Synthesis of lipid membranes for artificial cells

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Abstract | A major goal of synthetic biology is to understand the transition between non-living matter and life. The bottom-up development of an artificial cell would provide a minimal system with which to study the border between chemistry and biology. So far, a fully synthetic cell has remained elusive, but chemists are progressing towards this goal by reconstructing cellular subsystems. Cell boundaries, likely in the form of lipid membranes, were necessary for the emergence of life. In addition to providing a protective barrier between cellular cargo and the external environment, lipid compartments maintain homeostasis with other subsystems to regulate cellular processes. In this Review, we examine different chemical approaches to making cell-mimetic compartments. Synthetic strategies to drive membrane formation and function, including bioorthogonal ligations, dissipative self-assembly and reconstitution of biochemical pathways, are discussed. Chemical strategies aim to recreate the interactions between lipid membranes, the external environment and internal biomolecules, and will clarify our understanding of life at the interface of chemistry and biology.

Protocells
Primitive, abiotic 'original' cells. In synthetic biology, refers to self-assembling chemical systems with life-like characteristics.

The theory of evolution elicits questions of life's beginnings on Earth. Before the last universal common ancestor existed, there must have been an exact moment at which chemical building blocks became primordial, cell-like structures (protocells). This particular point in time, approximately 4 billion years ago[1], was considered by Charles Darwin. Darwin conceived of a "warm little pond" in which macromolecules assembled from "ammonia and phosphoric salts, light, heat, electricity etc."[2]. The modern NASA definition of life is "a self-sustaining chemical system capable of Darwinian evolution." This definition incorporates Darwin's principles of natural selection and implies life's emergence from a compartment like the "warm little pond". Owing to its focus on evolution, the NASA definition leads naturally to the assumption of compartmentalization as a key feature of life in individual organisms and cells[3]. Indeed, the ubiquity of compartments in extant life emphasizes their importance in sustaining far-from-equilibrium chemical systems and facilitating Darwinian evolution[4,5]. To better understand the origins of life on Earth, a more precise definition might describe the chemical conditions at which matter becomes alive. Revisiting the question of life, scientists are now asking "at what point does chemistry become biology?"[6]

Synthetic biologists are uniquely poised to answer this revised question. The complementary approaches of top-down and bottom-up synthetic biology investigate the border between chemistry and biology. Top-down synthetic biologists aim to create a minimal cell by sequentially deleting superfluous genes in a natural cell until a minimal genomic construct that sustains life is created[7,8]. Bottom-up synthetic biologists work from the other side of the border, with the goal of chemically synthesizing components of life and combining them to form life-like matter[8]. Constructing artificial cells from the bottom up is a promising and ambitious approach to redefining life in chemical terms.

In a landscape of infinite chemical combinations, artificial cell construction must begin by mimicking the most essential biological components of the earliest cells on Earth. Lipid compartments qualify as such components, because every living cell depends on lipid membranes to facilitate biological reactions and maintain a far-from-equilibrium state. Consequently, this Review highlights the bottom-up development of artificial lipid compartments. Specifically, we address the chemistries of natural and synthetic compartment self-assembly and function using three strategies for constructing artificial cells. First, we discuss chemistry for building protocells, with the aim of recreating steps thought to have been important in cellular evolution. Next, we assess the development of unnatural synthetic lipids that can assemble to form membranes that mimic characteristics of natural compartments, with both known and enhanced functions. Finally, we review recent efforts to study the interplay between synthetic compartments and natural biomolecules. Together, these strategies leverage and accentuate the central role of lipids in facilitating life-like processes.

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Reconstructing protocells

Before biology, there was chemistry. Whether delivered by extraterrestrial infall[9] or stewed by geochemical reactions of the Hadean Earth[10], organic compounds were present on Earth approximately 4 billion years ago[11]. Prebiotic atmospheric and geochemical reactions were likely major contributors to the synthesis of organic compounds on the early Earth[12–14]. It is thought that, under the extreme conditions of oceanic hydrothermal vents or land-locked hydrothermal pools, existing prebiotic organic compounds underwent a series of reactions to create the building blocks (nucleotides, amino acids and lipids) of life-forming biopolymers (RNAs, proteins and membranes)[15,16]. The chemical properties of these biopolymers (such as charge and hydrophobicity) enabled their spontaneous self-assembly into protocells.

A major goal of prebiotic chemists is to recapitulate the abiotic synthesis and self-assembly of protocell precursors in a laboratory. In 1953, Stanley Miller reported the foundational discovery that amino acids can be formed by exposing plausible vapours of the early Earth to an electrical discharge[17]. Since then, others have generated additional biogenic monomers from putative prebiotic precursors. For example, nucleobases have been synthesized from hydrogen cyanide or formamide under alkaline conditions[18,19], and it is thought that formaldehyde was produced by ultraviolet irradiation of carbon dioxide and water in the early Earth's atmosphere and fell as rain, providing the prebiotic source of carbohydrates[20,21]. More recent work from the Sutherland, Szostak and Powner groups has shown that activated, prebiotically plausible precursors can form nucleotides, amino acids and lipids[22–25], and that these prebiotic building blocks can be synthesized simultaneously in complex reaction mixtures[25–28]. This section highlights research in developing prebiotically plausible lipid syntheses, self-assembly and chemical selection.

