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In situ synthesis of artificial lipids

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Abstract

Lipids constitute one of the most enigmatic family of biological molecules. Although the importance of lipids as basic units of compartmental structure and energy storage is well-acknowledged, deciphering the biosynthesis and precise roles of specific lipid species has been challenging. To better understand the structure and function of these biomolecules, there is a burgeoning interest in developing strategies to produce noncanonical lipids in a controlled manner. This review covers recent advances in the area of *in situ* generation of synthetic lipids. Specifically, we report several approaches that constitute a powerful toolbox for achieving noncanonical lipid synthesis. We describe how these methodologies enable the direct construction of synthetic lipids, helping to address fundamental questions related to the cell biology of lipid biosynthesis, trafficking, and signaling. We envision that highlighting the current advances in artificial lipid synthesis will pave the way for broader interest into this emerging class of biomimetic molecules.

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Keywords

Artificial lipid, Phospholipid, Glycolipid, Chemoenzymatic, Enzyme-free.

Introduction

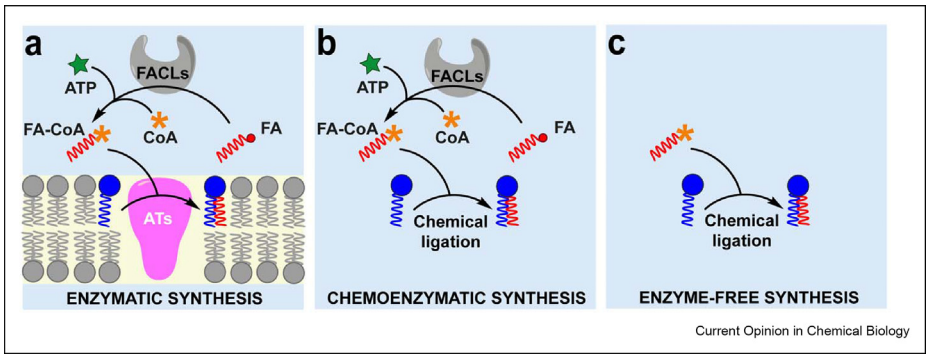
Lipid molecules are essential for maintaining the proper structure and functions of cells [1]. They play key roles in numerous biological processes, which includes membrane formation, cellular signaling, and energy storage [2,3]. However, the study of lipids is challenging and limited in scope not only because of their great structural diversity but also due to the difficulty of reconstituting their biosynthetic pathways [1]. To overcome these limitations and better understand the production, trafficking, functions, and interactions of lipid species, noncanonical lipids are increasingly being applied as a research tool. In particular, there has been a great interest in the formation of biomimetic phospholipids that can self-assemble into functional synthetic cells serving as models for origins of life, bioreactors, and smart drug delivery carriers [4,5]. Although most of the recent studies are focused on the development of *in situ* membrane-forming artificial phospholipids [1], it is worth mentioning that such lipid syntheses can be also important for producing lipid species with pharmacological properties [6], fluorescently-labeled lipid derivatives (i.e. tagged sphingolipids, gangliosides) [7], and inverted-headgroup lipids [8]. Moreover, artificial lipids generated *in situ* could shed light on key biological processes such as cell growth and division. This type of synthesis leads to a more biomimetic, straightforward and practical approach than conventional synthesis, thus facilitating the study of fundamental lipid biology. Additionally, *in cellulo* synthesis of artificial lipids may enable a better understanding of how natural lipids affect fundamental cell properties such as membrane curvature, signaling, and communication [9].

This short review describes the most prominent methodologies that have been recently employed for the construction of noncanonical lipids. Specifically, we focus our study on the *in situ* formation of artificial lipids following three distinct strategies, namely, enzymatic, chemoenzymatic, and enzyme-free synthesis (Figure 1). Additionally, given the growing importance of bottom-up minimal cellular systems, we specifically highlight the *in situ* formation of artificial phospholipids in the context of self-assembly and growth of biomimetic membranes (Figure 1).

