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# Replicating vesicles as models of primitive cell growth and division

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Primitive cells, lacking the complex bio-machinery present in modern cells, would have had to rely on the self-organizing properties of their components and on interactions with their environment to achieve basic cellular functions such as growth and division. Many bilayer-membrane vesicles, depending on their composition and environment, can exhibit complex morphological changes such as growth, fusion, fission, budding, internal vesicle assembly and vesicle-surface interactions. The rich dynamic properties of these vesicles provide interesting models of how primitive cellular replication might have occurred in response to purely physical and chemical forces.

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## Introduction

Several distinct pathways can be imagined for the replication of primitive vesicles (Figure 1). Model I shows discrete growth and division steps that could be executed in several ways (A–D). Vesicle growth could, in principle, occur by the incorporation of free amphiphilic molecules or micelles, or through vesicle–vesicle fusion (model IA). Membrane components may be delivered in their final state (model IA) or as precursors requiring chemical modification before incorporation (model IB). Division of the vesicles after growth could occur by roughly equal division into daughter vesicles in response to environmental conditions, or possibly spontaneously (model IC). A highly asymmetric budding of small daughter vesicles might also occur (model ID). Model II illustrates the assembly of new vesicles inside a parental vesicle followed by release and subsequent growth of the daughter vesicles. Finally, in model III, vesicle–surface interactions might facilitate flow-induced division or budding. Undoubtedly, other pathways for vesicle replication are possible. In this review, we discuss the experimental evidence (or lack thereof) in support of these models for vesicle replication, and discuss the plausibility of these pathways in pre-biotic scenarios.

## Vesicle growth and division

Model I depicts a two-stage process in which vesicles first grow and then divide, much as modern cells do during somatic or vegetative reproduction. Under conditions in which the bilayer lamellar phase is thermodynamically favoured, newly added amphiphilic molecules will either integrate into the membrane of an existing vesicle, resulting in vesicle growth, or self-associate to form new vesicles. Luisi and colleagues [1••] monitored a vesicle population before and after the addition of oleate micelles. The initial population of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) vesicles contained the electron-dense protein ferritin as a marker of the internal space. The sizes of the vesicles before and after micelle addition were determined by cryo-TEM (transmission electron microscopy). The diameters of the original ferritin-containing vesicles increased upon addition of the oleate micelles, suggesting that some of the fatty acid was incorporated into the existing vesicle membrane, leading to vesicle growth as in model I. In these experiments, a significant fraction of the final vesicles contained no ferritin and must have arisen through an alternate pathway that resulted in the formation of new vesicles. These results suggested that vesicles composed of simple single-chain fatty acids might serve to model primitive cellular replication.

Our laboratory has engineered a system of vesicle replication with discrete growth and division steps as depicted in model I [2••]. When one equivalent of myristoleate micelles was added to myristoleic acid/myristoleate vesicles (myristoleic acid is a 14 carbon, singly unsaturated fatty acid), we observed vesicle growth (model IA) as well as new vesicle formation. To promote vesicle growth over the formation of new vesicles, dilute micelles were introduced slowly over time. This resulted in preferential vesicle growth by eliminating the transient high micelle concentrations that favour self-association and the formation of new vesicles. Through both light scattering measurements and fluorescence techniques we confirmed that our initial vesicle population increased in size with ∼90% of the added fatty acid incorporated into the growing vesicles. Subsequent kinetic analysis of the growth process revealed an unexpectedly complex mechanism

