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Review Article

# Continuous expansion of a synthetic minimal cellular membrane

**Marten Exterkate** and **Arnold J.M. Driessen**

Department of Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747AG Groningen, The Netherlands

**Correspondence:** Arnold J. M. Driessen (a.j.m.driessen@rug.nl)

A critical aspect of a synthetic minimal cell is expansion of the surrounding boundary layer. This layer should consist of phospholipids (mimics) as these molecules assemble into a bilayer, creating a functional barrier with specific phospholipid species that are essential for membrane related processes. As a first step towards synthetic cells, an *in vitro* phospholipid biosynthesis pathway has been constructed that utilizes fatty acids as precursors to produce a wide variety of phospholipid species, thereby driving membrane growth. This now needs to be developed further into a sustainable expanding system, meanwhile keeping simplicity in mind. The non-enzymatic synthesis of phospholipid-like molecules forms a realistic alternative for natural enzymatic-based pathways, that nowadays can even support functional membrane proteins. Eventually, coupling to *in vitro* transcription/translation is required, for which efficient mechanisms of insertion and folding of the involved membrane proteins need to be developed. Such an integrated system will form a suitable foundation of a synthetic minimal cell that eventually can be coupled to other cellular processes such as division.

## Introduction

Over the past decades, there has been an increasing interest in the construction of an autonomous synthetic minimal cell [1–3]. Besides enabling us to fully understand the molecular basis of life and the interplay between individual processes that form a living entity, it will also potentially form a fundament for all sorts of new applications in a wide variety of disciplines (e.g. biotechnology, medicine, research etc.) [4]. In short, via bottom-up approaches, first various modules need to be built (protein synthesis, energy production, gene expression etc.), which should be combined to form a living entity [1,5]. A critical aspect of such an autonomous synthetic minimal cell is the ability to reproduce, which includes growth (and division) of the surrounding membrane boundary layer. Several achievements have advanced towards this goal. However, a truly continuous system that sustains growth has not yet been established. In this perspective article, we will focus on the design of such a system and discuss the requirements for its success.

## *De novo* vs pre-existing

In general, there are two starting points that need to be considered regarding the *in vitro* growth of membranes. One approach is to add newly (synthesized) building blocks into an already existing membrane. Alternatively, building blocks that are synthesized from pre-cursor molecules, self-assemble into *de novo* formed membranes that subsequently can further expand. The latter approach is more sophisticated as it truly represents synthesis from the bottom-up. However, it is not the most suitable method for growing membranes in the context of a synthetic cell, because encapsulation of all the necessary intracellular components would need to occur during compartment formation. As the construction of a synthetic cell will be complex and challenging, this extra layer of complexity should initially be avoided. Nevertheless, *de novo* membrane formation does form an interesting phenomenon, and is extremely suitable to study compartment formation in the context of the origin of life [6–8].

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## Building blocks of membranes

In nature, membranes are composed of phospholipids that assemble into a bilayer-like structure. However, for a synthetic cellular membrane, alternative building blocks are considered as well, such as the often-employed fatty acid [9]. Like phospholipids, fatty acids have the ability to form vesicular like structures in which the lumen has been used as a compartment for metabolic processes [10,11]. Their physical properties allow them to self-insert into vesicles, thereby spontaneously expanding the membrane [12]. At the same time, this destabilizes the vesicles making them permeable to small compounds. Moreover, fatty acid bilayers lack the ability to support other membrane related functions, such as membrane protein activity. Herein, often specific phospholipid (head group) species play an essential role [13]. Hence, the primary choice for a boundary layer of a synthetic minimal cell should be based on phospholipids or mimics thereof.

