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Marten Exterkate

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Membrane biology

# The role of lipid biosynthesis machinery in cellular membrane adaptation

MARTEN EXTERKATE
MEMBRANE BIOGENESIS AND LIPIDOMICS GRUPPE, BIOCHEMIE-INSTITUT, UNIVERSITÄT DÜSSELDORF

**Cells need to adapt their membranes to environmental changes. In this process the specific lipid composition of the membrane is of importance. The lipid biosynthesis machinery has direct influence on the membrane lipid content by 1) producing newly synthesized lipids, 2) remodeling already existing lipids, such as various cardiolipins. The interactions between components of the lipid biosynthesis machinery are crucial to obtain a specific lipid composition, but the interplay is largely unknown.**

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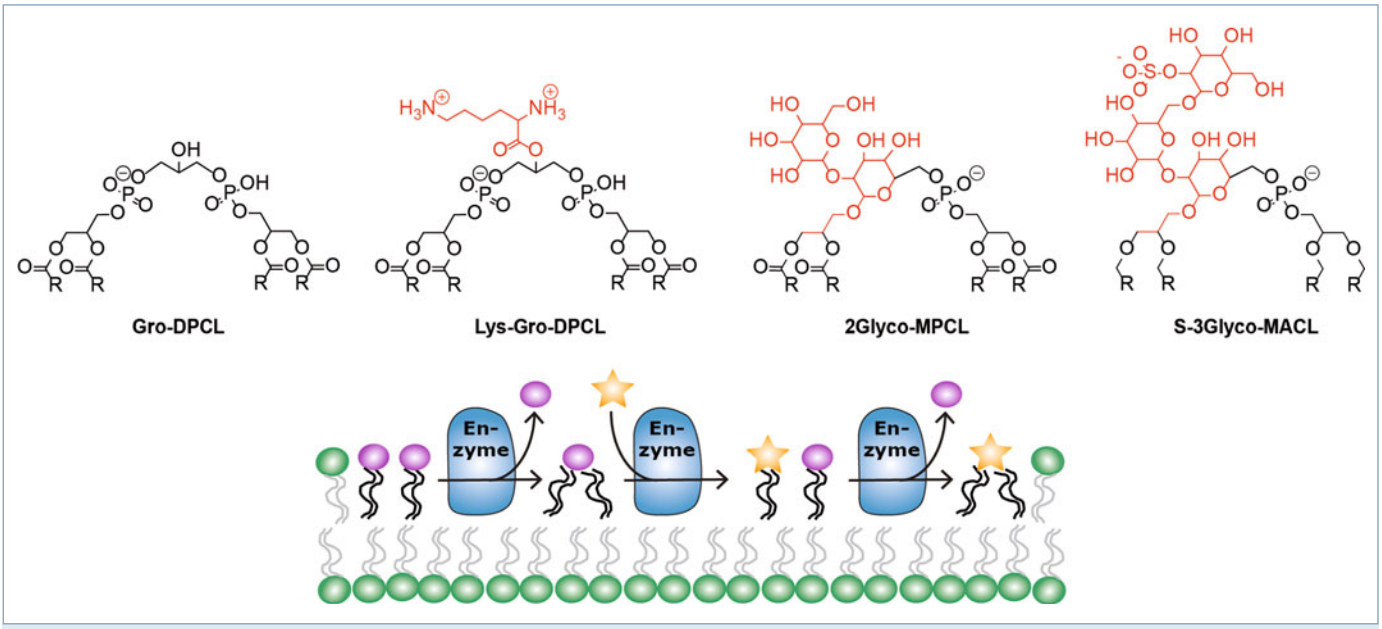
■ Cellular membranes are dynamic entities that do more than just separate the cell's interior from its external environment. They are integral to numerous cellular processes, such as solute transport, signaling, and stress adaptation, with membrane proteins playing essential roles in these functions. The activity of these proteins often relies on their interactions with specific lipids. Thus, lipids are not merely structural components forming the membrane bilayer; they also actively participate in various cellular processes, either directly or indirectly through their support of the embedded proteins.