Figure 1
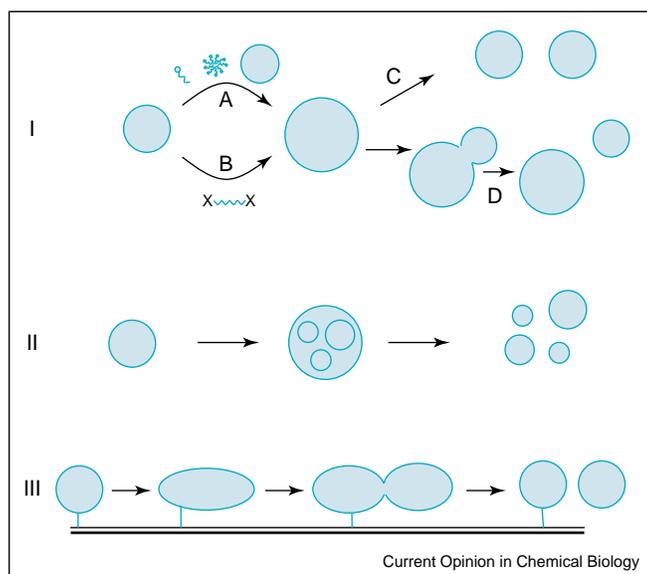


Models of vesicle replication. **I**: Vesicle replication occurs through discrete steps of vesicle growth and division. (A) Growth can occur although the incorporation of additional membrane components in the forms of free amphiphiles and micelles or through vesicle-vesicle fusion. (B) Alternatively, vesicle growth can occur although the introduction of precursors that will form additional membrane components upon chemical modification. (C) Division by vesicle fission can produce roughly equivalent daughter vesicle or (D) division can occur by asymmetric budding. **II**: New vesicles are assembled within a parent vesicle. Daughter vesicles are then released. **III**: A surface support may assist flow-induced vesicle growth and division.

involving fast and slow growth phases [3]. Fast growth is limited to an increase of ∼40% in vesicle surface area, possibly from the rapid formation of a coat of micelles around pre-formed vesicles, followed by incorporation of the micellar material into the vesicle membrane. The slow growth phase seems to result from the interaction of micelles to form larger aggregates, which can slowly exchange into the vesicle membrane or can form new vesicles. These kinetic experiments show that the rapid addition of limited amounts of new material can lead to efficient vesicle growth.

The rapid exchange of single-chain amphiphiles between vesicles allows vesicle growth to occur by competition between vesicles [4]. This pathway for vesicle growth is based on the fact that osmotically swollen vesicles are in a high energy state relative to relaxed (isotonic) vesicles. The overall energy of a mixture of swollen and relaxed vesicles can therefore be minimized by the transfer of membrane components from the relaxed vesicles, which shrink, to the swollen vesicles, which grow. This pathway is particularly attractive from a pre-biotic perspective, because the osmotically driven vesicle growth could result from the internal replication of a nucleic acid genome. Furthermore, faster replication of the internal genetic material could translate directly into faster membrane growth, through the mediation of the osmotic pressure generated by the counter-ions associated with a charged genetic polymer such as RNA.

Growth by vesicle–vesicle fusion (model IA) has the advantage that it brings both new encapsulated material and new membrane components to a growing vesicle. Content fusion could replenish encapsulated materials such as impermeable substrates, mineral grains, or enzymes in growing vesicles. Vesicle growth by micelle addition to fatty acid vesicles described above [2$^{\bullet\bullet}$] may be dependent upon the rapid exchange of single-chain amphiphiles. By contrast, growth by vesicle fusion is independent of this rapid exchange and thus allows for the growth of vesicles composed of more complex amphiphiles such as phospholipids. The mixing of membrane components following fusion is shown in Figure 2c–e [5]. Although the mechanism of fusion of phospholipid vesicles has received a great deal of attention because of its biological importance (e.g. see [6]), fatty acid vesicle fusion has not been explored. In biological systems, fusion events are often triggered by a local increase in $Ca^{2+}$ concentration, although a complex set of protein–protein and protein–membrane interactions also play essential roles. However, fatty acid vesicles are very sensitive to the presence of divalent cations. In the presence of $Ca^{2+}$, fatty acids aggregate and precipitate or crystallize, suggesting that $Ca^{2+}$-triggered fusion of fatty acid vesicles may be problematic. Significantly, it has been shown that vesicles made from a mixture of fatty acids and their glycerol monoesters can withstand low mM levels of $Ca^{2+}$ [7]. It will be interesting to see if vesicle–vesicle fusion events can be detected in such

