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Synthetic Minimal Cell: Self-Reproduction of the Boundary Layer

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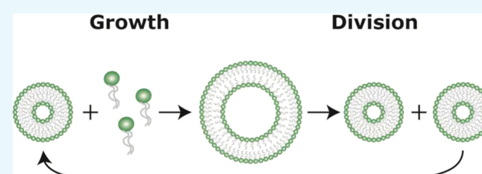
ABSTRACT: A critical aspect in the bottom-up construction of a synthetic minimal cell is to develop an entity that is capable of self-reproduction. A key role in this process is the expansion and division of the boundary layer that surrounds the compartment, a process in which content loss has to be avoided and the barrier function maintained. Here, we describe the latest developments regarding self-reproduction of a boundary layer with a focus on the growth and division of phospholipid-based membranes in the context of a synthetic minimal cell.



INTRODUCTION

One of the main challenges in the field of synthetic biology is the bottom-up construction of a minimal cell. Such synthetic systems would contain only a minimum of cellular components (nucleic acids, enzymes, lipids, etc.), thereby creating a "living cell" capable of executing basic functions among which are growth, replication, and division.[1] The interest in developing synthetic cells is manifold. It will provide a deeper understanding on how processes in cells interact, and define the minimal requirement for "life". Moreover, synthetic cells may open new fields of application. This is already evident for nonreproductive synthetic cells that are applied in cancer treatment, antibacterial studies, and directed evolution.[2−4] The ability to self-reproduce will further extend the functionality of such systems.

By using the bottom-up approach, first subcellular modules like adenosine triphosphate (ATP) generation, phospholipid biosynthesis, protein synthesis, etc. are created, which later are assembled together, finally resulting in a self-sustaining minimal cell mimic.[5] Essentially, a synthetic cell should be encoded by a minimal genome that specifies all essential functions and that allows the cells to thrive by coordinated transcription−translation. Such minimal systems do not contain complex networks and interactions that are present in living organisms, which creates an advantage as it allows to study biological processes with minimal undesired interference. At the same time, this also makes the system more vulnerable as it will lack the robustness and flexibility of a regulated cell. The bottom-up construction of a synthetic cell is to some extent similar to early life forms or protocells that emerged at the origin of life. Although a precise definition of such a minimal form of life remains elusive and is under debate, there is consensus regarding some critical elements for life, which include self-organization into a compartment.[6] A compartment defines a confined space that allows for crowding of molecules, which is essential for chemical reactions.[7] Furthermore, compartmentalization permits for distinct conditions in the interior of the synthetic cell, which is crucial for metabolism. Although compartmentalized metabolism is a reasonable description of a living entity, obviously a missing characteristic is the ability to self-reproduce, as the compartment should be able to grow and divide. Here, we will discuss the self-reproduction of compartments in the context of the bottom-up construction of a synthetic minimal cell. Specifically, we focus on the growth and division of the surrounding boundary layer and discuss the necessities providing communication across this barrier.

RESULTS AND DISCUSSION

Compartmental Self-Reproduction Based on Fatty Acids. In the development of a synthetic cell, the simplest design of a self-reproducing compartment would be growth by spontaneous insertion of new building blocks, resulting in expansion, followed by spontaneous division. Fatty acid-based vesicles are extremely suitable for this purpose.[8,9] Fatty acids can appear as monomers, micelles, as well as membranes (Figure 1A). Their chemical properties allow them to rapidly interchange between these different phases, thus resulting in compartmental growth by spontaneous insertion.[8,10] The group of Szostak pioneered the use of self-reproducing fatty acid-based vesicles in the context of the origin of life.[11,12] By simply feeding fatty acid vesicles with micelles, the vesicles grow by spontaneous integration of new fatty acids. This phenomenon has been extensively studied[13] and further developed. An active ribosome-like dipeptide catalyst encapsulated in such a vesicle was able to synthesize a new dipeptide. As binding of this dipeptide to the fatty acid vesicle membrane resulted in enhanced fatty acid incorporation, vesicle growth was stimulated, thereby providing an evolutionary advantage.[14] In another example, vesicular growth is linked to the initiation of enzymatic activity. By adding new fatty acids to overcrowded ribozyme- and oligonucleotide-containing fatty acid vesicles, compartment expansion caused internal dilution, which in turn activated ribozyme activity. Noteworthy, as the

