous for

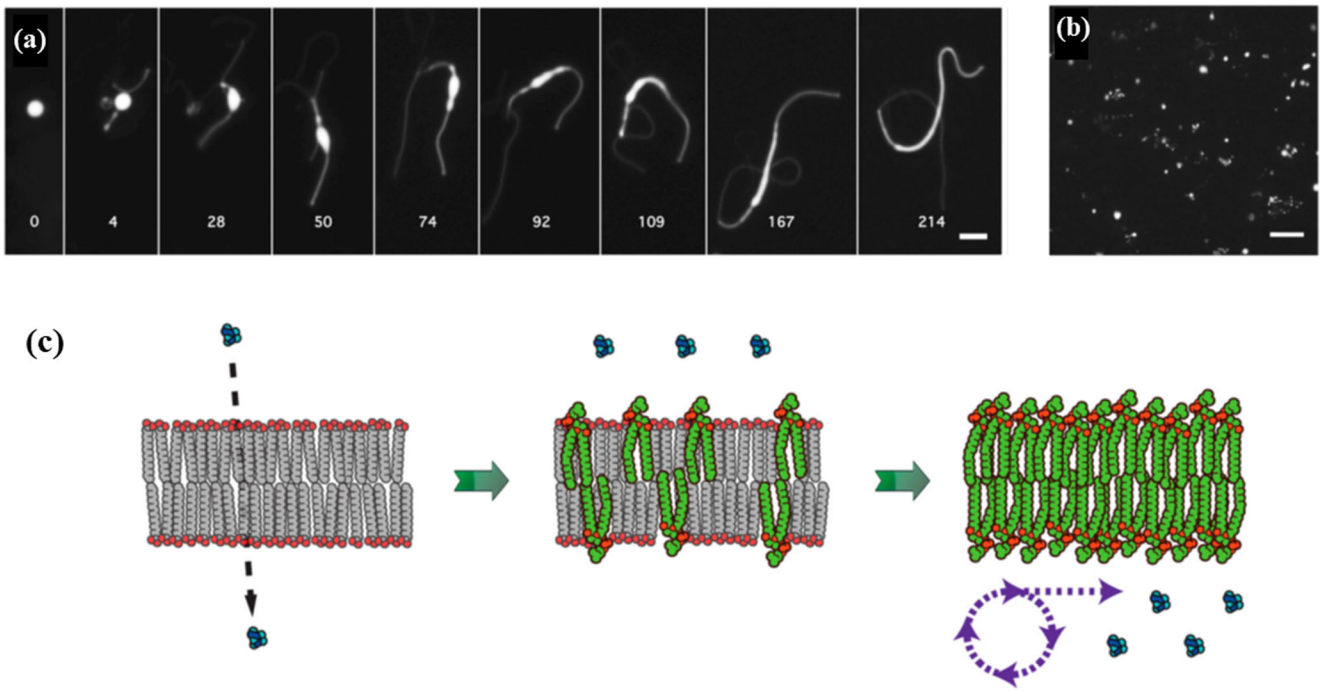


Fig. 5 **a** Shape transformation of 90:10 oleate: DOPA vesicles in presence of excess OA/oleate vesicle in different time (time in seconds); **b** division of vesicle into smaller spherical vesicle after gentle shear forces, **c** scheme for phospholipids driven cellular evaluation. The gradual evolution from permeable protocell membrane to lipid membrane is shown in the scheme and it is motivated by the selective growth advantage delivered by increasing phospholipid content in the membrane. Figure a and b and Scheme c are reprinted with the permission from Budin and Szostak (2011) (copyright 2011, National Academy of Science)

early protocells competing for a low supply of lipids. This phospholipid-driven competition can lead to an increase in the diacyl lipid content into the membrane and this transition would have come at the cost of membrane permeability (Fig. 5b).