**Figure 2**
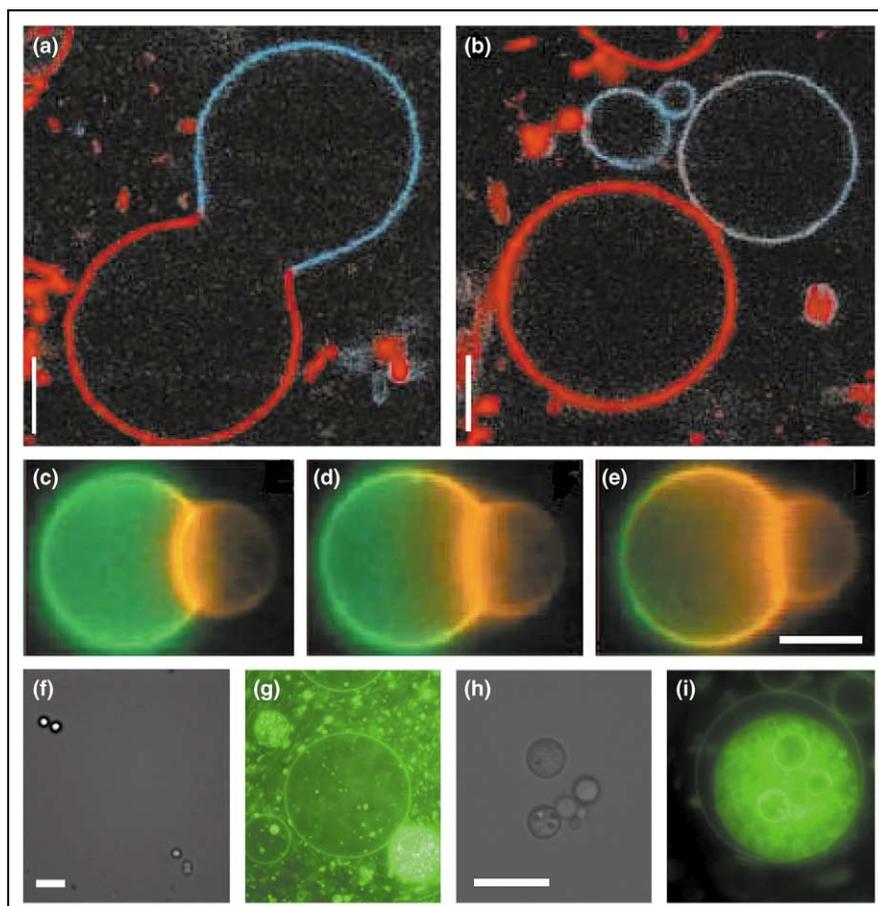


The dynamic morphology of giant vesicles. **(a,b)** Giant phospholipid vesicles were labeled with two different domain-specific dyes. A fission sequence triggered by heating the vesicles was captured by two-photon microscopy [9••]. Scale bar, 5 microns. **(c–e)** Panels taken from a video show the fusion of two charged phospholipid vesicles. The green fluorescence of the larger vesicle becomes quenched as the red dye from the smaller vesicle mixes within the fused membranes [5]. Scale bar, 10 microns. **(f–i)** Panels show alumino-silicate spheres by Nomarski optics (f,h) encapsulated within large dye-labeled myristoleate/myristoleic acid vesicles (g,i). Only the large vesicles containing the spheres become packed full of smaller vesicles presumably due to surface catalyzed vesicle assembly within the giant vesicles. Scale bar, 5 microns. Parts (a,b) reproduced from [9••] with permission. © *Nature* 2003 (http://www.nature.com/). Part (c–e) reproduced from [5] with permission. © 2003 *Biophysical Journal*.

systems. For primitive cells, even rare fusion events could have been important in allowing recombination between varying genotypes to occur. The origins of sex may thus trace back to environmentally triggered vesicle fusion events and the attendant mixing of encapsulated genetic molecules.