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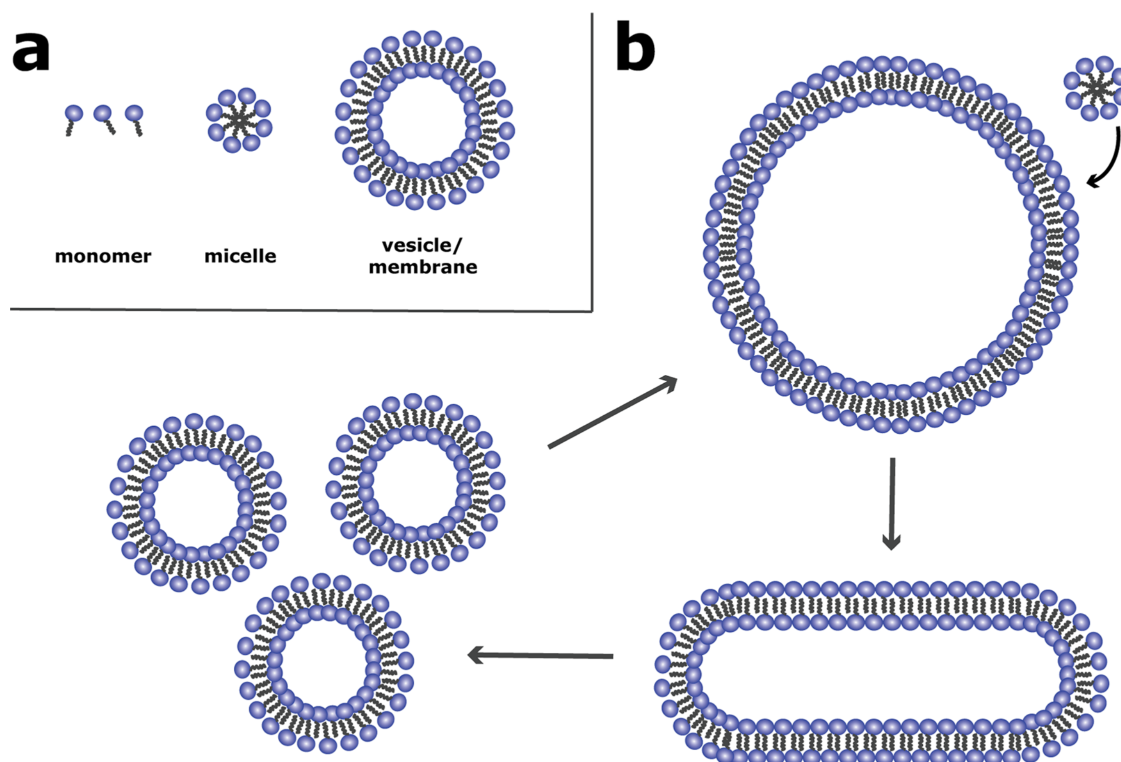

Figure 1. Schematic representation of compartment self-reproduction based on fatty acids. (a) Fatty acids appearing as monomers, micelles, and vesicles. (b) Fatty acid vesicles grown by a slow feed with micelles transform into long threadlike vesicles and ultimately divide.

ribozyme activity per unit volume during the protocell volume-change remained constant, the system shows homeostatic behavior.[15]

For self-reproduction, the growth of fatty acid vesicles could be combined with division (Figure 1B). By slowly growing these vesicles with fatty acid micelles, spherical vesicles transform into long threadlike vesicles, which minimizes content loss during division.[16] This was demonstrated with RNA-encapsulated vesicles. Daughter cells containing this RNA could be observed after multiple divisions, mimicking a possible early and simple form of life. The observed vesicle fission is caused by an imbalance of fatty acids between the two membrane leaflets.[17] In another approach, vesicles were fed with fatty acids formed by a simple nonenzymatic synthesis reaction, thereby coupling compartmental growth and division to a primitive form of lipid synthesis.[18]

Phospholipid-Based Membranes. Although fatty acid-based membranes are extremely useful in understanding the basics and principles of a self-reproducing system, the ability of fatty acids to rapidly migrate in and out of the membranes makes such compartments intrinsically unstable.[11] Furthermore, fatty acids are only able to encapsulate a rather small intravesicular volume, too small to support multienzymatic reactions and are, for this reason, unsuitable to function as compartments for a synthetic minimal cell. Indeed, biological membranes consist of more complex molecules, of which phospholipids are the major component. They are uniformly present in all life, and their amphipathic character allows them to self-assemble in bilayer-like structures, thereby forming the barrier between the interior cytoplasm and the exterior environment. Furthermore, phospholipid membranes are equipped with additional functions as they are involved in a wide variety of processes (signal transduction, solute transport, DNA replication, protein targeting, etc.).[19] Hence, the boundary layer of a synthetic minimal cell should exist out of phospholipids. For example, phospholipid vesicles would be suitable as they can be easily formed by multiple techniques and are already widely used.[20] A good prototype for such a phospholipid boundary layer is the inner membrane of the model organism *Escherichia coli*, which has been studied in great detail.[21] It exists almost completely out of the zwitterionic phospholipid phosphatidylethanolamine (PE, 70−75%), the anionic phosphatidylglycerol (PG, 20−25%), and, depending on the growth phase, varying amounts of cardiolipin (CL, 0−10%). While CL is nonessential, a proper balance between the bilayer forming PG and the nonbilayer PE is needed for proper functioning of membrane proteins.[22]

Expansion of Phospholipid Membranes. Unlike fatty acids, the chemical properties of phospholipids do not allow them to rapidly exchange between membranes and/or phases, meaning they are permanently localized. Hence, phospholipid membranes cannot self-reproduce via spontaneous incorporation of externally added phospholipid molecules. Instead, membrane expansion is a process in which phospholipid biosynthesis is combined with insertion. Phospholipid biosynthesis is a complex process, and most of the current knowledge is based on the pioneering biochemical research done by Kennedy and co-workers on *E. coli*.[23,24] This process can be divided into three different subsections: acyl chain biosynthesis, phosphatidic acid (PA) formation, and introduction of polar headgroup specificity (Figure 2).[25] In the next section, these processes are discussed within the context of the construction of a synthetic cell.

Acyl Chain Biosynthesis. Phospholipid synthesis begins with the synthesis of long-chain fatty acids that need to be covalently attached to the *sn*-glycerol-3-phosphate (G3P)