## Expansion of phospholipid-based membranes

Phospholipids do not spontaneously insert into bilayers, which means that their membrane incorporation must coincide with their synthesis. Indeed, in nature these two processes are coupled, in which simple metabolites (e.g. acetyl-CoA, malonyl-CoA, G3P, choline, etc.) are converted via an enzymatic cascade into a variety of phospholipid species located in the membrane [14]. Such a cascade involves numerous enzymes, of which most are involved in the attachment of specific polar head groups to the core structure [15]. Most of the phospholipid synthesis pathways share a common design/structure. Phospholipid synthesis in bacteria (more specifically in *E*scherichia *coli*) [16], starts with the synthesis of fatty acyl-chains by a fatty acid synthase (FAS) (Figure 1a). This large multi-subunit cytosolic enzyme links acetyl-CoA and malonyl-CoA moieties together, finally forming an acyl-CoA/ACP adduct that can be further enzymatically processed. Fatty acid synthesis is a complex and versatile process, which include many different reactions that can yield acyl-chains with different length and degrees of saturation [17]. Next, two acyl-CoA/ACP chains are coupled to a glycerol-phosphate via the enzymes glycerol-phosphate acyl-transferase (GPAT) and lysophosphatidic acid acyl-transferase (LPAAT), which results in the formation of the simplest phospholipid species phosphatidic acid (PA) (Figure 1a) [18]. In this process phospholipid synthesis and membrane insertion occur simultaneously. During the formation of lysophosphatidic acid (LPA) and PA, the CoA/ACP entity is detached to provide the energy to couple the fatty acids to the *sn*-1 and *sn*-2 position of glycerol-3-phosphate (G3P). GPAT and LPAAT are membrane (associated) proteins with one or more transmembrane segment(s), and thus catalysis occurs at the membrane surface. This ensures immediate incorporation of the products into the template membrane and hence growth is achieved. Although a membrane consisting of only PA will be able to fulfil some of the basic functions of a surrounding bilayer, further addition of specific polar head groups is essential for cellular functions. Moreover, PA forms non-bilayer structures in the presence of divalent cations, which influences the membrane stability, hence its concentration in natural membranes is usually low.

In the cytoplasmic membrane of *E. coli*, there are only two essential phospholipid species: phosphatidylethanolamine and phosphatidylglycerol (PE and PG), which are both formed through the intermediate CDP-diacylglycerol (CDP-DAG) (Figure 1b). This makes it a suitable template, as such membranes can have a simple composition and still will function as a suitable matrix in a synthetic cell.

Several attempts have been made to grow membranes by feeding fatty acids (derivatives) to the phospholipid biosynthesis pathway of *E. coli*. Starting from purified reconstituted proteins, membranes could be grown by insertion of newly synthesized PE and PG by an enzymatic cascade of eight membrane proteins (Figure 1c) [19]. Importantly, in this approach the soluble anabolic enzyme FadD (involved in the β-oxidation of fatty acids) was used for the synthesis of acyl-CoA thioesters, thereby avoiding the complex FAS-mediated synthesis of fatty acids (Figure 1a). This phospholipid biosynthesis system exhibits a high fidelity and efficiency, permits synthesis of a range of phospholipids whose acyl chain composition depends on the specific feed of fatty acids, and catalyzes the chemical conversion of simple building blocks into phospholipids concomitantly with membrane growth.

The same phospholipid biosynthesis pathway was also combined with an *in vitro* transcription/translation (IVTT) system [20]. Although individual phospholipid species could be observed, they were not synthesized at a level that allowed for membrane expansion. A major caveat with the coupled system is that enzymatic conversion was found to be slow and incomplete, which is most likely caused by the impaired structural integrity of the phospholipid synthesizing enzymes. As many of these enzymes are membrane proteins, often containing multiple membrane-spanning domains, correct folding and insertion in the membrane is essential for their function. These processes are mediated by the Sec-translocon which was not included in this IVTT system. Liposomes