Fatty acid and other amphiphile mixtures

Structural integrity of the vesicle can be enhanced by the mixing of two different single-chain amphiphiles such that the mixture provides better stability in high ionic strength, higher temperature media and over a wide pH range. Mixed vesicles are formed by mixing two single-chain amphiphiles with oppositely charged head groups. Preparation of mixed vesicles with negatively charged fatty acid has been conducted using different cationic amphiphiles, such as trimethyl ammonium-based amphiphiles, cationic surfactants and surface-active ionic liquids (SAILs) (Caschera et al. 2011; Xu et al. 2013; Suga et al. 2014; Kundu et al. 2017a; Douliez 2006; Fameau et al. 2017; Roy et al. 2018). Mixed vesicles composed of OA or linoleic acid (LA) and cationic surfactants, didecyldimethylammonium bromide (DDAB) have been reported in the literature and the physiochemical properties of the vesicle characterised by dielectric dispersion analysis (DDA) and fluorescence spectroscopy (Suga et al. 2014). Different fluorescent probes, such as DPH and Laurdan, were used to determine the fluidity and hydration state of the membrane, and the results suggested that mixing DDAB with LA vesicles induced the hydration of the membrane surface while the opposite effect was observed in the case of OA vesicle.

The self-assembly of fatty acids can also be modified with different SAILs. They exhibit the properties of both room temperature ionic liquids (RTILs) and surfactants. Roy et al. (2018) have prepared the mixed vesicle of OA and 1-hexadecyl-3-methyl imidazolium chloride (C_{16}mim-Cl) and these mixed vesicles were stable in the pH range of 2-12. The dynamic properties of the vesicles were investigated using FCS and FLIM techniques. Kundu et al. (2017b) further synthesized protic ionic liquid, oleate ethylamine (OEA) of OA with ethyl amine as a component (Fig. 6). The structural properties and dynamics were compared with OA/oleate vesicles using FCS measurements. They have used MW-FLIM technique to understand the solvation process in a single vesicle (Fig. 6b, c). For this, the images were taken at different emission wavelengths of dye molecule and it was suggested that the membrane surface of OEA was more dehydrated compared with OA/oleate vesicles and it was this property that was enabled the OEA membrane to fuse themselves in the presence of electrolyte, sodium chloride (NaCl). The different phases of vesicle fusion were studied by time scan FLIM measurements. The rich dynamic properties of the fatty acid modified vesicles provide better insight into the growth of the protocell membrane.