Prebiotic lipid synthesis

Single-chain amphiphiles. Fatty acids and monoacylglycerols are single-chain amphiphiles (SCAs). Fatty acids and glycerol, precursors to acylglycerols, have been found on extraterrestrial infall, such as comets, asteroids and other interplanetary particles[11]. SCAs can self-assemble in controlled laboratory experiments and have been proposed as likely candidates for the compartment-forming amphiphiles of protocells. The Fischer–Tropsch synthesis of hydrocarbons through catalytically mediated reactions between carbon monoxide and hydrogen has been adapted to synthesize SCAs under prebiotically relevant conditions[29]. Nooner and Oró's seminal Fischer–Tropsch-type synthesis[30] of $C_{5–18}$ fatty acids depended on a catalytic combination of nickel and iron from Canyon Diablo meteorite powder and potassium carbonate or rubidium carbonate promoters (FIG. 1a). Although using geochemically relevant materials, the experiment omitted water, which is a necessary component in both the hydrothermal vent and hydrothermal pool scenarios of the origin of life[30]. In aqueous conditions, simplified Fischer–Tropsch-type syntheses using formic acid and oxalic acid precursors as prebiotic carbon sources have produced a mixture of alkanes, alkenes, long-chain monocarboxylic acids and alcohols[31,32] (FIG. 1b). $C_{3,5,7,9}$ monocarboxylic acids have also been synthesized by combining an aqueous suspension of prebiotically plausible nickel sulfides with acetylene and carbon monoxide[33] (FIG. 1c).

Phospholipid precursors. Phospholipids are the major constituent of membranes in modern cells. Monoacylglycerols and diacylglycerols are key phospholipid precursors[34–37]. In the early 1970s, Oró and colleagues showed that, under prebiotic conditions, cyanamide can be used as a condensing agent for the polymerization of both nucleotides and amino acids to form oligonucleotides and peptides, respectively[18,38,39]. It was reasoned that, if the synthesis of nucleic acids and lipids could be unified through their starting materials, then these biological molecules may have originated from similar or simultaneous chemical reactions. Using cyanamide and imidazole as catalysts, a dried film of ammonium palmitate was reacted with glycerol at elevated temperatures to produce monopalmitoylglycerols, dipalmitoylglycerols and tripalmitoylglycerols[40] (FIG. 1d, top route). Condensation reactions of lipids can also occur under hydrothermal conditions: monoacylglycerols, diacylglycerols and triacylglycerols have been synthesized from various fatty acids ($C_7–C_{16}$) and a stoichiometric excess of glycerol under aqueous, salt-free conditions[41] (FIG. 1d, bottom route). Poor yields of the extracted products prevent the formation of acylglycerol membranes, but purified monoacylglycerol products can form protocellular membrane structures. To lend further experimental support to the theory that life may have started in hydrothermal pools, future efforts could be devoted to understanding the determinants of membrane formation in complex product mixtures containing low concentrations of acylglycerols.

Building on the investigations into a universal condensing agent, the Sutherland group identified commonalities in intermediates used for the prebiotic synthesis of biomolecular (RNA, protein and lipid) precursors. Investigation of the synthesis of ribonucleotides from a hydrogen cyanide feedstock revealed that by-products of the RNA assembly chemistry are precursors to the Strecker synthesis of four amino acids: glycine, serine, alanine and threonine[42,43]. One RNA intermediate, glyceraldehyde, can convert into a more stable isomer, dihydroxyacetone, by keto–enol tautomerization under general acid–base phosphate catalysis. Photoreduction of dihydroxyacetone by hydrogen sulfide forms glycerol, a key starting material for the synthesis of acylglycerols (FIG. 1e). Moreover, further conversion of glycerol produces glycerol-1-phosphate and glycerol-2-phosphate, which are hydrophilic precursors of phospholipids[28] (FIG. 1f). This study and subsequent work focused on geochemically feasible sequential reactions, describing a scenario in which RNA, protein and lipid may have been created simultaneously[28,44,45]. To demonstrate that membrane-forming lipids could have originated from feedstocks similar to those for RNA and DNA, further work must show the feasibility of acylation under these environmental conditions and membrane formation in the resulting mixed product environment.