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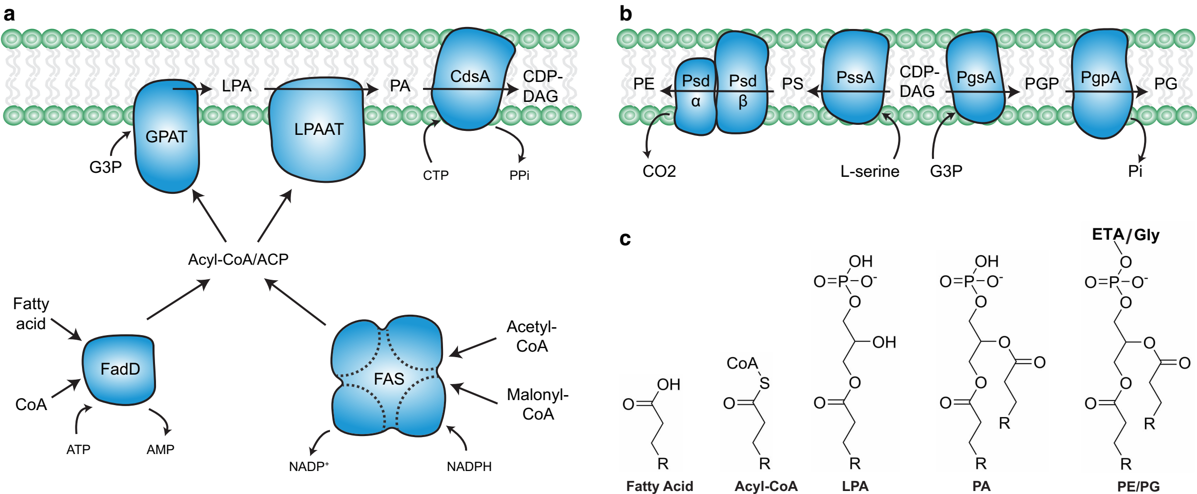


**Figure 1. The main phospholipid biosynthesis pathway in *E. coli* yielding phosphatidylethanolamine (PE) and phosphatidylglycerol (PG).**
**(a)** First, fatty acids are synthesized and coupled to an acyl-carrier moiety (CoA/ACP) via the FAS complex. Alternatively, acyl-CoA can be produced from supplied fatty acids via the enzyme FadD. Subsequently, two acyl-CoA molecules are enzymatically coupled to glycerol-3-phosphate (G3P) via GPAT and LPAAT, resulting in the formation of LPA and PA respectively. PA is then converted into CDP-DAG by CdsA, which serves as pre-cursor for both PE and PG. **(b)** PG is synthesized from CDP-DAG through the intermediate PGP, which is catalyzed by the enzymes PgsA and PgpA. Alternatively, CDP-DAG is converted into PS by PssA, after which Psd catalyzes the synthesis of PE. **(c)** Structural build-up of phospholipid from a fatty acid building block. Figure adapted from Exterkate and Driessen [35].

reconstituted with the Sec-translocon may function as a nucleation point to facilitate membrane insertion of newly synthesized phospholipid biosynthetic enzymes and thus function to kick-start membrane expansion.

# Chemical phospholipid synthesis

An interesting alternative for natural phospholipid biosynthesis is based on the chemical synthesis of synthetic molecules that are structurally very similar to phospholipids [21]. By making use of chemically reactive building blocks, phospholipid-like molecules can be synthesized in a non-enzymatic manner, which could be utilized for boundary layer self-reproduction [22,23]. In this way, the use of a complex enzymatic pathway containing membrane proteins can be avoided and complexity can be reduced, as coupling with an IVTT system is not needed. Until recently, a major disadvantage of chemical phospholipid analogues has been their poor support of membrane protein activity. However, recently a method has been developed that allows for the reconstitution of multiple functional proteins into a synthetic lipid bilayer [24,25]. This is a main step forward in the field of chemical synthesis of phospholipid analogues, and make them into promising building blocks for a synthetic cell. Noteworthy, synthesis of phospholipids in a non-enzymatic or enzymatic manner is not mutually exclusive, which is exemplified by a hybrid system in which an enzymatically synthesized fatty acid derivative acts as a substrate for chemical synthesis of a phospholipid analogue. In this hybrid approach, the membrane proteins GPAT and LPAAT are replaced by a chemical synthesis step, whereas the soluble enzyme FadD10 is maintained, which makes this setup compatible with IVTT [26]. Moreover, this setup shows how chemical synthesis can provide a short-cut for naturally occurring enzymatic pathways, thereby forming an interesting alternative approach for the construction of a synthetic minimal cell.