Enzymatic synthesis

The Baskin group achieved the synthesis of artificial lipids using phospholipase D (PLD), an enzyme that catalyzes the hydrolysis and transphosphatidylation of phosphatidylcholine to form phospatidic acid (PA) and

Figure 1



Strategies for the *in situ* formation of artificial phospholipids. (**a**) Enzymatic lipid synthesis following a natural pathway. (**b**) Chemoenzymatic lipid synthesis combining an enzymatic reaction with a chemical ligation step. (**c**) Enzyme-free synthesis to produce lipids in the absence of proteins via a chemoselective reaction [AT: acyltransferase, FACL: fatty acyl-CoA ligase, FA: fatty acid, FA-CoA: fatty acyl-coenzyme A].

phospatidyl alcohol, respectively. They treated HeLa cells with five different alkynols (propynol, 3-butyn-1-ol, 4-pentyn-1-ol, 5-hexyn-1-ol, and 6-heptyn-1-ol), obtaining the corresponding artificial phosphatidyl alcohols. After Cu-catalyzed azide—alkyne cycloaddition (CuAAC) labeling, this approach allowed to identify several intracellular sites of PLD-mediated PA synthesis, which is a potent secondary messenger involved in several diseases [10].

Canonical phospholipids are enzymatically generated by complex membrane-dependent proteins as part of the Kennedy lipid synthesis pathway [11,12]. However, these enzymatic routes are usually difficult to fully reconstitute. In order to construct a synthetic cell capable of growing autonomously, the Danelon group developed a technique that reconstituted such natural phospholipid biosynthetic route using a minigenome of seven enzyme-encoding genes, thus allowing the cell to produce its own membrane components [13]. Specifically, phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) were synthetized in liposomes already containing both lipids. Although they mainly synthesized natural phospholipids, this enzyme-catalyzed technology would be valuable for the fabrication of artificial lipids with biological interest, including phospholipids with asymmetric acyl chain compositions and acyl-labelled phospholipid analogues.

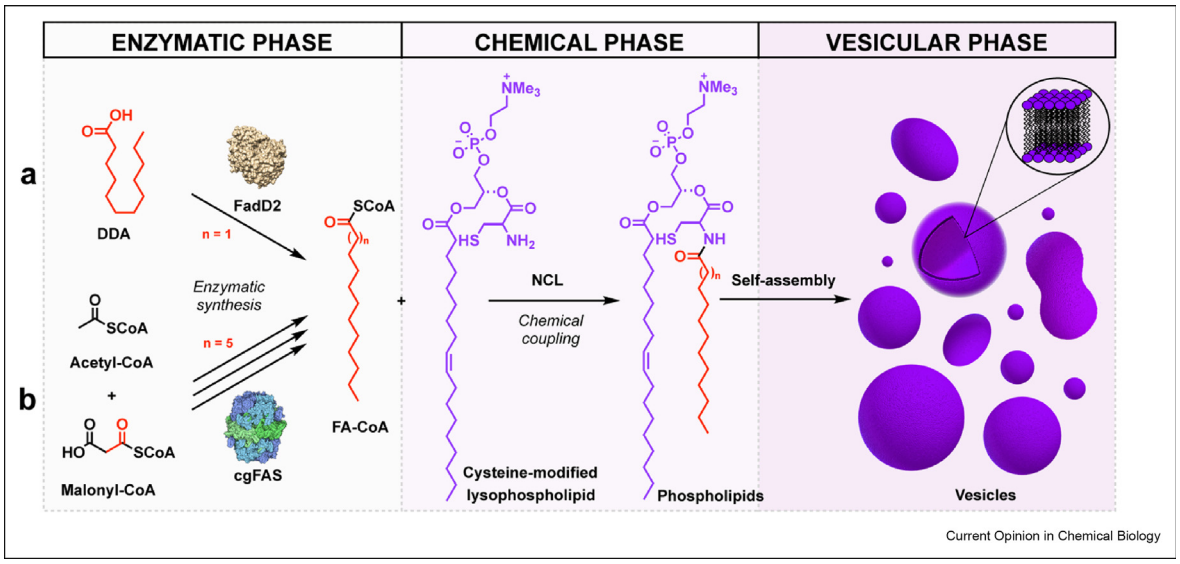
Since integral membrane protein enzymes are difficult to reconstitute [14], it would be interesting to avoid the integration of those complex systems, thus facilitating the straightforward production of artificial lipids. Moreover, it is likely that primitive building blocks capable of generating membranes were simpler than the present ones [15]. Therefore, origin of life studies would benefit from methodologies that do not involve enzymes.