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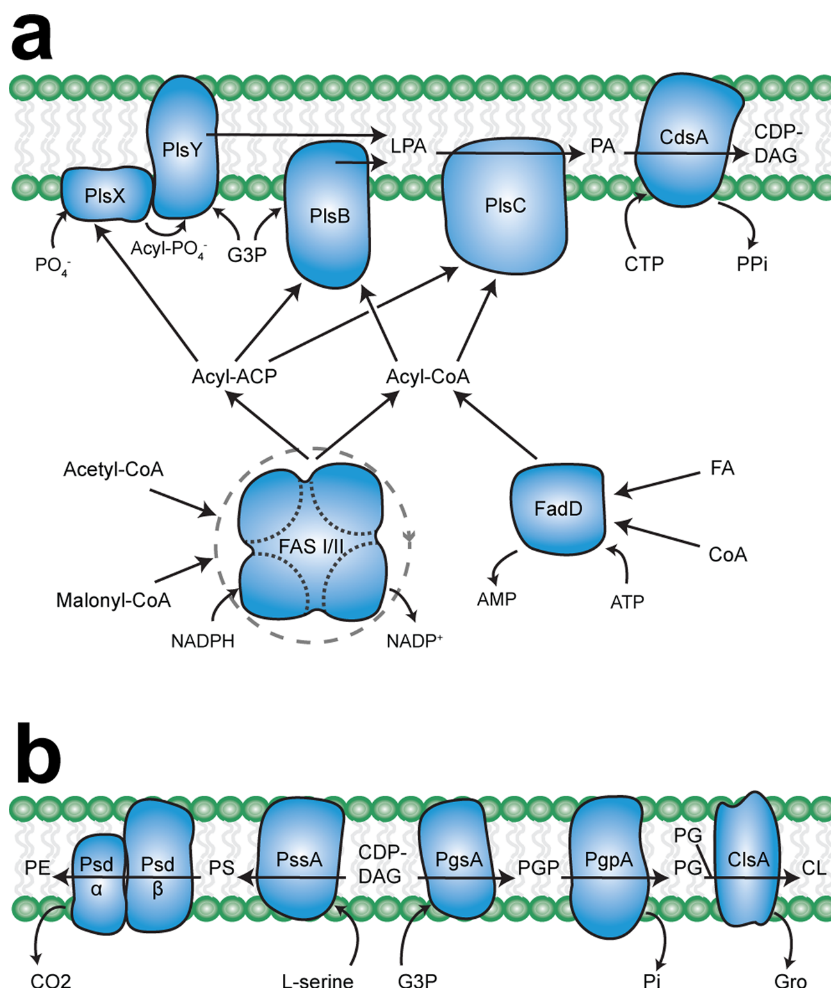

Figure 2. Phospholipid biosynthesis in *E. coli* as a template for general phospholipid synthesis in bacteria. (a) Acyl chain synthesis, followed by lysophosphatidic acid (LPA), PA, and cytidine diphosphate diacylglycerol (CDP-DAG) formation. (b) Polar headgroup incorporation. Note: the phosphatidylserine synthase A (PssA) enzyme is displayed integrally in the membrane as most commonly in bacteria; however, in *E. coli*, this is a soluble protein attaching to the membrane.

backbone, which occurs via formation of a thioester. As this coupling requires energy, the fatty acid is initially linked to an energy-containing group: acyl-carrier protein (ACP) or coenzyme A (CoA). In *E. coli*, these acyl–acyl donor complexes are mainly produced by the fatty acid synthetase-II (FAS-II), a multicomplex enzyme that combines the construction of long fatty acid chains, together with the linkage toward ACP (Figure 2A).[26] As a first step toward in vitro fatty acid biosynthesis, purified FAS from *E. coli* was shown to be capable of producing acyl-ACP and fatty acid derivatives from the substrates acetyl- and malony-CoA.[27,28] Later, in vitro fatty acid synthesis could be combined with glycolytic enzymes and pyruvate dehydrogenase, to complete an in vitro reconstituted system capable of converting glucose into fatty acid.[29] This system could potentially form a module in a synthetic minimal cell; however, it comprises a large amount of enzymes, which complicates its applicability. As an alternative for the multicomplex enzyme FAS-II, FAS-I could be used, a huge single enzyme that consists of multiple subdomains, thereby simplifying its in vitro applications.[30] The corresponding enzyme from *Brevibacterium ammoniagenes* (*Corynebacterium ammoniagenes*) has been purified, reconstituted into liposomes, and shown to synthesize fatty acids.[31] The fatty acids inserted spontaneously into the lipid bilayer, thus showing compartment expansion via enzymatic fatty acid synthesis.[32] Although the in vitro application of fatty acid synthetases (FAS) can yield substantial product, the complexity of the FAS enzyme makes it difficult to control the composition of the fatty acid products and is therefore less suitable for the development of synthetic cells.

A more efficient route for the biosynthesis of acyl–acyl donor complexes comprises the β-oxidation of free fatty acids. Here, fatty acids are directly coupled to a CoA moiety by the enzyme FadD, thereby bypassing the complex synthesis of fatty acids (Figure 2A).[33] By mixing fatty acids, ATP and CoA with purified FadD, a wide variety of FA-CoA could be produced in vitro.[34] Due to the simplicity and versatility of this enzymatic reaction, FadD is a suitable candidate for the initial step in in vitro phospholipid biosynthesis as demonstrated in coupled enzymatic reactions that resulted in the synthesis of phosphatidic acid, with a fatty acid composition dictated by the feed with free fatty acids.[35]

Phosphatidic Acid Biosynthesis. Phosphatidic acid (PA) comprises a G3P attached to two fatty acids, which makes it the simplest form of a phospholipid. It is an essential intermediate in phospholipid biosynthesis. In *E. coli*, two membrane-associated proteins are responsible for these acylation reactions, i.e., glycerol-3-phosphate acyltransferase

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(PlsB) and lysophosphatidic acid acyltransferase (PlsC) (Figure 2A).[36,37] PlsB attaches the acyl chain from both acyl donors acyl-CoA and acyl-ACP to the 1-position of G3P via formation of a new thioester, finally resulting in the synthesis of lysophosphatidic acid (LPA).[38] PlsB is an inner membrane acyltransferase that requires the presence of a phospholipid membrane for activity.[35,39] Hence, for the development of a synthetic cell, preexisting vesicles need to be supplied, as membranes cannot be formed de novo. As an alternative for PlsB, the enzyme tandem PlsX−PlsY, commonly found in bacteria, could be used (Figure 2A).[40] However, as PlsY is an integral membrane protein, it is expected to require a membrane for its activity as well.[41]