# Sustainable membrane expansion in a synthetic minimal cell

In the current systems, phospholipid synthesis has been employed to drive membrane growth, but they are limited in their expansion. To further develop these systems into an expanding membrane module, that can be readily implemented in a synthetic cell, sustainable growth is essential (Figure 2). A critical condition for such a continuously expanding system is the constant and limitless availability of new phospholipid substrate. As synthetic compartments (e.g. liposomes) can only capture a limited amount of molecules, constant import of new building blocks is essential. In that respect, the already developed fatty acid-based *in vitro* phospholipid biosynthesis pathway has an

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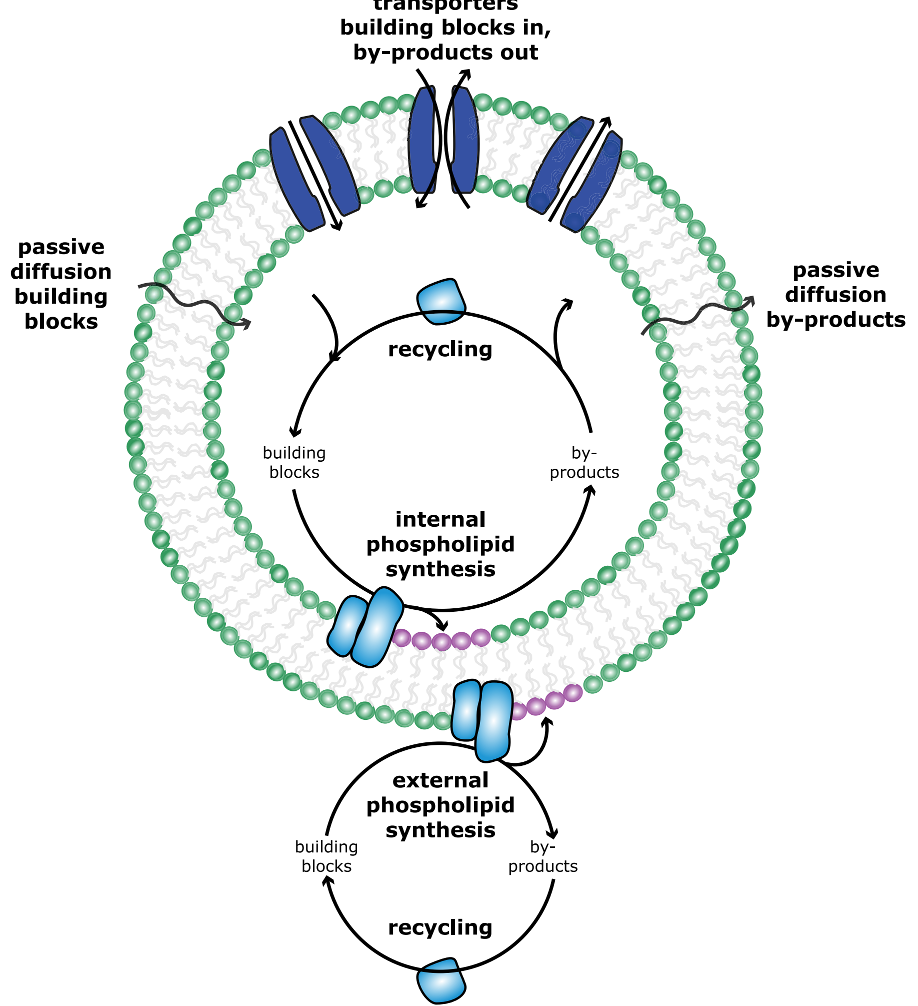