Chemoenzymatic synthesis

Drawing inspiration from natural pathways, several research groups have developed simpler strategies for the spontaneous construction of artificial phospholipid membranes by combining an enzyme with a chemical reaction [14,16]. For instance, the Devaraj lab has reported a chemoenzymatic methodology to generate synthetic membrane-forming phospholipids *de novo*, thus shedding light on how the synthesis machinery of these molecules arose in the cell [17]. The approach took advantage of FadD10, a soluble mycobacterial ligase that catalyzes the formation of fatty acid adenylates (FAAs) from fatty acids, Mg^{2+}, and ATP. FadD10 displays an open conformation of its active site, enabling the FAA product to be easily obtained and diffused away. Once the FAA was formed, it reacted chemoselectively with a synthetic amine-functionalized lysophospholipid to generate a new class of noncanonical amidophospholipids, which spontaneously self-assembles into membrane-bound vesicles. Interestingly, this method made it possible to synthesize membranes without the need for a preexisting one. Additionally, due to the importance of gene expression in obtaining functional synthetic cells, the authors were able to successfully couple enzyme expression to lipid membrane formation by using a minimal transcription-translation (TX-TL) system. We foresee future applications of this chemoenzymatic methodology on the synthesis of specific artificial lipids in a cellular milieu by expressing FadD10 in the presence of appropriate reactive precursors.

Taking into consideration the previous approach, Bhattacharya et al. designed an efficient one-pot route to synthesize membrane-forming noncanonical phospholipids by repurposing the activity of a soluble ligase and using fatty acids as precursors (Figure 2**a**) [18]. In an initial enzymatic step, fatty acids were converted into

Figure 2



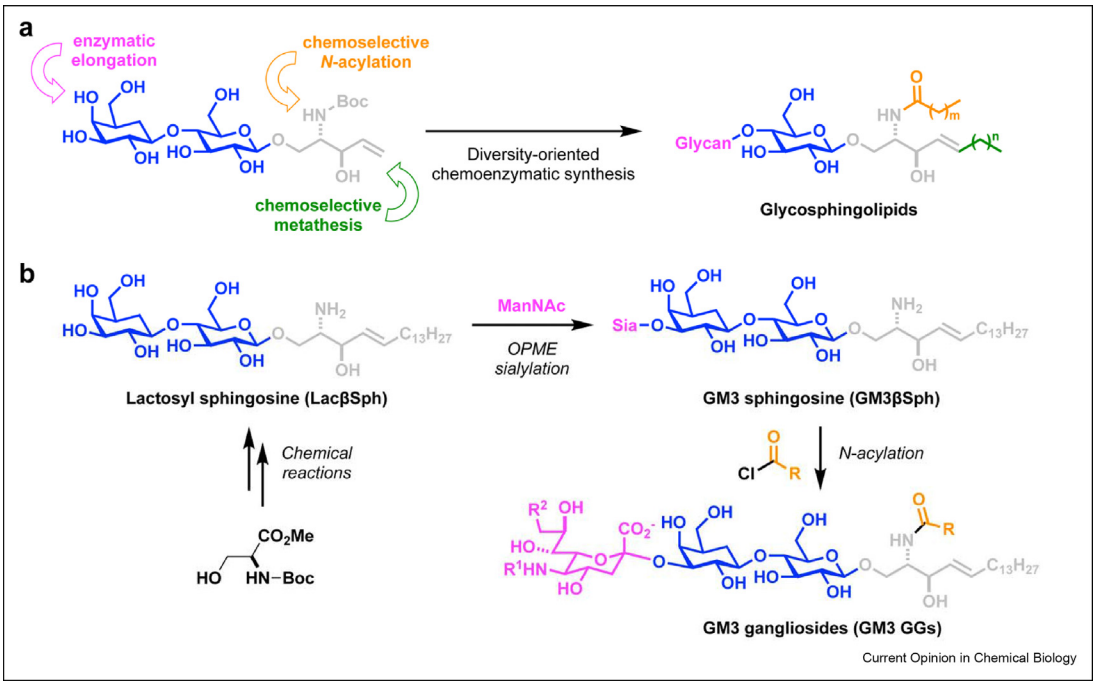
Chemoenzymatic synthesis of membrane-forming noncanonical phospholipids. (**a**) Synthetic route to artificial phospholipids in which a cysteine-modified lysophospholipid undergoes chemical coupling with fatty acyl-CoA thioesters generated enzymatically by a fatty acyl-CoA ligase (FadD2) using dodecanoic acid (DDA) as precursor. The corresponding amidophospholipids spontaneously self-assemble into micron-sized vesicles. Adapted with permission from A. Bhattacharya, C. J. Cho, R. J. Brea and N. K. Devaraj. *J Am Chem Soc* 2021, **143**, 11235–11242 [18]. (**b**) Schematic representation of a fatty acid synthase (FAS)-mediated phospholipid synthesis. A bacterial type I FAS generates palmitoyl-CoA *in situ* using acetyl-CoA, malonyl-CoA, and NADPH. Such palmitoyl derivative subsequently reacts with a cysteine-modified lysophospholipid through native chemical ligation (NCL) to form a new class of membrane-forming synthetic amidophospholipids. Adapted with permission from S. Khanal, R. J. Brea, M. Burkart and N. K. Devaraj, *J Am Chem Soc* 2021, **143**, 8533–8537 [19].