PlsC is a membrane protein that attaches another acyl−acyl donor moiety to the 2-position of LPA, resulting in the formation of PA (Figure 2A).[42] Unlike PlsB, this enzyme does not strictly require a membrane for its activity, although the presence of a membrane seems to enhance its activity.[35]

An in vitro system based on PlsB and PlsC should in principle be capable of mimicking an expanding phospholipid-based boundary layer. Indeed, by combining the PlsB- and PlsC-based enzymatic reactions with FadD-mediated formation of acyl-CoA, phospholipids could be produced from simple fatty acid building blocks, resulting in expansion of the membrane.[35] In this system, PlsB was found to incorporate both saturated and unsaturated fatty acids, whereas PlsC shows a preference for unsaturated fatty acids. A similar observation was made by analyzing the lipidome of *E. coli*, in which the sn-1 site is mainly occupied by oleic acid (C18:1) and palmitic acid (C16:0), whereas mostly unsaturated fatty acids are present at the sn-2 position.[26,43] Notably, PlsB- and PlsC-mediated production of PA has also been established through in vitro-based transcription−translation of these enzymes.[44,45] Currently, however, the yield of those reactions is too low to observe any appreciable membrane expansion.

Polar Headgroup Incorporation. As final steps in phospholipid biosynthesis, alcohols are introduced onto PA as polar head groups. While there is a huge variety in nature, all key phospholipid species are derived from the central precursor CDP-diacylglycerol (CDP-DAG) (Figure 2B). CDP-DAG synthesis is catalyzed by the Mg^{2+}-dependent enzyme CDP-diacylglycerol synthase (CdsA), which hydrolyzes a pyrophosphate from cytidine triphosphate, thereby coupling a cytidine monophosphate (CMP) to PA (Figure 2B).[46] CdsA consists mostly of transmembrane spanning segments.[47] The structure of Cds from *Thermotoga maritima*, which is a homologue of the *E. coli* CdsA, shows the presence of nine transmembrane helices that are arranged into a novel fold with three domains.[48] CdsA has been reconstituted into liposomes, and in conjunction with FadD, PlsB, and PlsC, CDP-DAG can be synthesized from simple building blocks.[35] However, since CDP-DAG is an inhibitor of CdsA activity, bulk production of phospholipids is only possible when the downstream polar headgroup enzymes are included as well. Here, the biosynthesis pathway splits into different directions depending of the phospholipid species produced.

To synthesize PE from CDP-DAG, two additional conversions are necessary (Figure 2B). First, CDP-DAG is converted into phosphatidylserine (PS) by PS synthase (PssA), which replaces the CMP moiety with a serine.[49,50] Next, PS can be converted into PE by the membrane protein PS decarboxylase (Psd), by decarboxylation of PS.[51] PG is synthesized via the conversion of CDP-DAG into phosphatidylglycerol-3-phosphate (PGP) by PGP synthase (PgsA), followed by the removal of the 3-phosphate by a PGP phosphatase, most notably by PgpA[52] (Figure 2B). PG serves as a substrate for the synthesis of cardiolipin, a conversion that in *E. coli* is catalyzed by one of the three cardiolipin synthetases (ClsA, ClsB, or ClsC) (Figure 2B). Like most bacterial cardiolipin-synthesizing enzymes, ClsA and ClsB use two PG molecules as substrate.[53] In this process, the glycerol head group of one PG molecule is coupled to the phosphate of the other PG molecule that serves as a phosphatidyl donor. This results in the formation of cardiolipin and glycerol. However, ClsC uses PE instead of PG as phosphatidyl donor, which results in an ethanolamine leaving group.[54] Interestingly, cardiolipin-synthesizing enzymes appear to be responsible for the production of multiple other phospholipid species as well, most likely via incorporation of an alcohol during the reverse reaction of cardiolipin hydrolysis.[55,56] The aforementioned enzymes (CdsA, PssA, Psd, PgsA, and PgpA) have been purified and coreconstituted into liposomes allowing for the biosynthesis of PE and PG from PA.[57] Moreover, this enzymatic cascade could be coupled to the in vitro synthesis of PA from fatty acids and G3P, yielding the essential phospholipid species PE and PG in a membrane-expanding system.[35] In a similar approach, the in vitro biosynthesis of multiple phospholipids species was demonstrated by cell-free gene-encoded enzymes,[58] albeit with low efficiency.

Homeostasis of the Membrane Composition. In *E. coli*, the ratio between the phospholipid species PE and PG is crucial for membrane functioning and is maintained constant under all conditions.[25,59] Therefore, phospholipid homeostasis may be critical for the synthetic cell as well. How this balance is maintained is unknown, but it likely involves transcriptional regulation. By varying the concentrations of the enzymes involved in the synthesis of PE and PG, a liposomal membrane containing a 70:30 ratio of these two phospholipid species could be obtained that mimics the features of the *E. coli* inner membrane.[35] Obviously, in a transcription−translation-based synthetic cell, the elements that ensure phospholipid homeostasis most likely will be absent. Therefore, a proper balance of the expression of the various phospholipids will be critical. However, some self-regulatory mechanisms at the protein level are present as well, as the PssA enzyme from *E. coli* can only associate with the membrane in the presence of sufficient anionic lipid, thereby stabilizing the balance between PG and PE.[60,61]