**Figure 2. Schematic conceptual representation of a sustainable expanding compartment based on phospholipid biosynthesis.**

Phospholipid building blocks enter the compartmental lumen either passively via diffusion across the membrane, or actively via membrane protein-mediated transport. In conjunction with the phospholipid biosynthetic pathway, this enables the internal synthesis of new phospholipids and their insertion into the membrane, thereby expanding the compartment. During this process, there will be a constant generation of by-products, which have to be either recycled or excreted in a passive or active manner. A more simple alternative is presented by (partial) external phospholipid synthesis that would avoid the need for specific transport.

advantageous feature [19]. Fatty acids spontaneously insert into membranes and can flip-flop towards the inner-leaflet, thereby making this building block directly available in the compartment interior [27]. Nevertheless, other required substrates (e.g. G3P, ATP, CTP etc.) cannot passively diffuse across the membrane and would require either active transport via specific transporter proteins, or internal synthesis for constant availability (Figure 2).

A consequence of continuous phospholipid synthesis is the production of by-products. Their accumulation will eventually inhibit growth and their processing will be essential to maintain a sustainable system (Figure 2). Although, this can be achieved by active excretion, it would require the presence of additional transport mechanisms, thereby adding another layer of complexity. Therefore, the preferred way of processing is recycling. By introducing short recycling pathways, it would be possible to convert by-products, such as cytidine monophosphate (CMP), adenosine monophosphate (AMP) and pyrophosphate (PPi), back into cytidine triphosphate (CTP) and adenosine triphosphate (ATP), thereby circumventing the import of these building blocks as well.

Altogether, sustainable membrane expansion cannot be achieved by simply feeding substrates to a phospholipid biosynthesis pathway, but requires a far more complex solution that includes building block transport and

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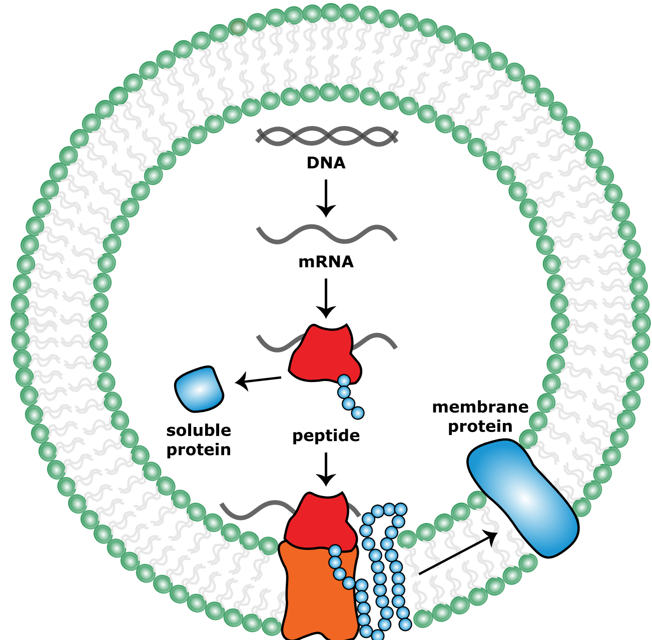


**Figure 3. A schematic representation of an *in vitro* transcription/translation (IVTT)-based sustainable membrane system.**
Proteins involved in phospholipid biosynthesis, recycling and transport are synthesized inside the interior of the compartment. In particular, the synthesis of membrane proteins is coupled to their folding and membrane insertion, resulting in a fully functional enzyme.

by-product processing to maintain the system its continuity (Figure 2). Due to the complexity of reconstitution of the many enzymes involved, this may quickly escalate into a system that is very difficult to build from the bottom-up. Therefore, an alternative would be to utilize and incorporate (some) building blocks from outside of the compartment (Figure 2), in which the input of new substrates and disposal of by-products can be regulated by a continuous flow system.