the corresponding coenzyme A thioesters (FA-CoA) by FadD2, a mycobacterial fatty acyl-CoA ligase (FACL). First, the fatty acid is activated to the corresponding adenylate with ATP and Mg^{2+}. Next, it is converted to the corresponding FA-CoA and released from the active site of the enzyme. The enzymatically-generated FA-CoA subsequently undergoes NCL with cysteine-modified lysophospholipids to rapidly produce membrane-forming artificial phospholipids. By combining enzymatic and chemical steps, the authors precluded the need for a membrane-bound acyltransferase enzyme, which is necessary for phospholipid synthesis in cells. Interestingly, the synthesis of noncanonical sphingolipids was also possible by using an analogous cysteine-modified lysosphingomyelin as precursor. This chemoenzymatic lipid synthesis is characterized by a high efficiency, allowing to optimize artificial lipid formation in a cell-free TX-TL system. This fact, together with the possibility to coexpress membrane-interacting proteins with the FACL and localize them onto the *in situ* synthesized lipid membrane, constitutes a critical step to broaden the knowledge about the functioning of cell-free systems.

Recently, Khanal et al. established a novel chemoenzymatic method for the generation of noncanonical phospholipids from minimal reactive precursors, using native chemical ligation (NCL) as a key chemoselective reaction (Figure 2**b**) [19]. In this biomimetic route, a bacterial fatty acid synthase (cgFAS I) was utilized to catalyze the *in situ* formation of palmitoyl-CoA from acetyl-CoA and malonyl-CoA, precursors that had never been used to directly reconstitute phospholipid membranes. Palmitoyl-CoA subsequently reacted with a synthetic cysteine-functionalized lysophospholipid by NCL, generating an amidophospholipid that spontaneously self-assembles into micron-sized membrane-bound vesicles. Interestingly, addition of guanidine hydrochloride (GuHCl), cholesterol or 1-decanol led to the formation of more stable vesicles. This chemoenzymatic methodology would enable the *in situ* synthesis of diverse phospholipid species, which could facilitate fundamental studies of how lipid membrane composition affects membrane assembly, growth and division.

Inspired by the elegant use of the chemoenzymatic strategy for the fabrication of synthetic phospholipids, numerous researchers have applied similar approaches to produce a diverse class of artificial lipids (Figure 3) [20–23]. Given their fundamental, biological, and clinical importance, main efforts have been focused on the generation of glycosphingolipids (GSLs) [20,24]. GSLs are glycolipids consisting of a glycan and a ceramide moiety with relevant functions as key signaling molecules [25], thus playing essential roles in many

Figure 3



Chemoenzymatic synthesis of artificial glycosphingolipids. (**a**) Diversity-oriented strategy combining enzymatic glycan assembly and lipid remodeling through chemoselective cross-methatesis and *N*-acylation, which enables the synthesis of relevant glycosphingolipids (GSLs). Adapted with permission from Q. Li, M. Jaiswal, R. S. Rohokale and Z. Guo. *Org Lett* 2020, **22**, 8245–8249 [20]. (**b**) Total synthetic methodology for the preparation of a diverse library of structurally defined GM3 gangliosides containing different sialic residues. Adapted with permission from H. Yu, M. R. Gadi, Y. Bai, L. Zhang, L. Li, J. Yin, P. G. Wang and X. Chen. *J Org Chem* 2021, **86**, 8672–8682 [28].