Just as there are many possible pathways for vesicle growth in model I, there are many potential mechanisms for vesicle division. When Luisi and colleagues [1••] demonstrated the growth of ferritin-labeled vesicles, they also saw a few very small vesicles containing ferritin that may have been produced through spontaneous vesicle fission (model IC). It is possible that these small vesicles result from some instability of rapidly growing vesicles, but this approach to spontaneous division needs more study. To obtain more efficient and reproducible vesicle

division, we extruded grown vesicles through small pores, so that the extruded vesicles have the same diameter as the initial population [2••]. By monitoring a fluorescent dye that was encapsulated in the initial vesicles, we were able to show that all of the dye remained encapsulated during growth and only a little more than expected was lost during the division step due to the geometrical constraint of division with constant surface area. The growth and division cycle was completed five consecutive times and presumably could be repeated indefinitely. This simple system serves as a proof of principle that primitive cellular replication could have occurred through purely physico-chemical forces. Fluid flow through porous rocks near hydrothermal vents has been proposed as a possible natural setting in which vesicle division by extrusion might take place [8]. Experimental tests of this idea are important, because it is far from clear that there

would be sufficient fluid flow through small channels in fractured rock to lead to significant vesicle division.

Division by budding (model ID) has been observed and studied in giant vesicles composed of phospholipids and other amphiphiles for many years [9••,10–12]. A variety of shape changes including budding can result from osmotically induced surface to volume changes, and thermally induced changes in relative leaflet area. Several recent papers illustrate new approaches to vesicle division by budding.

Takakura and Sugawara [13] described the generation of unusual myelin-like giant multi-lamellar vesicles, which exhibit budding upon electrolyte addition. These giant vesicles exhibited changes in morphology including both growth and division by budding, upon addition of a lipid precursor that hydrolyzes to generate both an amphiphile and an electrolyte (model IB,D). This system requires the presence of a membrane-incorporated catalyst of the hydrolysis reaction, and this catalyst would have to be replenished to allow for continued cycles of growth and division. It will be interesting to see if the multi-lamellarity of these vesicles can be maintained over many cycles of growth and division.

A new approach to the induction of budding makes use of the spontaneous separation of certain lipid mixtures into distinct phases. There is significant energy associated with the phase boundaries, and minimization of this energy can lead to budding and complete division. Giant vesicles made with sphingomyelin, dioleoyl-phosphatidylcholine (DOPC) and cholesterol form discrete domains with two distinct compositions that can be labeled with different dyes [9••] (Figure 2a,b). When the temperature is raised, the domains increase in curvature, thus decreasing the length of their common boundary, until complete separation is achieved. Of course, the daughter vesicles differ in composition from the parental vesicle, and selective uptake of new lipid or re-equilibration of the lipid composition would have to occur to enable repeated cycles of budding.

Budding can also result from the induction of bilayer asymmetry. By loading one leaflet with components that cannot flip to the other leaflet, the membrane may acquire an intrinsic curvature that favours budding. Giant phosphatidylcholine/sphingomyelin vesicles have been induced to bud by the asymmetrical sphingomyelinase-catalyzed generation of ceramide in the inner leaflet [10]. Vesicle fission and budding (model IC,D) can also be achieved through the introduction of surfactants [11] and through the action of enzymes such as phospholipase [12].

## Internal production of vesicles
Model II illustrates a different approach to vesicle replication involving the internal synthesis of new vesicles.

Wick, Walde and Luisi [14] observed the formation of new vesicles inside a giant vesicle microscopically when new amphiphiles were enzymatically synthesized inside the vesicle. More recently, Takakura et al. have devised a chemical system in which new amphiphiles are synthesized inside a giant vesicle [15]. In both cases, these new vesicles were sometimes released from the original vesicle without rupture. The mechanism by which a vesicle can pass through the membrane of a giant vesicle (sometimes referred to as 'birthing') is unclear.