Single-chain amphiphiles
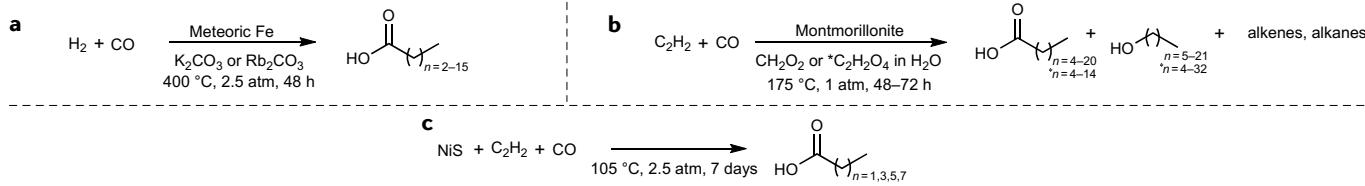
Phospholipid precursors
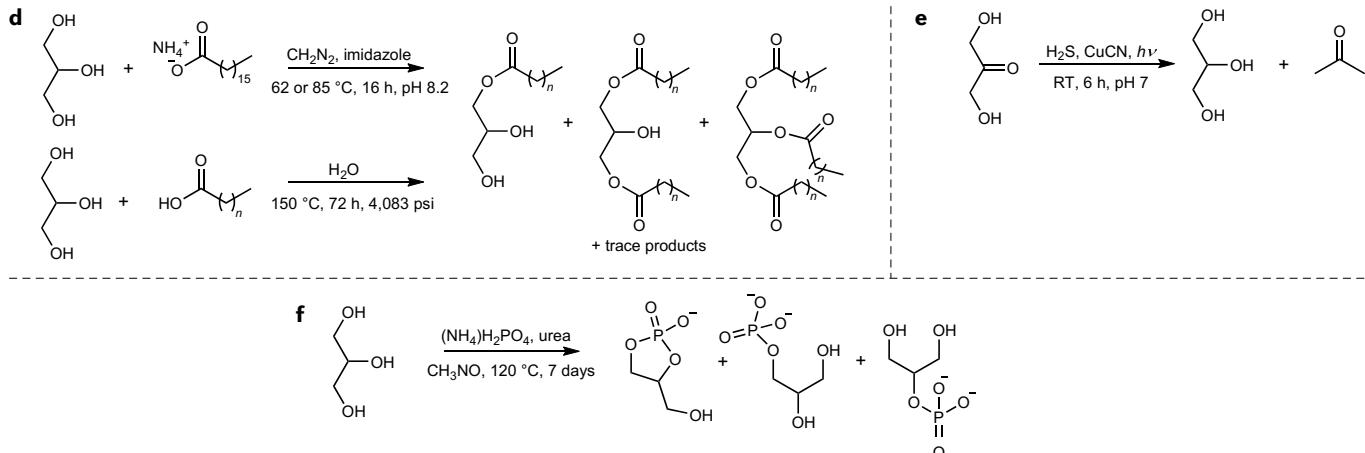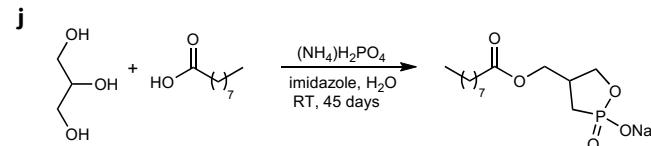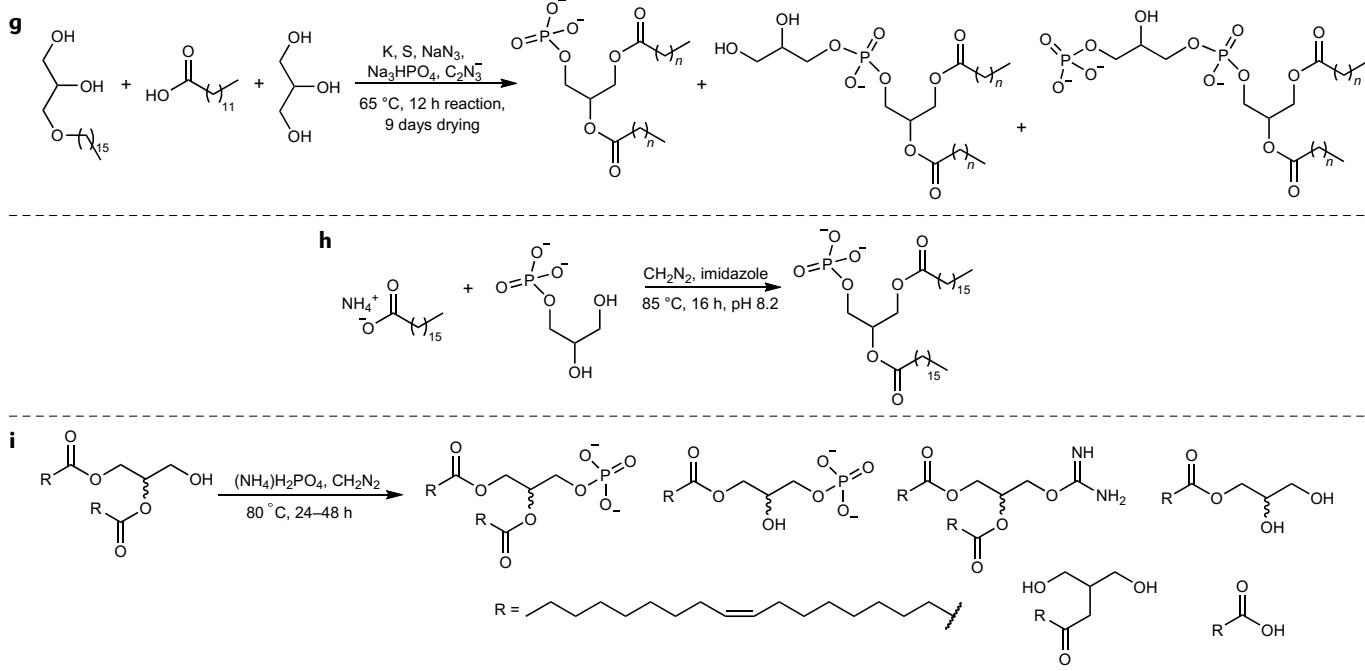
Phospholipids

Fig. 1 | **Prebiotic lipid synthesis.** Prebiologically relevant abiotic reaction schemes and conditions for the synthesis of single-chain amphiphiles (**a**–**c**)[30,32–34], phospholipid precursors (**d**–**f**)[29,40–43] and phospholipids (**g**–**j**)[46,47,49,50]. RT, room temperature.

Phospholipids. In 1977, Deamer and colleagues hypothesized that phospholipids could be synthesized in evaporating tide pools that contain glycerol, phosphate and cyanamide on hot sand under wet–dry cycling conditions, mimicking a hydrothermal pool scenario of protocell formation. They experimentally verified that

ether monoglycerols could be made under wet–dry cycling only when evaporated to dryness. A resulting ether monoglycerol, chimyl alcohol, was then mixed with various phospholipid precursors and clay, producing very low yields of phosphatidic acid, phosphatidylglycerol and phosphatidylglycerol phosphate[46] (FIG. 1g). In 1978, using different precursors, Oró and colleagues obtained yields of up to 45% of phosphatidic acid and other phospholipids under aqueous conditions evaporated to dryness[47] (FIG. 1h). Additionally, phosphatidylcholine, one of the most abundant phospholipids in eukaryotic cell membranes, was produced in a dry film of phosphatidic acid and choline in the presence of cyanamide[48]. More recently, two additional phospholipids, dioleoylphosphatidic acid (DOPA) (FIG. 1i) and dioleoylphosphatidylethanolamine (DOPE), were abiotically synthesized from a racemic mixture of dioleoylglycerol phosphorylated in the presence of cyanamide or urea with ammonium dihydrogen phosphate or 2-aminoethylphosphate, respectively, by evaporating the reaction mixtures to dryness[49]. The crude products containing 30–60 mol% DOPA formed stable giant vesicles capable of encapsulating the water-soluble dye calcein, whereas crude products containing DOPE did not.