biological processes [26] and diseases [27]. Remarkably, Li et al. have developed a robust methodology for the rapid production of glycosphingolipids (GSLs) containing different lipid tails, glycans and tags (Figure 3**a**) [20]. The approach takes advantage of a diversity-oriented strategy combining enzymatic glycan assembly and chemical lipid remodeling. Starting from a simple glycoside, the corresponding ceramide was created by on-site lipid remodeling via chemoselective cross-metathesis and *N*-acylation based on the sphingosine headgroup. Interestingly, chemical lipid remodeling may be carried out after each enzymatic glycosylation using both natural and functionalized lipids. Moreover, either natural or modified sugars can be utilized for glycosylation. Since the reaction conditions for lipid remodeling are mild and compatible with various functionalities, this methodology was appropriate for the incorporation of fluorescent labels such as nitrobenzoxadiazole (NBD) in the lipid chains. Most notably, through two-way diversification of the glycan head and the lipid tails in each step, a large array of synthetic GSLs with diverse biological applications can be rapidly accessed.

Similar methodologies have been recently employed to efficiently synthesize artificial gangliosides (GGs) (Figure 3**b**) [28,29]. GGs are sialic acid-containing glycosphingolipids that play key roles in cellular signaling, recognition, communication, and viral binding [30]. Interestingly, Chen group have reported the synthesis of various monosialodihexosylgangliosides (GM3 GGs) using a chemoenzymatic methodology constituted by three main steps (Figure 3**b**) [28]. In the first step, lactosyl sphingosine (LacβSph) was generated from a commercially available L-serine derivative by combination of several traditional chemical reactions. The second step was the enzymatic step, in which GM3 sphingosines (GM3βSph) were synthesized using a one-pot multienzyme (OPME) sialylation system containing *Pasteurella multocida* sialyltransferase 3 (PmST3). The OPME system thus facilitates the introduction of diverse sialic forms to LacβSph to *in situ* generate the corresponding GM3βSph derivatives. In the final step, chemical fatty acid acylation of GM3βSph and subsequent C-18 cartridge purification process allowed them to obtain a library of GM3 GGs with fatty acyl chains of varying lengths and different modifications. Construction of a wide variety of structurally diverse GM3 gangliosides containing various sialic forms and fatty acyl chains represents a powerful synthetic strategy and open new avenues to explore their functions in a simple manner.

Chemoenzymatic strategies have been also used to *in situ* generate noncanonical terpenes [31,32]. Terpenoid natural products are highly diverse secondary metabolites with relevant applications in fields as diverse as fragrances, flavorings, pharmaceuticals, biofuels, and agrochemicals [33,34]. All known terpenes are produced from two universal five-carbon precursors: dimethylallyl diphosphate (DMADP) and isopentenyl diphosphate (IDP) [35]. Despite the importance of non-natural terpenoids, their chemical syntheses are often long and complex. Therefore, the development of simpler methodologies for the construction of products beyond the natural terpenome would offer rapid access to noncanonical terpenoids with improved chemical properties and altered biological activities. The Allemann lab has recently designed an efficient modular chemoenzymatic approach to generate non-natural terpenoids from modified DMADP and IDP intermediates [31]. Combining phosphorylation and ATP recycling steps with prenyl transferases resulted in excellent yields of natural farnesyl diphosphates, key intermediates in the synthesis of sesquiterpenoid products. It is worth mentioning that adding prenyl transferases and terpene synthases to the reaction mixture enabled the direct construction of both natural and non-natural terpenes. Interestingly, the addition in different combinations of prenol analogues allowed the unprecedented production of modified linear terpene precursors.