An alternative approach to the enzymatic formation of new internal vesicles involves the effects of encapsulated mineral surfaces (model II). We have shown that a dispersion of montmorillonite clay in buffer can accelerate the assembly of vesicles from fatty acid micelles [2••]. When the vesicles form, some of the clay becomes encapsulated in large vesicles, which in turn become packed full of smaller vesicles (Figure 2f–i). This striking effect is probably due to some of the added fatty acid crossing the membrane, interacting with the surface of the encapsulated clay, and forming new vesicles that are then trapped inside the original vesicle. Repeated cycles of internal vesicle synthesis, release and growth would require some means by which clay particles could be introduced into newly formed vesicles. While this could potentially occur through vesicle–vesicle fusion, a more interesting possibility would be internal mineral synthesis by precipitation from a super-saturated solution of silicates, perhaps facilitated by some aspect of the internal vesicle environment.

## Surface-mediated vesicle replication
An intriguing but still hypothetical scheme for vesicle replication invokes a role for fluid flow past surface-attached vesicles, as shown in model III. Surface-attached vesicles would grow through the uptake of additional membrane components, eventually becoming unstable to the shear gradient leading to the budding off of daughter vesicles. Vesicles can be anchored to a surface by either a specific integral membrane-bound linkage or through adsorption. For example, vesicles may be tethered to an avidin-coated surface via biotinylated phospholipids [16]. Vesicles adsorbed to a surface have been shown to fuse with additional vesicles introduced in fluid flowing over the surface [17••]. Giant fatty acid vesicles have also been observed to assemble by the fusion of smaller vesicles in regions of a glass surface coated by hydrocarbons [18]. Microfluidic devices in which fluid flow can be precisely controlled may well prove ideal for the study of vesicle deformations in a flow-field.

## A direct link between vesicle growth and energy production
As models of primitive cellular life, replicating vesicles must also provide a means of harvesting energy and small molecules to fuel a primitive metabolism. Work in our

laboratory has recently demonstrated that a pH gradient can form spontaneously across the membrane of the growing vesicles [19••]. When vesicles are grown by the addition of fatty acids in the form of micelles, the additional fatty acid is initially incorporated into the outer leaflet of the vesicle membrane. The fatty acid then equilibrates within the membrane by flipping from the outer to the inner leaflet. Protonated fatty acid molecules flip more rapidly than ionized molecules, but re-equilibrate on the vesicle interior, releasing on average 0.5 protons per fatty acid molecule and therefore acidifying the interior of the vesicle. With vesicles composed of fatty acids, this gradient can only be maintained in the absence of permeable cations. However, vesicles built from other amphiphiles (e.g. phosphorylated lipids) may accumulate and maintain pH gradients as a result of growth. The generation of pH gradients due to vesicle growth suggests that it may be possible to capture some of the energy released during growth in a form that could be used for other processes such as substrate uptake. Alternatively, because vesicle growth is limited by the build-up of the pH gradient, the evolution of membranes able to maintain a pH gradient may have required the co-evolution of mechanisms for the release of the gradient, such as proton ionophores or pumps.

## Conclusions

The growing interest in experimental models of vesicle replication has produced a variety of interesting systems that reach beyond chemical evolution to begin exploration of dynamic supramolecular self-organization in relation to simple replicating cell-like compartments. This body of work sets the stage for future efforts to generate systems capable of continuing replication under plausibly prebiotic conditions. Additional challenges include the development of replicating vesicles compatible with ribozyme activity and therefore the internal replication of genetic material, while retaining permeability to small molecule substrates such as nucleotides. Continued exploration of the properties of vesicles made from small, simple amphiphiles may eventually provide clues to the identity of the actual pre-biotic constituents of the earliest cells.

## Acknowledgements

## References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest

1. Berclaz N, Muller M, Walde P, Luisi PL: **Growth and
•• transformation of vesicles studied by ferritin labeling and cryotransmission electron microscopy**. *J Phys Chem* 2001, **105**:1056-1064.
Luisi and colleagues have published several papers on self-replicating systems composed of amphiphiles. In this work, phospholipid vesicles containing ferritin as a marker were grown by adding fatty acid micelles.