Phospholipids in the form of monoalkyl phosphates are plausible prebiotic membrane components. Adapting Deamer's phospholipid synthesis (FIG. 1g) in the presence of diammonium phosphate and minimal water, cyclophospholipids have been synthesized under aqueous conditions and can form multilamellar vesicle structures of up to $20\,\mu m$ in diameter[50] (FIG. 1j). Furthermore, oligonucleotides and peptides are formed under the same phosphorylation conditions, signifying another reaction in which nucleotides, amino acids and lipids can be simultaneously created.

Prebiotic self-assembly

Ionic strength, pH and temperature. In controlled laboratory experiments, the self-assembly of prebiotically plausible SCAs into liquid-crystalline membranes is adversely affected by high pH and salt concentrations[51–54]. The pH and salt sensitivities of SCAs could indicate whether prebiotic protocellular formation was more likely to have occurred in hydrothermal pools on lava beds or in hydrothermal vents at the ocean floor. The Deamer group investigated whether SCAs self-assemble more readily in hydrothermal water of different pH or in seawater[55]. Two hydrothermal water samples were gathered from hot springs in Yellowstone National Park: one from an acidic, low-salt, clay-lined pool in the Midway Geyser Basin area and another from the slightly alkaline, high-salt, silica-rich Bison Pool of the Lower Geyser Basin area. Short-chain fatty acids preferentially formed vesicles in the acidic, low-salt environment that mimics a hydrothermal pool scenario (FIG. 2a, top). By contrast, longer-chain fatty acids formed vesicles in both hydrothermal water samples (FIG. 2a, middle), most likely because of their higher stability than membranes composed of their more permeable, shorter-chained counterparts. Mixed vesicles of fatty acids and their monoglycerols, irrespective of acyl chain length, were stable under a broader range of conditions (FIG. 2a,

bottom). For each of the SCAs, the high ionic strength of seawater inhibited membrane formation and promoted lipid aggregation, owing to interactions between divalent cations and the carboxyl and phosphate groups of the SCAs. Vesicle formation in seawater, however, has been observed with more complex mixtures of SCAs. The Lane group found that mixtures of different numbers of SCAs, including C_{10}–C_{15} fatty acids and 1-alkanols, form vesicles in aqueous solutions between pH 6.5 and 12 at modern seawater salinity[56]. This work has been extended to other prebiotically plausible lipid monomers, showing that vesicles formed from cyclophospholipids and fatty alcohols are more stable in buffers containing high concentrations of Na^+, Mg^{2+} and Ca^{2+} ions than in vesicles formed from fatty acids and alkyl phosphates[57]. Taken together, these findings demonstrate that the composition of a lipid mixture dictates its formation into membranes in seawater. Further analysis of the effects of charge, acyl chain length and the ratios of SCAs on vesicle formation in various prebiotically relevant environmental conditions will hone our understanding of where compartmentalization occurred.

The spontaneous assembly of fatty acids into vesicles often results in the formation of nanoscale small unilamellar vesicles (SUVs) or large multilamellar vesicles[58]. Although challenging to control vesicle morphology, fatty acid giant unilamellar vesicles (GUVs) can be formed by altering the pH of the solution[59]. When oleate, palmitate or myristate micelles formed at pH 10 are dispersed in an aqueous solution with a pH just below the fatty acid pK_a, abundant cell-sized GUVs form. These GUVs can encapsulate a range of particles from sugars and RNA to nanoparticles, and exhibit membrane division driven by the addition of oleate to the outer membrane leaflet.

Mineral-surface-mediated self-assembly. Minerals can facilitate the micelle-to-vesicle transition of fatty acids and phospholipids[60,61], with membrane-formation kinetics depending strongly on the surface charge of the minerals[62–65]. The crustal rocks of the Hadean and Eoarchean Earth were composed of silica (SiO_2), quartz (α-SiO_2), pyrite (FeS_2), siderite ($FeCO_3$), zincite (ZnO), γ-alumina (Al_2O_3) and goethite (α-FeOOH). To examine the effects of mineral surface chemistry on the assembly of SCAs, pure dodecanoic acid or a 2:1 mixture of dodecanoic acid and dodecanol was combined with amorphous nanoparticles of these minerals and others[66]. All the minerals increased the rate of vesicle formation, although the rates were strongly dependent on the isoelectric point of the minerals (FIG. 2b). Positively charged minerals led to the greatest rate enhancement, owing to the electrostatic association with the negatively charged SCA head groups. By contrast, van der Waals forces and hydrogen bonding facilitate binding of the SCAs to negatively charged and neutral mineral surfaces. The stronger electrostatic interactions result in 6–10 times more adsorption on positively charged alumina than on negatively charged silica.