Enzyme-free synthesis

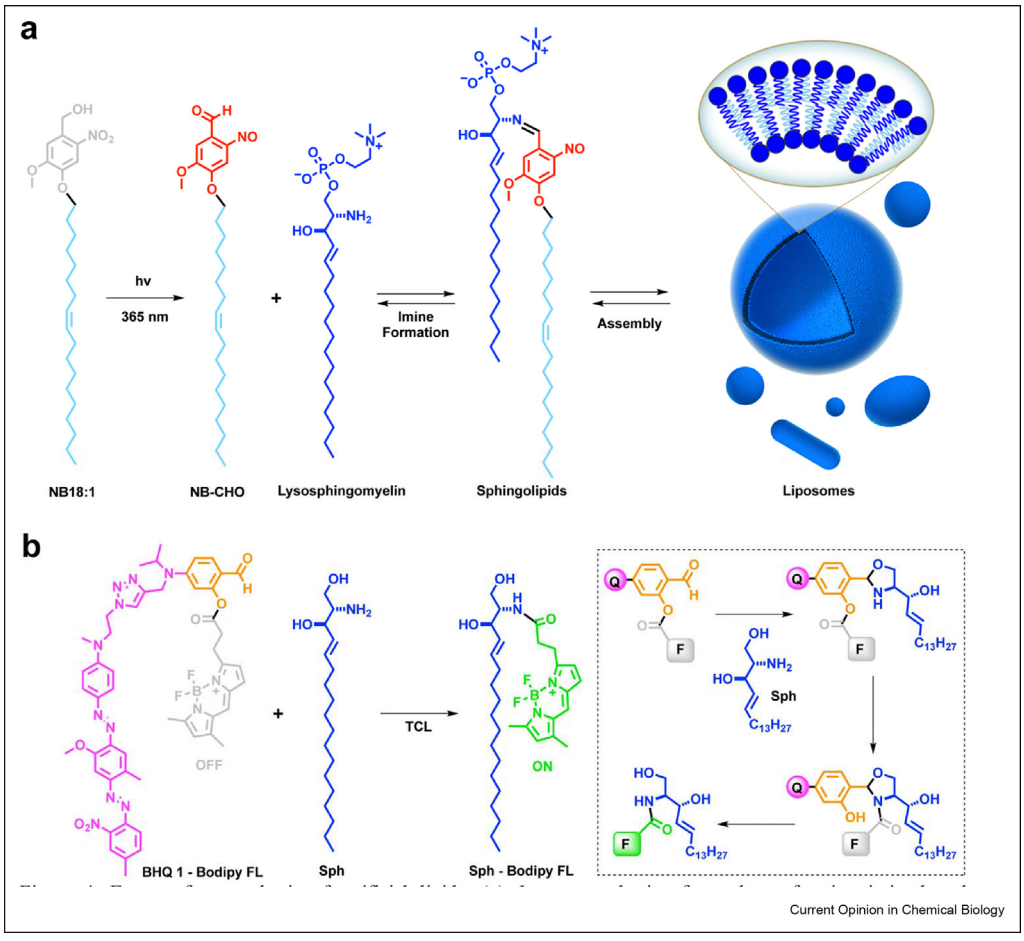
An ambitious goal of biomimetic chemistry is to develop strategies to produce lipids in the absence of enzymes [14,16]. In particular, numerous efforts have been made on the construction of cell-like systems by *in situ* generating membrane-forming lipids from simple non-membrane forming precursors [1,16,36–42]. For instance, the Bowman group developed an enzyme-free method to generate phospholipids *in situ* by covalent addition via thiol-Michael reaction between a thiol-functionalized lysophospholipid and an acrylate-functionalized lipid tail [43]. Interestingly, Liu et al. have recently designed a non-enzymatic high-yielding approach to obtain natural diacylphospholipids in water via direct transacylation [44]. This methodology could be useful for the precise construction of a wide collection of noncanonical phospholipids through simple esterification using reactive water-soluble precursors.

More recently, Zhou et al. were able to obtain biomimetic membrane-forming phospholipids *in situ* using light as a stimulus (Figure 4**a**) [45]. Lipid synthesis was successfully achieved by using a conceptually new technology based on a photoligation chemistry between two different non-membrane-forming precursors: lysosphingomyelin and 2-nitrobenzyl derivatives (NBs). Light triggers the conversion of alkyl-substituted NBs into reactive aldehyde intermediates (NB–CHO). These photogenerated species selectively react with the amine of lysosphingomyelin to produce the corresponding imine-based sphingomyelins, which spontaneously self-assemble into vesicular structures. It is worth noticing that the amphiphilic character of lysosphingomyelin promotes the formation of mixed aggregates with NBs in aqueous solution, increasing the proximity of the precursors and leading to the accelerated formation of the desired phospholipid. The light-controllable lipid synthesis transformation represents the use of an external energy stimulus to trigger *in situ* membrane formation, which will enable novel applications in synthetic biology.