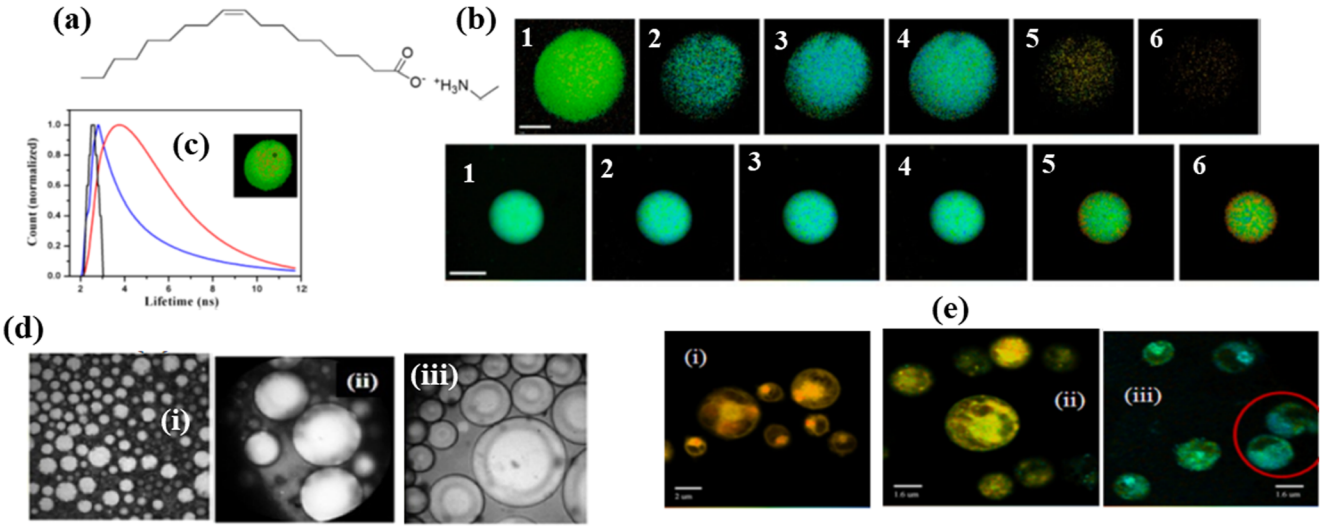


Fig. 6 **a** Chemical structure of oleate ethylamine (OEA); **b** MW-FLIM images of OEA vesicle (upper panel) and OA/oleate vesicle (lower panel). Images are collected in six different emission wavelengths of fluorophore: (1) 606 − 620 nm, (2) 630 − 645 nm, (3) 655 − 669 nm, (4) 680 − 694 nm, (5) 706 − 720 nm and (6) 730 − 744 nm; **c** fitted fluorescence lifetime decay of fluorophores in OEA membrane. The blue and red lines indicate that the decays are collected in 606–620 nm and 730–744 nm region. The red decay signifies the solvation of fluorophores in single vesicle; **d** images of formation of different sized vesicles of OEA in presence of 0.1 M Bmim-BF_4. **e** FLIM images of K-562 cell in presence of (i) 0 M, (ii) 0.2 M and (iii) 0.8 M Bmim-BF_4. The red circle in **e** (iii) indicates the disrupted cell membrane. **a**, **b**, **c**, **d** and **e** are reprinted with the permission from J. Phys. Chem. B 2017, 121, 24 and J. Phys. Chem. B 2017, 121, 8162 respectively (copyright 2017, American Chemical Society)

The fusion kinetics of OEA membrane was further studied in presence of hydrophilic ionic liquid, 1-butyl-3-methyl imidazolium tetrafluoroborate (Bmim-BF_4) (Kundu et al. 2017b). Ionic liquid cation can insert into the bilayer of the vesicle because of the strong electrostatic and hydrophobic interaction between $Bmim^+$ and fatty acid. In presence of high concentration of IL, the permeability of the membrane is increased and membrane is ruptured (Fig. 6e).

Genetic replication in early protocell

A fundamental requirement for the origin of life is the replication of genetic polymers and a key requirement would be the ability of the membrane to transport such large, polar molecules across the membrane without any protein machinery (Hanczyc et al. 2003). A primary criterion for the protocell model is the stability of the membrane and the replication of nucleic acids, and for this, the membrane system must be compatible. Modern phospholipid-based cell membranes are impermeable to polar and charged molecules ranging from metal ions to complex nutrients. Therefore, they require protein channels and pumps to exchange molecules with the environments. Such low permeability to polar solute is consistent with the desolvation model of permeation: for the diffusion of a solute molecule into the hydrophobic bilayer bound, water must be dissociated, and for large polar molecules, this is practically impossible. Furthermore, phospholipid membrane lacks the dynamic properties which are required for membrane growth. Fatty acid vesicles are stable in the range between 7.5 and 9. However, this pH range can be extended by mixing them with fatty alcohols and glycerol monoesters and this pH range is suitable for both non-enzymatic RNA polymerization and RNA polymerase ribozyme activity. Furthermore, fatty acid-based vesicles are stable upto 100^0C (Mansy and Szostak 2008). These features allow the thermal denaturation of nucleotides encapsulated in the vesicles and enhance the permeation of nucleotides (Meierhenrich et al. 2010). Therefore, nucleotides may spontaneously cross barrier composed of fatty acid in a protocell membrane and take part in efficient template copying in the interior of the vesicles. These permeability properties suggest that different active nutrients can be acquired in the interior of the vesicle from the environments in absence of any transport machinery (Fig. 7).

Fatty acid membranes are not stable in the presence of low concentrations of divalent cations. However, non-enzymatic RNA replication and RNA polymerase ribozyme activity require at least 50 mM of Mg^{+2} (Shechner and Bartel 2011). Recently, it has been found that fatty acid membranes can be preserved in the presence of tricarboxylic acid citrate which can chelates Mg^{+2} (Adamala and Szostak 2013a). Thus, it is possible to encapsulate RNA primer-template complex and primer extension has been observed in the presence of activated ribonucleotides. Further development of model protocells based on RNA encapsulation and copying is possible. For the fully integrated protocells, activated nucleotides must be encapsulated into the vesicle, waste must be able to leave and the self-replicating genetic system must be trapped in the water