Phospholipid Flip-Flop. A crucial aspect of a continuously growing phospholipid bilayer is the insertion of newly synthesized phospholipids into both membrane leaflets. Since phospholipid biosynthesis occurs asymmetrically at the membrane, i.e., at the inside of the cell, phospholipid flip-flop may play a crucial role in membrane expansion. Spontaneous flip-flop of phospholipids from one membrane leaflet to the other is an extremely slow process[62] and cannot keep up with the rate of biosynthesis. Therefore, it is the general view that this process is catalyzed either by specific enzymes termed flippases, floppases, or scramblases, or less specifically along the surface of integral membrane proteins. Flippases do not just balance the lipid composition on both leaflets of the membrane, but also allow for an asymmetric distribution of a specific phospholipid between those leaflets.[63] Although in the bacterial membrane, the enzymes responsible for phospholipid flip-flop have not yet been identified, it is known that membrane-spanning proteins in general can

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stimulate this process.[62,64] Moreover, introduction of certain phospholipid species in one of the leaflets creates an unequal distribution of phospholipid species, which can stimulate flip-flop as well.[65]

In the in vitro phospholipid-synthesizing systems, phospholipid flip-flop does not seem to play a role.[35] Possibly, the partitioning of fatty acids in the membrane causes local defects in the bilayer, thereby stimulating flip-flop.[66,67] At high concentrations, however, fatty acids interfere with the membrane permeability barrier, which is an undesired phenomenon for a synthetic cell. As recently a family of phospholipid flippases has been discovered in fungi,[68,69] introduction of such membrane proteins may alleviate the potential asymmetry issues with localized phospholipid biosynthesis in the synthetic cell once the production of high levels of phospholipid is reached.

Chemical Phospholipid Analogues and Compartmentalization. An alternative for the natural synthesis of phospholipids is formed by the nonenzymatic or chemical synthesis of phospholipid analogues. Their synthesis can be relatively simple compared to biological phospholipid synthesis, which provides a possible advantage. This is illustrated by the coupling of an amphiphilic aldehyde to a lipophilic aniline derivative, which forms a phospholipid-like amphiphile.[70] As the synthesis occurred inside a vesicle consisting of the same amphiphile, the formation of new vesicles represents self-reproduction. Moreover, vesicle self-reproduction could be combined with intravesicular DNA amplification, thereby coupling compartment self-reproduction with self-replication of information. As the amplification of DNA accelerated the division of the giant vesicles, this event provides an evolutionary benefit.[71] Interestingly, the chemical synthesis of phospholipid opens the field of de novo membrane formation as well. In nature, de novo membrane formation appears to be impossible, as many of the enzymes involved in the synthesis of phospholipids are membrane-associated or integrated proteins that require the bilayer as a matrix to function properly. Chemical phospholipid synthesis can circumvent the requirements of a complex enzymatic pathway. Furthermore, the chemical substrates out of which a synthetic phospholipid is built can be water-soluble, which makes them ideal candidates for the de novo construction of membranes. One example showing de novo bilayer formation is based on hybrid micelles consisting of a dodecylamine-containing amphiphilic imine and an amphiphilic aldehyde. Transfer of the dodecylamine onto the amphiphilic aldehyde within the hydrophobic environment of the amphiphilic aggregates resulted in the transformation into phospholipid-like giant vesicles.[72]

From a more biological point of view, synthetic lipids with great similarity to a specific natural phospholipid species were created as well. A copper-catalyzed azide−alkyne cycloaddition resulted in the formation of a triazole-containing phospholipid analogue of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), thereby mimicking the attachment of a second acyl chain to a lysophospholipid.[73,74] To create even more biological relevance, POPC-like phospholipids could be synthesized from thioesters by applying native chemical ligation, thereby mimicking biological acyl chain coupling.[75] Moreover, the synthesis of these POPC analogues could be combined with integral membrane protein reconstitution, as the detergent analogue used for solubilizing the membrane protein is part of the phospholipid synthesis.[76,77] Altogether, these examples show that synthetic phospholipid analogous have a great potential to form the boundary layer of a synthetic minimal cell. Their synthesis can be simple compared to biological phospholipid synthesis and not limited to a given subset of enzymes, which opens a world for new phospholipid species. Nevertheless, the current variety in chemical phospholipid analogues is still limited, and their functioning in membrane-related processes is still barely resolved.

Functional Phospholipid Membranes and Synthetic Cells. Cellular membranes do not only function as impermeable barriers for ions and other molecules, but also provide a matrix for membrane proteins to act as an anchor site, promote folding, and support activity.[19,78] Proteins embedded in, or associated with, the membrane play a pivotal role in membrane functions. They facilitate transport of solutes (nutrients, ions, signaling molecules, etc.) and macromolecules (proteins, oligosaccharides, etc.) across the membrane. This enables regulatory processes of exchange and communication between the interior of the cell and the environment, processes which are critical for homeostasis and growth. Moreover, membrane proteins function in intracellular processes as well, such as DNA replication, protein targeting, etc..[19] Evidently, a synthetic minimal cell should comprise membranes containing membrane proteins.

Often membrane proteins rely on specific physicochemical properties of specific lipids for proper functioning, which is illustrated by the role of the two essential *E. coli* phospholipids PE and PG.