In an innovative approach to tracking newly formed vesicles, giant dioleoylphosphatidylcholine (DOPC) vesicles were supported on a glass bead and then fed with

oleic acid[67]. The supported giant vesicles subsequently bud and produce giant vesicles composed of DOPC and oleic acid. With immobilized budding vesicles large enough to detect with light microscopy, this approach is not only a useful tool for observing vesicle replication but also has implications for lipid templating on mineral


a

Monomers	Geyser Basin sample	Bison Pool sample
	• pH 3.3 • Location temperature: 53.0 °C • Cationic eq.: 1.12 mM • Anionic eq.: 0.67 mM	• pH 7.9 • Location temperature: 68.4 °C • Cationic eq.: 12.90 mM • Anionic eq.: 13.08 mM

b

Nanometre-sized mineral particles
Micrometre-sized mineral particles
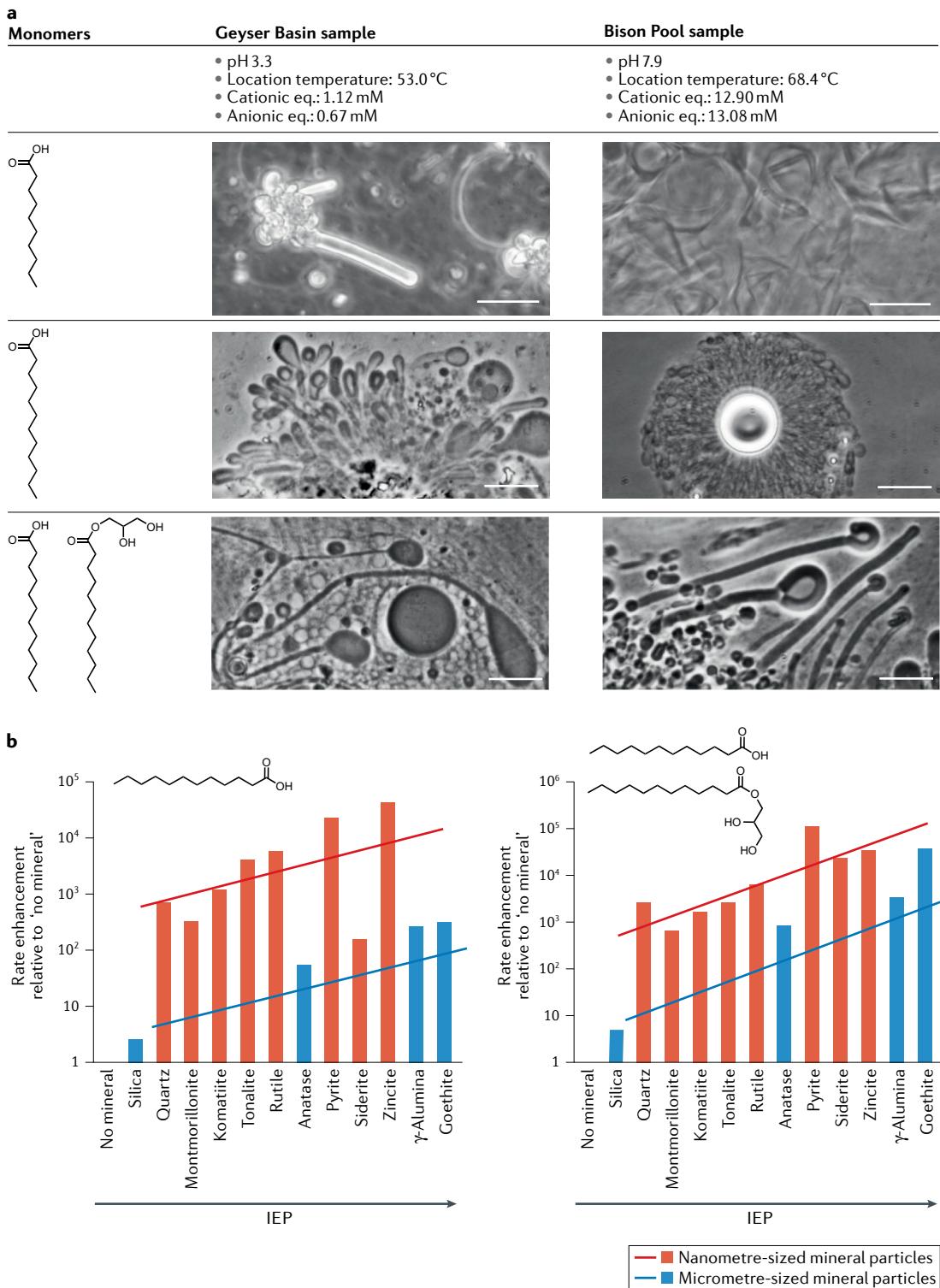
Fig. 2 | **Prebiotic self-assembly of single-chain amphiphiles. a** | Comparison of the self-assembly of a short-chain fatty acid (decanoic acid), longer-chain fatty acid (dodecanoic acid) and heterogeneous mixtures of dodecanoic acid and its monoglycerol in hydrothermal water samples of different pH and temperature. Heterogeneous single-chain amphiphile (SCA) vesicles are more stable under varying pH and temperature than homogeneous SCA vesicles[55]. Scale bars are 20 μm. **b** | Minerals in solution enhance the rate of SCA vesicle formation relative to solutions with no mineral present[66]. Compared with fatty acids alone (left), vesicle formation of SCA mixtures is enhanced by negatively charged minerals (right). The isoelectric points (IEPs) of the minerals increase from left to right. Part **a** adapted from REF.[55], CC BY 4.0. Part **b** adapted from REF.[66], CC BY 4.0.

surfaces. Further experiments could be performed to encapsulate biomolecules or metabolic precursors. For a more in-depth discussion of mineral-assisted lipid self-assembly, readers are referred to REF.[68].

Membrane composition, stability and lipid preference. Early investigations of protocell self-replication focused on SCA-based vesicle growth and division by changing the environmental conditions, such as pH (REFS[69–71]), shear force[72] and lipid concentration[71,72]. Abiotic syntheses of fatty acids produce acyl chains of heterogeneous length, and small kinetic and thermodynamic differences between lipid interactions in a heterogeneous mixture could also drive life-like function. Indeed, it has recently been shown that SCA composition can drive protocell division. Mixing populations of protocells composed of longer-chain or shorter-chain fatty acids results in membrane division, owing to growth-induced curvature driven by differences in fatty acid chain length. Intriguingly, the mixtures tend to form multilamellar vesicles of a single lipid composition[73]. Similarly, starting from a mixture of activated fatty acids with various chain lengths, iterative cycles of non-enzymatic phospholipid formation selectively produce long-chain acylglycerol phosphates[74]. These findings indicate that the chemical synthesis of lipids with varying acyl chain lengths could possibly drive the life-like phenomenon of lipid membrane fission.