2. Hanczyc MM, Fujikawa SM, Szostak JW: **Experimental models
•• of primitive cellular compartments: encapsulation, growth, and division**. *Science* 2003, **302**:618-622.
We demonstrate that primitive cellular growth and division could occur purely through physico-chemical forces. Also, we suggest that mineral surfaces may have played a key role in both the synthesis of biopolymers and the assembly of the membrane compartments in a prebiotic environment.

3. Chen IA, Szostak JW: **A kinetic study of the growth of fatty acid vesicles**. *Biophysical J* 2004, **87**:988-998.

4. Chen IA, Roberts RW, Szostak JW: **The emergence of competition between model protocells**. *Science* 2004, **305**:1474-1476.

5. Lei G, MacDonald RC: **Lipid bilayer vesicle fusion: intermediates captured by high-speed microfluorescence spectroscopy**. *Biophysical J* 2003, **85**:1585-1599.

6. Muller M, Zschornig O, Ohki S, Arnold K: **Fusion, leakage and surface hydrophobicity of vesicles containing phosphoinositides: influence of steric and electrostatic effects**. *J Membr Biol* 2003, **192**:33-43.

7. Monnard PA, Apel CL, Kanavarioti A, Deamer DW: **Influence of ionic inorganic solutes on self-assembly and polymerization processes related to early forms of life: implications for a prebiotic aqueous medium**. *Astrobiology* 2002, **2**:139-152.

8. Russell M: **On the importance of being alkali**. *Science* 2002, **302**:580-581.

9. Baumgart T, Hess ST, Webb WW: **Imaging coexisting fluid
•• domains in biomembrane models coupling curvature and line tension**. *Nature* 2003, **425**:821-824.
Two-photon microscopy was used to produce striking images of giant vesicles with distinct membrane domains.

10. Holopainen JM, Angelova MI, Kinnunen PK: **Vectorial budding of vesicles by asymmetrical enzymatic formation of ceramide in giant liposomes**. *Biophys J* 2000, **78**:830-838.

11. Mavcic B, Babnik B, Iglic A, Kanduser M, Slivnik T, Kralj-Iglic V: **Shape transformation of giant phospholipid vesicles at high concentrations of C12E8**. *Bioelectrochemistry* 2004, **63**:183-187.

12. Staneva G, Angelova MI, Koumanov K: **Phospholipase A2 promotes raft budding and fission from giant liposomes**. *Chem Phys Lipids* 2004, **129**:53-62.

13. Takakura K, Sugawara T: **Membrane dynamics of a myelin-like giant multilamellar vesicle applicable to a self-reproducing system**. *Langmuir* 2004, **20**:3832-3834.

14. Wick R, Walde P, Luigi PL: **Light microscopic investigations of the autocatalytic self-reproduction of giant vesicles**. *J Am Chem Soc* 1995, **117**:1435-1436.

15. Takakura K, Toyota T, Sugawara T: **A novel system of self-reproducing giant vesicles**. *J Am Chem Soc* 2003, **125**:8134-8140.

16. Pignataro B, Steinem C, Galla HJ, Fuchs H, Janshoff A: **Specific adhesion of vesicles monitored by scanning force microscopy and quartz crystal microbalance**. *Biophys J* 2000, **78**:487-498.

17. Johnson JM, Ha T, Chu S, Boxer SG: **Early steps of supported
•• bilayer formation probed by single vesicle fluorescence assays**. *Biophys J* 2002, **83**:3371-3379.
Vesicles adsorbed to a surface were shown to fuse with vesicles introduced in a flow field. This study may form the basis for surface-mediated vesicle growth and division.

18. Morigaki K, Walde P: **Giant vesicle formation from oleic acid/ sodium oleate on glass surfaces induced by adsorbed hydrocarbon molecules**. *Langmuir* 2002, **18**:10509-10511.

19. Chen IA, Szostak JW: **Membrane growth can generate a
•• transmembrane pH gradient in fatty acid vesicles**. *Proc Natl Acad Sci USA* 2004, **101**:7965-7970.
The growth of fatty acid vesicles produces a pH gradient. This energy source may be used in future studies to drive essential functions such as the transport of small molecules.