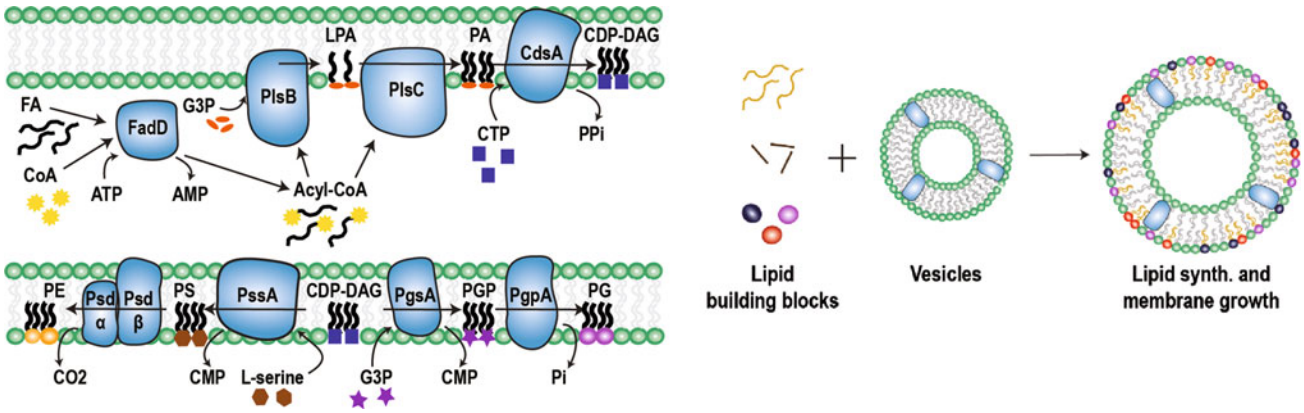
In living cells, new lipids are continuously synthesized and integrated into the membrane according to the cell's needs. Additionally, existing lipids can be modified in response to environmental changes, highlighting the dynamic nature of biological membranes. Here, I will discuss the synthesis and remodeling of cardiolipin, a lipid class consisting of four lipid tails connected to a single headgroup, as an example of dynamic membrane adaptation. Furthermore, I will describe our efforts to understand the interactions between the components of the bacterial phospholipid biosynthesis machinery in the context of the bottom-up construction of a synthetic cell; i.e., a continuously self-replicating unit, surrounded by a membrane, that contains a simple genetic system and metabolism.

## The cardiolipin family: a versatile lipid class

Membranes can consist of various lipid species, including cardiolipins. Cardiolipins are universally found in energy-transducing




▲ **Fig. 1:** The cardiolipin lipid class. **Top:** schematic representation of several cardiolipins with varying headgroups (in red). The "R" represents a lipid tail that forms the hydrophobic part of the membrane bilayer. For simplicity, stereochemistry is not included. **Bottom:** schematic overview of cardiolipin synthase enzymatic promiscuity.




▲ **Fig. 2:** Schematic representation of an engineered phospholipid biosynthesis pathway [3]. Simple building blocks are converted into phospholipids via a membrane reconstituted enzymatic cascade, resulting in membrane growth. Two fatty acids (FA) serve as lipid tails that are stepwise linked to a glycerol-3-phosphate (G3P) backbone, resulting in phosphatidic acid (PA), thereby forming the core of any phospholipid. Next, through the intermediate CDP-DAG, head group diversity is implemented, which results in a variety of phospholipids species. Coenzyme A (CoA); adenosine mono/tri-phosphate (AMP/ATP); cytidine mono/tri-phosphate (CMP/CTP); pyrophosphate (PPi); Phosphate (Pi), lysophosphatidic acid (LPA); cytidine di-phosphatidyl diacylglycerol (CDP-DAG); phosphatidylglycerol-phosphate (PGP); phosphatidylglycerol (PG); phosphatidylserine (PS); phosphatidylethanolamine (PE).