A strain in which the Pss and Psd genes were inactivated, thus lacking PE, is unable to grow and exhibits impaired motility and chemotaxis.[79] However, this strain can be rescued by the presence of high concentrations of divalent cations.[80] This has been associated with the need for a nonbilayer type of phospholipid for activity and functionality, as the high concentrations of divalent cations can induce a conelike structure in PG, which is the main phospholipid in this PE-deficient strain. Furthermore, some membrane proteins require PE for folding as exemplified by the lactose permease.[81,82]

Also, the anionic lipid PG is critical for membrane protein functioning, which in most cases can be attributed to the negative charge of the head group. For instance, DNA associates with the membrane in a PG-dependent manner, which involves the protein DnaA that in turn regulates the initiation of DNA replication at the initiation site oriC. In this process, DnaA associates with the membrane through the interaction of an amphipathic helix with the anionic lipid head group of PG, which is essential for replication.[83,84] PG can further act as a chaperone in protein folding during the insertion of proteins into the membrane. This is illustrated by the PG-dependent folding of co-translationally membrane-inserted MraY translocase[85] and the assembly of the potassium channel KcsA.[86]

In *E. coli*, most proteins are inserted into or translocated across the membrane by the Sec translocase.[87,88] Translocation of proteins through the SecYEG pore, i.e., translocon, is driven by SecA. Functioning of this ATP-ase is strictly dependent on anionic phospholipids,[89] which in *E. coli* mostly concerns PG. SecA interacts with the membrane through its N-terminal amphipathic helix that inserts into membranes containing anionic phospholipids. The aforementioned interaction induces a conformational change onto SecA, which in turn promotes the high-affinity interaction with the translocon concomitantly with the activation of the SecA ATPase activity.[90] Furthermore, the positively charged signal sequences

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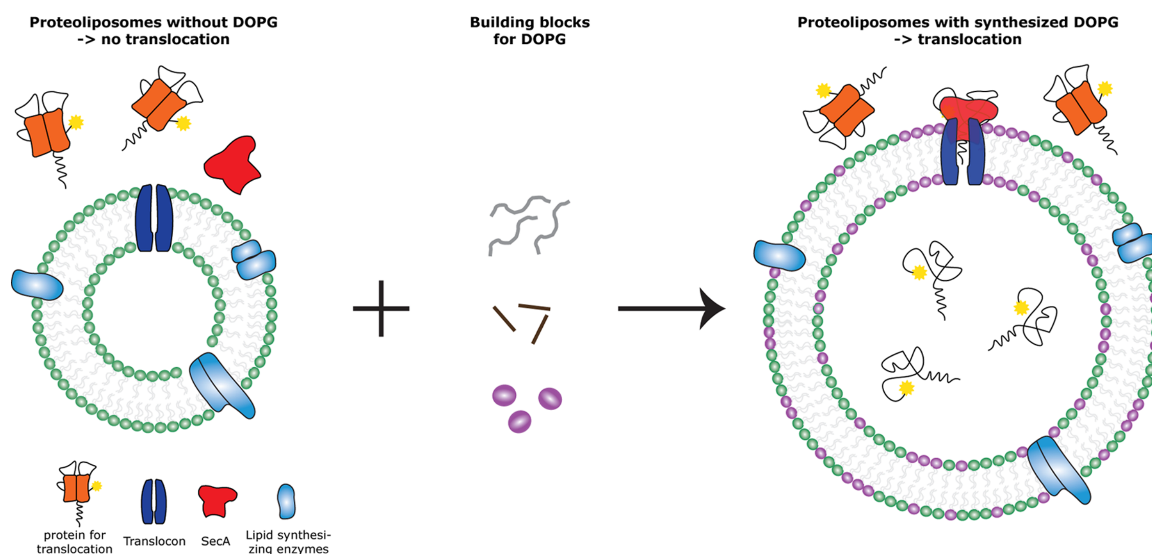

Figure 3. Schematic representation of anionic lipid-dependent translocon activation. Anionic lipid-depleted proteoliposomes reconstituted with SecYEG and enzymes involved in PG synthesis cannot translocate preproteins. Introduction of the building blocks for PG allows for synthesis and incorporation of this molecule into the liposomal membrane, thereby activating the translocon for SecA-mediated protein translocation.