Hypothesizing that a mixture of lipid tail lengths could increase stability in the dilute prebiotic environment of Earth, the Szostak group showed that mixed fatty acid vesicles have a lower critical aggregation concentration than uniformly short-chain fatty acid vesicles[75]. The aggregation of mixtures of varying decanoic acid and oleic acid stoichiometries was monitored by NMR spectroscopy, revealing that higher concentrations of isotopically labelled oleic acid partition into membranes compared with decanoic acid, owing to the lower solubility of oleic acid. These findings suggest that modulation of the fatty acid chain lengths in vesicles could impart compositional dynamics that might be advantageous to artificial cells. Namely, the addition of long-chain fatty acids could impart stability to artificial protocells, whereas shorter acyl chains may increase permeability and chemical uptake. Mixed vesicles of oleic acid and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) also have high stability to divalent cations and permeability to nucleotides and divalent ions[76]. This finding is intriguing, because, at some point in evolutionary history, there would need to have been a transition from SCA-based vesicles to phospholipid-dominated cells, with both potentially intermixing. In this regard, Dalai et al. showed that Mg^{2+} can act as an environmental selection pressure in fatty acid membranes. Mixed fatty acid phospholipid membranes treated with increasing amounts of Mg^{2+} eventually transitioned to phospholipid-enriched membranes that are more stable in the presence of Mg^{2+} (REF.[77]). The trade-off between membrane stability and permeability is important for the protection and maintenance of biochemical processes. At the expense of decreased permeability, increasing the content of phospholipids for more stable membranes

was clearly prebiotically advantageous[78]. Promising avenues of investigation in this area, namely, determining the contribution of additional prebiotic environmental factors (such as the high pressure of the deep ocean), will help elucidate the composition of compartments amenable to life on the early Earth.

Assembling artificial compartments

The main goal of artificial cell development is not necessarily to understand how life on Earth emerged but to create systems that serve as models for understanding universal biological characteristics. Freed from the constraints of prebiotic geochemistry, synthetic systems can be controlled through an expanded repertoire of available functional groups and chemistries. Synthetic, unnatural lipid precursors can non-enzymatically react and assemble into biomimetic cell boundaries. It is possible to design synthetic molecules with properties that can be tuned by external inputs such as salt concentration, heat or light. By exploiting mutually compatible bioorthogonal chemistries, multifunctional synthetic membranes can be made. Creating and assembling cell boundaries from the bottom up provides insight into which physicochemical characteristics lead to life-like attributes. This section discusses the generation and morphology of unnatural lipid compartments from synthetic precursors.

Compartment formation and dissolution
De novo formation and growth of synthetic lipid compartments. Natural lipid membranes are constantly created and maintained by enzymatic machinery. To construct eukaryotic cell membranes, the Kennedy pathway of de novo phospholipid synthesis involves multiple enzymatic steps to synthesize phosphatidylcholine or phosphatidylethanolamine. Similarly, phospholipid remodelling is maintained by enzymes and tunes the mechanical properties of a membrane in response to internal or external stimuli. In the Lands cycle, an acyl chain is removed from a phospholipid, and the resulting lysophospholipid is combined with a thioesterified fatty acid of different chain length or saturation in a single enzymatic step[79,80].