membranes, such as archaeal and bacterial cytoplasmic membranes, mitochondrial inner membranes, and hydrogenosome membranes, where they play a crucial role in stabilizing and supporting the function of membrane proteins. The most common cardiolipin species is 1,3-bis(*sn*-3'-phosphatidyl)-*sn*-glycerol, also known as glycerol-di-phosphatidyl-cardiolipin (Gro-DPCL) (**Fig. 1**). This molecule comprises two phosphatidyl moieties bridged by a glycerol headgroup. The polar headgroup can carry up to two negative charges and is relatively small compared to the four bulky acyl chains, giving the molecule a distinctive cone-like shape associated with membrane curvature in locations like the cell pole, mitochondria, and division sites.

Other cardiolipin species have also been identified, differing in their polar head groups (**Fig. 1**, [1]). Some, such as lysyl-glycerol-di-phosphatidyl-cardiolipin (lys-Gro-DPCL), show structural similarities to Gro-DPCL with additional modifications. Others, like the bacterial di-glycosyl-mono-phosphatidyl-cardiolipin (2Glyco-MPCL) and the archaeal S-tri-glycosyl-mono-archaetidyl-cardiolipin (S-3Glyco-MACL), have a single phosphate moiety combined with a much bulkier sugar polar headgroup. These belong to the subclass of glycosyl-mono-phosphatidyl cardiolipins (glyco-cardiolipins), which have entirely different intrinsic properties, such as molecular shape, polarity, and charge.

Various molecules (exemplified by the yellow star) can be incorporated as lipid/cardiolipin headgroup.

While the classical cardiolipin Gro-DPCL has been extensively studied, little is known about other cardiolipin species. However, some of these species are associated with specific cellular properties in bacteria and archaea, such as virulence and osmotic resistance, indicating their role in cellular responses by altering membrane identity. In certain bacteria, cardiolipin levels significantly increase under specific environmental conditions like nutrient depletion, osmotic shock, or antibiotic exposure. These observations suggest that cardiolipins play a role in the membrane's direct response to environmental conditions, with the unique chemistry of the headgroup being pivotal in these processes. This makes the cardiolipin family an intriguing subject for further research. Besides further unraveling the cellular function of cardiolipins, we are also focusing on how the different cardiolipins are synthesized and under which conditions.

## Biosynthesis of cardiolipins

The enzyme responsible for classical cardiolipin synthesis (Gro-DPCL) was identified decades ago in eukaryotes and bacteria. Recent research has linked many of these ‚classic' cardiolipin synthases to the production of other cardiolipin species as well. This suggests that ‚classic' cardiolipin synthases might be responsible for synthesizing a diverse range of cardiolipins. For this reason, we focused on characterizing known and putative cardiolipin synthases, and specifically analyzed the ability to synthesize a wide range of cardiolipin species. Our efforts led to the first report of cardiolipin synthesis in archaea, including a comprehensive *in vitro* characterization of the cardiolipin synthase from *Methanospirillum hungatei* [2]. Surprisingly, the enzyme exhibited remarkable promiscuity, capable of producing various phospholipids, including multiple cardiolipin species with different polar head groups (**Fig. 1**). Further characterization of this cardiolipin synthase revealed a preference for producing cardiolipins with two phosphate moieties. Trace amounts of glyco-cardiolipins with a single phosphate group and a single glycosyl headgroup were however also synthesized. Similarly, the cardiolipin synthase A from *Escherichia coli* demonstrated broad substrate promiscuity, though with different substrate specificity compared to the archaeal enzyme. My research group is currently focusing on the structural aspects that enable the promiscuity of cardiolipin synthases. Moreover, we are unravelling the cellular function of the wide variety of cardiolipins, in which we are particularly interested in the archaeal cardiolipins.