To test the ability of these liposomes to act as biomimetic membranes they used an enzymatic cascade reaction. They found that the enzymes were indeed encapsulated, and the reaction occurred, indicating that these liposomes served as bioreactors.

Non-enzymatic approaches are extremely powerful for the *in situ* synthesis of ceramides [7]. Ceramides play role in cell signaling as second messengers, and their modulation has been recently found to be involved in diseases such as diabetes, Alzheimer's, cardiovascular disorders, and cancer [47]. It is worth mentioning that there are up to 26 different enzymes that regulate ceramide levels [48]. Additionally, such class of lipids have low membrane permeability [49]. All these factors make ceramides challenging to study in cells. To avoid the use of short-chain artificial ceramides, which do not have the same biological roles as natural ceramides, Rudd et al. developed an enzyme-free methodology described as traceless ceramide ligation (TCL) [9]. This chemical strategy allows ceramides to be obtained *in situ* from two membrane-permeable ligation partners. Thus, alkyl-based salicylaldehyde esters react with sphingosine chemoselectively to form both natural and non-natural ceramides. Interestingly, the authors further demonstrated that the TCL-based ceramide formation occurs in HeLa cells, inducing apoptosis. Recent work using an analogous approach enabled the construction of a small molecule capable of labeling sphingosine in living cells (Figure 4**b**) [46]. This turn-on probe consists of a fluorophore ester of salicylaldehyde that detects endogenous concentrations of sphingosine and sphinganine. Using this fluorogenic probe, sphingosine accumulation in cells from patients with Niemann-Pick type C1 (NPC1) was successfully detected. Therefore, this strategy shed light on sphingosine signaling in biology and disease.

Figure 4



Enzyme-free synthesis of artificial lipids. (**a**) *In situ* synthesis of membrane-forming imine-based phospholipids by photoligation reaction between lysosphingomyelin and alkyl-substituted NBs. Adapted with permission from Y. Zhou, H. Yang, C. Wang, Y. Xue, X. Wang, C. Bao and L. Zhu. *Chem Sci* 2021, **12**, 3627–3632 [45]. (**b**) Proposed mechanism for the TCL reaction between a salicylaldehyde-containing probe and sphingosine (Sph). During the reaction, the fluorescent dye (F) is covalently attached to the sphingolipid base and separated from quencher (Q), resulting in a highly fluorescent noncanonical lipid product [BHQ 1: black hole quencher 1]. Adapted with permission from A. K. Rudd, N. Mittal, E. W. Lim, C. M. Metallo and N. K. Devaraj. *J Am Chem Soc* 2020, **142**, 17887–17891 [46].

Conclusion

In this concise review, we provided an account of the recent methodologies for the synthesis of non-canonical lipids, as well as some methods that involve the production of natural lipids in artificial environments. Specifically, we described the most distinctive strategies (enzymatic, chemoenzymatic, and enzyme-free synthesis) for the *in situ* formation of artificial lipids. These robust approaches enable the direct preparation of artificial lipids with potential applications on therapeutics, drug delivery carriers, imaging, remodeling, cell signaling, and even origin of life studies [1,14,16,50,51]. Given the biological importance of mimicking cellular systems, we highlighted the *in situ* synthesis of artificial phospholipids capable of driving self-assembly of biomimetic membranes. The use of specific chemical reactions can be linked to the *de novo* formation of such lipid membranes, a phenomenon that is not known to occur in biology, where membrane synthesis requires machinery dependent on preexisting membranes.

We strongly believe that advances in artificial lipid synthesis will pave the way for broader investigations into these relatively new classes of biomimetic molecules. We also envision several exciting emergent methods that will offer a deeper understanding of membrane biophysics and lipid biology. Interestingly, biophysical studies may be necessary to evaluate structure–function relationships of the artificial lipids to understand if they can adequately serve as alternatives to natural lipids and whether they have any perturbative roles. We believe that one area where *in situ* artificial lipid synthesis can be valuable is the incorporation of isotopically-labeled fatty acids. Such species

are important for methods which do not rely on additional tags (fluorophores), including Raman spectroscopy/microscopy and nanoscale secondary ion mass spectrometry (NanoSIMS).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could appeared to influence the work reported in this article.

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