Our group has been interested in adapting non-enzymatic bioorthogonal reactions to mimic lipid synthesis pathways and has demonstrated the utility of bioorthogonal reactions for forming synthetic lipid membranes. The copper-catalysed azide–alkyne cycloaddition (CuAAC) between an alkyne derivative of a lysophospholipid and oleyl azide mimicked the remodelling pathway of the Lands cycle[81] (FIG. 3a). Coupling results in a close analogue of a natural phospholipid containing an unnatural triazole linker. Triazole phospholipids assemble into lipid bilayers with physical characteristics (melting temperature and fluidity) that are similar to those of their natural counterparts. Spatiotemporal control of lipid formation would be an interesting feature of an artificial cell, as it would enable localization of bilayer growth, budding and division to specific areas of the cell at certain times, mimicking biological systems[82,83]. To demonstrate that light can initiate and control in situ vesicle formation, the
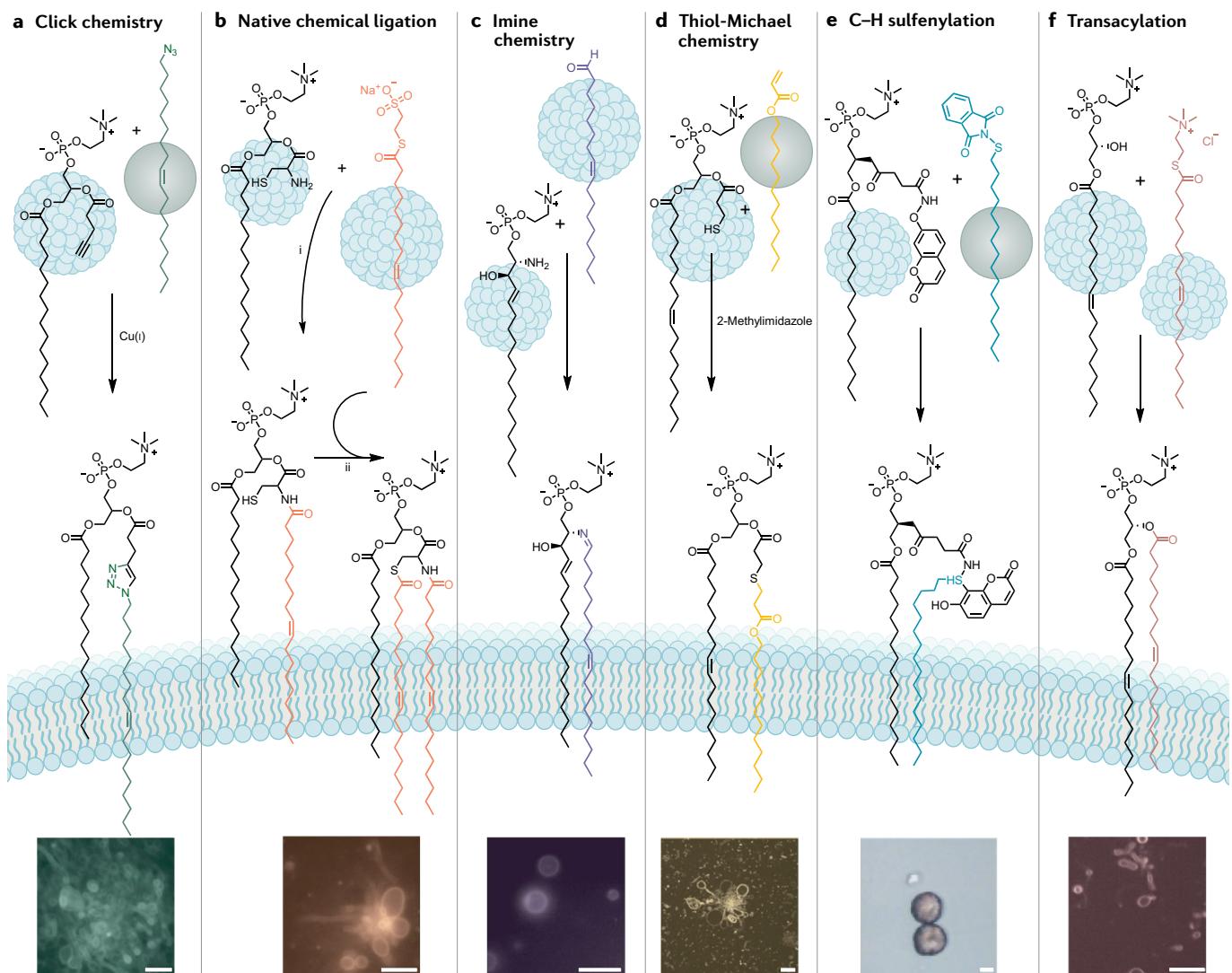
Fig. 3 | **De novo formation of lipid compartments.** Unnaturally functionalized lipid precursors react to form phospholipids that self-assemble into vesicles without a pre-existing membrane template. Images of the de novo-formed synthetic phospholipid membranes are shown below the respective reaction scheme. Parts **a–d**[81,85,89,100] and **f**[102] show fluorescence images of dye-stained membranes, whereas part **e**[101] shows a bright-field image. Scale bars are 10 μm. Part **a** (bottom) adapted with permission from REF.[81], ACS. Part **b** (bottom) adapted with permission from REF.[86], Wiley. Part **c** (bottom) adapted with permission from REF.[90], ACS. Part **d** (bottom) adapted with permission from REF.[101], Royal Society of Chemistry. Part **e** (bottom) adapted from REF.[102], CC BY 4.0. Part **f** (bottom) adapted from REF.[103], Springer Nature Limited.

Bowman lab synthesized a visible-light-sensitive photoinitiator copper(II) acylphosphinate-*N,N,N′,N″,N″*-pentamethyldiethylenetriamine (CAP) complex for CuAAC catalysis[84]. Normally, catalytic Cu(I) is generated in situ by reducing Cu(II) (REF.[82]). Light activation of the CAP complex results in the radical-based reduction of Cu(II) to Cu(I), leading to the formation of vesicles from functionalized alkyne lysolipids and azide aliphatic chains. The technique demonstrated control over where the vesicles formed (with higher densities of vesicles in well-lit areas) and vesicle size (an increase in light intensity resulted in larger vesicles).

The use of redox-active metal ions, such as copper ions, can lead to oxidative damage of biomolecules and motivates development of alternative strategies for forming artificial cell membranes. Native chemical ligation

(NCL) is a biocompatible, chemoselective coupling reaction that is normally used to ligate two polypeptide fragments[85]. We adapted this technique for the synthesis of phospholipids, showing that NCL spontaneously occurs when a cysteine-modified, single-chain phospholipid and a charged thioester fatty acid derivative are combined in water under reducing conditions[86] (FIG. 3b). The SCAs are initially dispersed in aqueous solution as micelles, and de novo formation and growth of vesicles occurs upon NCL. Chemoselective histidine peptide ligation can also be used for the de novo formation of phospholipid membranes from functionalized histidine-modified lysolipids and long-chain acyl thioesters[87].