## The phospholipid biosynthesis machinery

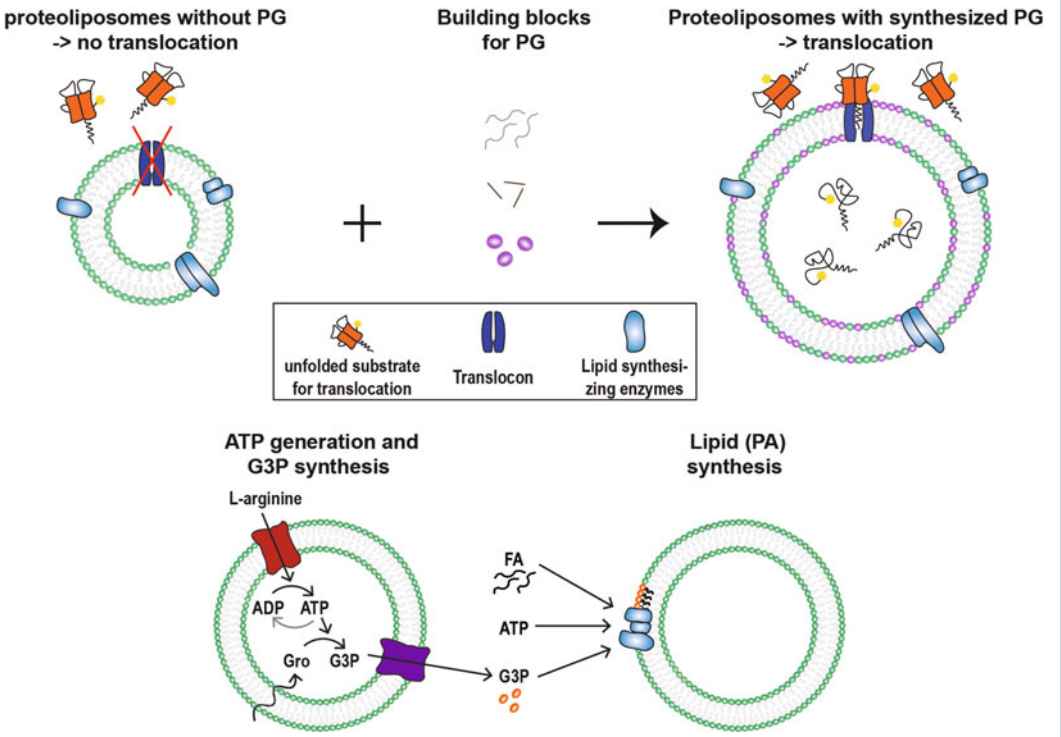
Cardiolipins are just one of many lipid types synthesized by cells. During cellular growth, lipids are continuously produced and incorporated into the membrane, a process finely

tuned to meet the cell's current needs. Decades ago, extensive research uncovered the general lipid-synthesizing enzymes and pathways in bacteria and eukaryotes. More recent studies have identified lipid-synthesizing enzymes in archaea, with the discovery of the synthase responsible for the production of the membrane spanning tetraether lipid being particularly noteworthy. While research has primarily focused on identifying and characterizing individual enzymes, the interactions between different lipid-synthesizing enzymes remain largely unexplored. Understanding these interactions is crucial, as the interplay between the numerous components of the lipid synthesis and remodeling machineries ultimately determine the membrane's composition and characteristics.

As a first step toward unraveling the complete phospholipid biosynthesis machinery in bacteria, we constructed an *in vitro* enzymatic pathway for the bottom-up bulk synthesis of phospholipids [3]. Using a stepwise enzymatic cascade, simple building blocks were converted into two essential phospholipid species of the *E. coli* inner membrane: phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (**Fig. 2**). Initially, acyl-CoA moieties are synthesized from supplied fatty acid (FA) building blocks, forming the lipid tails. Two acyl-CoA molecules are then coupled to the lipid backbone glycerol-3-phosphate (G3P) via the enzymes PlsB and PlsC, producing lysophosphatidic acid (LPA) and subsequently phosphatidic acid (PA). Next, PA can be converted into CDP-diacylglycerol (CDP-DAG) by the enzyme CdsA, serving as a precursor for both PE and PG. Through a two-step enzymatic reaction, PE is then synthesized through the intermediate phosphatidylserine (PS), catalyzed by the enzymes PssA and Psd. Similarly, the enzymes PgsA and PgpA produce PG via phosphatidylglycerol-phosphate (PGP). This work not only produced a variety of lipids in bulk, but also facilitated vesicle growth by membrane expansion (**Fig. 2**). Understanding how cells control and regulate membrane expansion is fundamental to comprehending the mechanisms behind cellular proliferation and development. Our work therefore represents a crucial first step towards mimicking the growth of a synthetic compartment, with the ultimate goal to construct a living synthetic cell from non-living components.