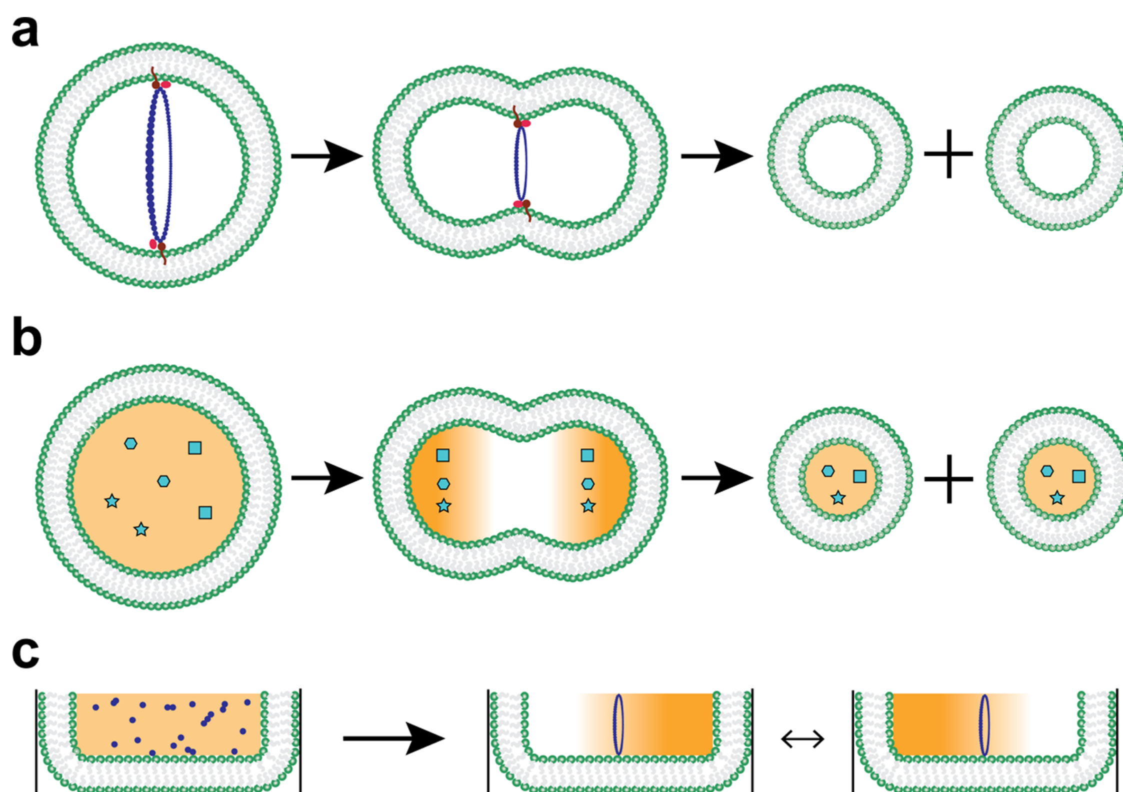

Figure 4. Division of phospholipid-based compartments. (a) Contraction of a membrane-anchored FtsZ invaginates the compartment, finally resulting in division. (b) Oscillating Min-system ensures binary fission. (c) Reconstitution of FtsZ in the presence of the Min-system allows for proto-ring formation at midcell.

of secretory proteins convert into an α-helical configuration upon interaction with the anionic phospholipids.[91] This dual function of PG was exploited to demonstrate a functional connection between phospholipid biosynthesis and protein translocation in an in vitro reconstituted system. Herein, the translocon was reconstituted into a membrane lacking anionic phospholipids, whereupon the synthesis of bulk quantities of PG was found to reactivate the translocon for protein translocation (Figure 3) (Koch et al., unpublished data). The latter represents an example of the functional integration of two biochemical modules in the development of a functional expanding boundary layer of a synthetic cell.

Division of Phospholipid Vesicles. Although fatty acid vesicles can spontaneously divide, phospholipid-based membranes need to be divided to complete compartmental self-reproduction. While the division of vesicles can be based on physical mechanisms or driven by the chemical synthesis of materials,[92] cellular life makes use of an enzymatic division

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machinery. In bacteria, division occurs via contraction of a membrane-interacting ring located at midcell, ultimately splitting the cell into two daughter cells by invagination (Figure 4A).[93] In bacteria and some archaea, division is initiated by the formation of a proto-ring, which in its simplest form comprises three proteins: filamenting temperature-sensitive mutant Z (FtsZ), filamenting temperature-sensitive mutant A (FtsA), and ZipA. FtsZ is a guanosine triphosphate-dependent protein that can assemble into a protein polymer and form the so-called Z-ring. This ring can associate with the membrane via an interaction of FtsZ with either FtsA or ZipA, which have overlapping roles but are both essential for subsequent division events. Interestingly, gain of function mutants of FtsA can compensate for the loss of ZipA, thereby allowing for functional proto-ring formation by only two enzymes.[94,95]

The positioning of the proto-ring at midcell is crucial. In conjunction with binary fission, this allows for the formation of the two daughter cells with intracellular content identical to that of the mother cell (Figure 4B). This process ensures continued proliferation. The middle of a compartment can be identified via polarization. In bacteria, correct placement of the proto-ring is most commonly regulated by the Min-system. In *E. coli*, this system comprises the proteins Min C, D, and E that creates polarity via formation of oscillating patterns from cell-pole to cell-pole.[96]

The in vitro division of phospholipid-based compartments starts with reconstitution of the minimal divisome in liposomes. Some pioneering work has been performed by the group of Schwille regarding in vitro proto-ring formation and reconstitution of an oscillating Min-system in the presence of a membrane.[97,98] By simple reconstitution of the Min-system, the proteins self-organize, which results in ATP-driven oscillating waves.[99] A more detailed study on the dynamic behavior of the Min-system revealed that the observed pole-to-pole oscillations are caused by the shape of the compartment, as Min-systems reconstituted in other geometrical chambers allow for spiral rotations and traveling waves as well.[100,101] Noteworthy, the fraction of anionic lipid alters the oscillation speed and width, thereby emphasizing the role of phospholipids in membrane-related events.[102] Although Min-oscillations were well studied in vitro, only recently they have been successfully reconstituted into giant unilamellar vesicles. As a result, several different spatiotemporal patterns could be observed, in which the oscillations applied such a force on the lipid bilayer that the vesicles repeatedly showed to divide and form periodic buds.[103]

Unlike polarization, in vitro proto-ring formation is far more challenging, as the in vitro reconstitution of FtsZ and FtsA into liposomes mostly results in the formation of fibers and clumps.[104] Nevertheless, by using FtsZ fused to a membrane-targeting amphipathic helix (FtsZ-mts), tubular membrane-associated structures could be observed.[105] Although no division took place, these proteoliposomes were able to constrict, indicating that FtsZ can generate contraction forces on its own. Remarkably, replacement of FtsA with the FtsA gain of function mutant enabled FtsZ to form proto-rings to some extent, and even a few dividing liposomes could be detected in vitro.[106] In an ambitious attempt to extend FtsZ-based vesicle division, the proteins FtsZ, FtsA, and ZipA were synthesized inside liposomes via cell-free translation. Production of active protein was confirmed, as some FtsZ polymerization as well as membrane localization was observed. However, this did not result in Z-ring formation and/or division.[107] To localize proto-ring formation to the middle of a compartment, the in vitro assembly has to be combined with the polarizing oscillating Min-system. Reconstitution of self-organized oscillations of Min CDE proteins, resulting in a distinct protein concentration gradient, directed the localization of FtsZ-mts to the middle of the compartment, thereby mimicking Z-ring formation at midcell (Figure 4C).[108] Although this in vitro reconstituted system is not able to divide, it provides a good starting point toward the controlled binary division of synthetic cells.