## *In vitro* transcription/translation-based membrane expansion

Eventually, all modules in a synthetic minimal cell should rely on proteins derived from an IVTT system, including phospholipid-based membrane expansion. However, as briefly mentioned before, phospholipid biosynthesis involves mostly membrane proteins often with complex structures where correct folding and insertion is crucial for activity [28]. Currently, IVTT can be sub-divided into two different categories. *In vitro* protein synthesis from the bottom-up based on purified recombinant elements (PURE [29]), and the top-down approach which utilizes cell-free extract [30]. Although current IVTT systems (such as PURE) are suitable for the synthesis of more simple soluble proteins, most of them are not yet compatible with the production of functional membrane inserted proteins. Therefore, any approach toward IVTT-based membrane expansion should first focus on correct folding and insertion of membrane proteins (Figure 3), and thus implement insertion machineries such as the Sec-translocon [31] and the membrane insertase YidC [32,33]. To convert this system in a high-fidelity membrane biogenesis module is still a formidable challenge when combined with IVTT-based protein production. Ultimately, in a synthetic cell, such an IVTT system should be genome encoded.

## Conclusion/outlook

The realization of synthetic cellular membrane expansion is a complex process, in which many different facets should be considered. Initial steps towards membrane expansion have been taken by successfully reconstituting an entire phospholipid biosynthetic pathway, resulting in limited membrane growth. However, these advances now need to be transformed into a sustainable growing system. A key aspect in this development is to maintain

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simplicity, possibly also by introducing non-enzymatic synthesis and/or an (partial) inside-out based model. Nevertheless, the development of such continuous systems will be complex in which encapsulation of sufficient protein into the current liposomal systems seems not feasible to sustain a high synthesis activity. One approach towards the assembly of this complex system is the use of fusogenic liposomes. In this approach, small unilamellar liposomes containing subsets of enzymes are fused together into one giant unilamellar vesicle, which could be utilized to combine the different sub-modules [34]. Although, this would be a feasible approach towards a sustainable growing membrane, it is not compatible with compartmental self-reproduction. Coupling with an *in vitro* transcription/translation system (IVTT) would form a more logical solution, but this cannot succeed without the inclusion of a membrane protein insertion system and chaperones that guide folding of the newly synthesized proteins. Therefore, the first initial sub-modules should be developed that can still rely on reconstituted purified proteins. For example, a nucleotide recycling module that can be used to recycle nucleoside mono-phosphate by-products, coupled to transport or the generation of ATP. Meanwhile, IVTT-based insertion of the involved membrane proteins in a functional form can be developed simultaneously.

## Summary

- A phospholipid-based membrane forms an efficient permeability barrier and provides a functional matrix for membrane proteins in synthetic cells.
- In synthetic cells, membrane growth must co-occur with phospholipid (bio)synthesis.
- Phospholipid biosynthesis in expanding synthetic cells should also contain substrate/by-product transport and recycling.
- Folding and insertion of membrane proteins are essential for *in vitro* transcription/translation-based membrane expansion in synthetic cells.

### Abbreviations

ACP, acyl-carrier protein; AMP, adenosine monophosphate; ATP, adenosine triphosphate; CDP, cytidine diphosphate; CDP-DAG, cytidine diphosphate-diacylglycerol; CMP, cytidine monophosphate; CoA, coenzyme A; CTP, cytidine triphosphate; FAS, fatty acid synthase; G3P, glycerol-3-phosphate; GPAT, glycerol-phosphate acyl-transferase; IVTT, *in vitro*transcription/translation; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyl-transferase; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PPi, pyrophosphate; PURE, protein synthesis using recombinant elements; Sec, secretory.

### Author Contribution

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

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### Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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