## Functional lipid membranes in synthetic cells

One of the major challenges in synthetic biology is constructing a synthetic (minimal) cell using a bottom-up approach, i.e., creating a continuously self-replicating unit, surrounded by a membrane, that contains a simple genetic system and metabolism. Synthetic cells provide a unique platform to research the fundamental biological processes that underpin life. Current efforts in constructing synthetic cells have revealed that engineering a minimal synthetic cell is even more challenging than initially anticipated. Issues such as encapsulation limitations, efficient coupling of information-encoding systems with basic metabolism, and controlled self-replication have proven difficult to overcome.




◀ **Fig. 3:** Schematic representations of bottom-up engineered complex membrane systems. **Top:** protein translocation across an expanding liposomal membrane is activated by synthesis and insertion of the anionic lipid phosphatidylglycerol (PG). **Bottom:** a continuous ATP-production system drives glycerol 3-phopshate (G3P) synthesis, which activates synthesis of the precursor lipid phosphatidic acid (PA), in a vesicle feeder-utilizer setup.

Additionally, it remains largely unknown how different sub-cellular machineries (such as energy generation, DNA replication, and cellular growth and division) interact to form a living entity. The bottom-up approach offers an excellent opportunity to study these interactions in a simple, yet controlled environment, and the engineering process itself will lead to a deeper understanding of the fundamentals of cellular life.

As mentioned above, membranes not only act as permeability barriers but also support the functioning of membrane proteins through specific interactions with phospholipids. In the context of engineering a synthetic cellular membrane from the bottom-up, we expanded our earlier *in vitro* growing membrane model system by integrating the *E. coli* translocon. The translocon is a conserved membrane protein complex essential for protein translocation across, or insertion into the membrane, and is universally present across all three domains of life. Its activity relies on anionic lipids. Utilizing the previously described phospholipid biosynthesis cascade, we synthesized and inserted the anionic phosphatidylglycerol (PG) into a neutral membrane, thereby activating the translocon (**Fig. 3**, [4]). To further increase complexity, we coupled lipid synthesis to an *in vitro* ATP-synthesis machinery engineered by the Poolman lab [5]. In this setup, imported L-arginine is degraded via deamination to generate ATP. The ATP produced from the arginine breakdown pathway is then used to synthesize glycerol-3-phosphate, a central building block in phospholipid biosynthesis, effectively coupling ATP production and lipid biosynthesis (**Fig. 3**). Altogether, this work represents only a first step towards the integration of cellular sub-modules that are involved in membrane functionality. The next aim will be to keep expanding and add more functionality, with the ultimate goal to engineer a fully functional synthetic membrane. Although this will be a challenging task, it will provide us with novel insights into the complex interplay between cellular membrane processes, which will be vital to fully understand the basics of all cellular life. ■

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**Corresponding author:**
Dr. Marten Exterkate
Universität Düsseldorf
Universitätsstraße 1 Gebäude: 26.44
Etage/Raum: 01.070
D-40225 Düsseldorf
marten.exterkate@hhu.de

AUTHOR

**Marten Exterkate**
**2011–2014** Masterstudium Biomolecular Sciences an der University of Groningen, Niederlande. **2019** Promotion bei Prof. Dr. A. J. M. Driessen an der University of Groningen, Niederlande. **2019–2021** PostDoc im "Building a Synthetic Cell" (BaSyC)-Programm, Niederlande. Seit **2022** Leiter der *Membrane Biogenesis and Lipidomics*-Gruppe, Teil des Biochemie-Instituts an der Universität Düsseldorf.