Alternatively, a division machinery derived from the archaeal domain of life could be employed to divide liposomes. While the FtsZ-based division system is predominantly found in Euryarchaea, Thaumarchaea, and Korarchaea, at least two other division machineries have been identified in other archaea. This concerns Cdv-mediated division in Sulfolobales and Desulfurococcales (belonging to the Crenarchaea), and fission based on the archaeal actin homologue crenactin, which is present in Thermoproteales.[109] The latter system is poorly understood as most of the components involved still need to be discovered.[110] In contrast, Cdv-mediated division is much better understood, as it is a paralogue of the well-studied eukaryal endosomal sorting complex required for transport III (ESCRT-III) complex, involved in, e.g., membrane abscission during cytokinesis, exosome biogenesis, and multivesicular body formation. The specific similarities and differences between these two systems will not be discussed here, as it has been extensively reviewed.[111] Essentially, the Cdv-division machinery comprises three proteins: CdvA, CdvB, and CdvC, which, during constriction, organize into a ringlike structure at the invagination site.[112,113] To explore the in vitro potential of this system, initially CdvA was reconstituted into liposomes, which was found to polymerize at the membrane.[114] Addition of CdvB deforms the membrane as CdvB is recruited by CvdA, which finally results in a network of connected membrane tubes, instead of a liposome.[115] Although this partial reconstitution of the Cdv-machinery into liposomes did not result in membrane division, it is a promising alternative for FtsZ-based division. Perhaps proteins of the eukaryal ESCRT-III complex can be used for division as the liposomal encapsulation of the core proteins comprising this complex induced the formation of internal vesicles by constraining and scission of the liposomal membrane.[116]

CONCLUSIONS AND OUTLOOK

In this review, we discussed the self-reproduction of boundary membrane layers, with respect to the bottom-up construction of a synthetic minimal cell. A first step was made by the spontaneous growth and division of fatty acid-based vesicles with minimal content loss. Although engineering of these self-reproducing fatty acid vesicles provided new insights, especially related to the origin of life, they are intrinsically unstable and barely encapsulate a "cytosol", which make them unsuitable to function as barriers in a synthetic minimal cell. Moreover, boundary layers do not only separate the interior from the exterior, but are involved in a wide variety of processes, wherein specific phospholipid species play a pivotal role. Hence, any approach toward the engineering of a synthetic minimal cell should be based on phospholipids or phospholipid-like structures. Expansion of phospholipid-based liposomes could be realized by the insertion of multiple phospholipid species, produced by a designed and engineered

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enzymatic phospholipid biosynthesis pathway. Moreover, this could be coupled to the process of protein translocation across the membrane, thereby introducing transport across the membrane. Although these are promising developments toward a functional boundary layer, a next challenge would be to establish growth via a constant feed of precursor fatty acids, thereby allowing for continued self-replication. Moreover, introduction of proteins involved in transport across the membrane of other precursors involved in phospholipid synthesis should allow for continued phospholipid biosynthesis from within and mimicking growth of a synthetic minimal cell.

Besides growth, a self-reproducing compartment should be able to divide. Initial attempts were based on co-reconstitution of a minimal divisome with the polarizing Min-system. Recent developments allowed for Z-ring formation at the midcell, thereby providing a promising start toward binary fission, although alternatives to FtsZ such as the Cdv-system should be considered as well. The current research illustrates that in vitro engineering of division is not as easy as simply reconstituting the enzymes, but is a complicated process that requires detailed knowledge and a robust design. Linking continuous growth of phospholipid liposomal membranes to division, based on the reconstitution of purified enzymes, would provide an excellent starting point for a self-reproducing compartment.

Eventually, a synthetic minimal cell should rely on a DNA/RNA-based information storage system. Currently, the limited performance and complexity to synthesize many proteins simultaneously in a functional state within a liposomal compartment presents a formidable challenge in the bottom-up approach for building a synthetic cell. Moreover, events involved in transcription and translation will influence the enzymatically compartmental self-replication and vice versa. This creates a complex interplay in which there will not only be beneficial cross-talk, but also competition and incompatibilities are to be expected. Obviously, by adding new modules in a synthetic minimal cell, the complexity of controlling these processes will increase, and adequate tuning is required. Hence, first simple systems based on purified enzymes should be created, prior to their coupling with in vitro transcription/translation. By establishing partially functional synthetic cells, complete integration may become more feasible in the long run.

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Both the authors conceived and designed the manuscript. The manuscript was written by M.E. and corrected by A.J.M.D.

Notes

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ABBREVIATIONS

ATP, adenosine triphosphate
CDP-DAG, CDP diacylglycerol
CdsA, CDP-DAG synthetase
CL, cardiolipin
ClsA, cardiolipin synthase A
CMP, cytidine monophosphate
CoA, coenzyme A
E. coli, *Escherichia coli*
FadD, long-chain fatty acid-CoA ligase
FAS, fatty acid synthetase
FtsZ, filamenting temperature-sensitive mutant Z
FtsA, filamenting temperature-sensitive mutant A
G3P, glycerol-3-phosphate
LPA, lysophosphatidic acid
PA, phosphatidic acid
PC, phosphatidylcholine
PE, phosphatidylethanolamine
PG, phosphatidylglycerol
PlsB, glycerol-3-phosphate acyltransferase
PlsC, 1-acyl-*sn*-glycerol-3-phosphate acyltransferase
PgsA, phosphatidylglycerophosphate synthase A
PgpA, phosphatidylglycerophosphatase A
Psd, phosphatidylserine decarboxylase
PssA, phosphatidylserine synthase A

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