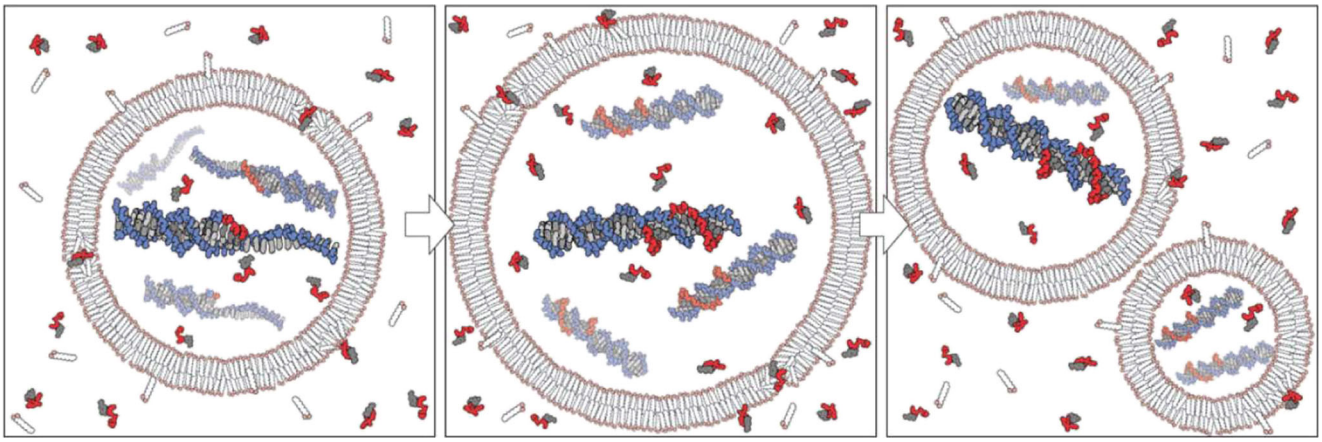


Fig. 7 Growth of protocell membranes due to the incorporation of nutrients and division can be guided by different physical forces. The permeation of active nucleotides to the protocell membrane should be high and membrane should act as a template for non-enzymatic copying of internal templets. Daughter vesicles are formed after complete template replication. This figure is reprinted with the permission from Mansy et al. (2008) (copyright 2008, Nature Publishing Group)

pool of the vesicle. In this aspect, fatty acid vesicles have certain advantages because small molecules, such as nucleotides, can cross the membrane, whereas others, such as oligonucleotides, cannot. For lipid vesicles, nutrients can be encapsulated into the vesicles using vesicle fusion process (Blumenthal et al. 2003). However, in most of the cases, such fusion processes are ineffective and induce considerable content leakage. Substantial progress on this topic has been nicely reviewed by Joyce and Szostak (2018).

Conclusions and outlook

The origin of life is a rich field that is filled with several possibilities, mature for discovery. The present review attempts to synthesize current knowledge from the fields of chemistry and biology and focuses on potential different complex pathways. The prebiotic environment produces the conditions necessary for the development of more complex chemistry and finally to the synthesis of biological building blocks. Simple amphiphilic molecules, such as fatty acids, can self-assemble into vesicles in a manner that depends on their surroundings, and these vesicles have properties which are well suited for model protocells. During the growth process, the shape of the vesicles is transformed from spherical to filamentous and under mild agitation vesicles can divide into spherical daughter vesicles. The kinetics and dynamics associated with each step have been discussed. The transition from protocell membrane to modern cell membrane in the presence of low concentrations of phospholipids has also been discussed. Fatty acid vesicles are permeable to charged nutrients, such as nucleotides and therefore do not require any membrane transport proteins to transfer the nutrients and vesicles and are thus also compatible with nucleic acid template copying chemistry.

Recent progress in the design of model protocells is very encouraging, although numerous challenges still persist. The first major challenge is the development and synthesis of genetic materials which are capable of repetitive cycles of chemical replication. A future goal is to identify the simple processes by which model cells can synchronize the replication of nucleic acid genome and replication of membrane compartment. If the overall protocell replication can be achieved, then it will be possible to demonstrate the spontaneous evaluation of adaptive innovation in simple chemical systems. Such adaptation may provide us with valuable information about the transition of modern cells from the earlier ancestors.

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