The acyl chains of lipids in cell membranes are constantly being enzymatically remodelled to suit specific

a

b

c
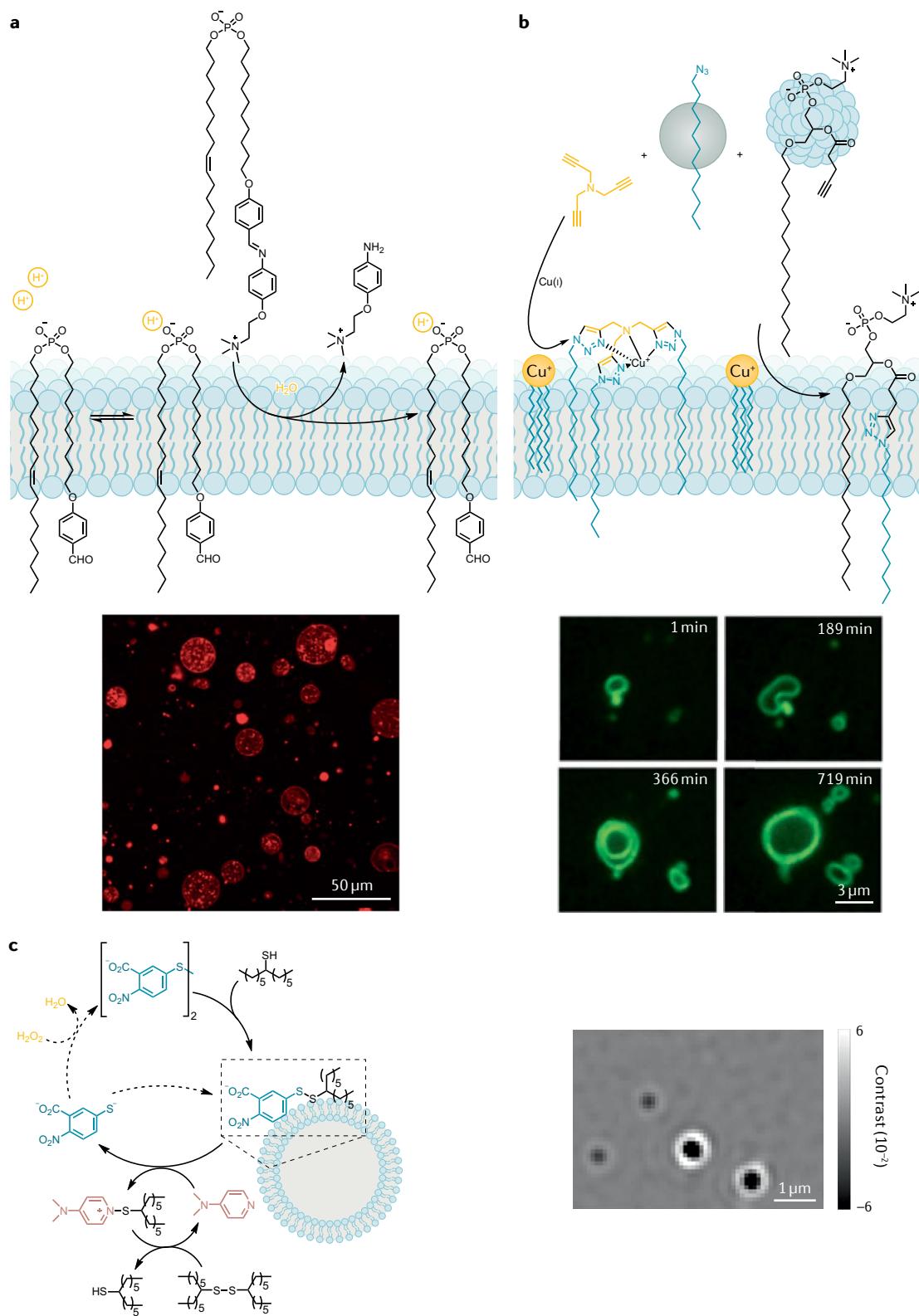
cellular functions. To mimic this process in artificial lipid membranes, the phospholipid NCL reaction can be made reversible by use of an *N*-methylcysteine-modified single-chain phospholipid precursor[88]. This system can undergo subsequent additions of alternative methylcysteine-modified single-chain phospholipids for continual reversible remodelling[89]. The addition of

saturated cysteine-containing phospholipid fragments to GUVs formed from unsaturated precursors triggered membrane remodelling that could be visualized by fluorescence microscopy. Remodelling led to the spontaneous formation of microdomains in artificial membranes, mimicking the formation of lipid rafts in natural membranes. Similarly, lipid remodelling can be

◀ Fig. 4 | **Autocatalysis and dissipative self-assembly. a** | Synthetic, anionic phospholipids recruit protons (yellow, circled H⁺) to membranes, creating a pH gradient around the vesicles[108]. This gradient increases the rate of hydrolysis of the phospholipid precursor tail at the membrane surface, forming additional catalytic membrane. A confocal laser scanning microscopy image (bottom) shows vesicle products stained with Texas Red-DHPE (1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; 0.1 mol%). **b** | The reaction of a long-chain alkyl azide (3 eq.) and tripropargylamine generates membrane-embedded tris-(lauryl triazole)amine (TLTA). Upon addition of Cu(I), the TLTA trimer catalyses the de novo membrane-forming reaction between azide and alkyne reactive precursors and the synthesis of more TLTA[109]. Time-lapse fluorescence microscopy images (bottom) of lipid-synthesis-mediated growth of vesicles stained with Rh-DHPE. **c** | The catalytic cycle and reaction scheme for the out-of-equilibrium formation of self-reproducing vesicles[119]. Discontinuation of the supply of chemical fuel, H_2O_2, shifts the system towards thermodynamic equilibrium (dashed lines). An interferometric scattering micrograph (right) shows vesicles formed through the reaction (with the chemical structure shown in the inset). Part **a** (bottom) adapted with permission from REF.[109], Elsevier. Part **b** (bottom) adapted with permission from REF.[110], National Academy of Sciences. Part **c** (right) adapted from REF.[120], CC BY 4.0.

driven by other reversible coupling reactions, such as imine formation using amine-containing lysosphingomyelin and long-chain aldehydes[90,91] (FIG. 3c). De novo synthetic membrane assembly and remodelling could be used for programmable compartment reorganization to facilitate functions such as biomolecule sequestration or division and fusion[92–100].

Control over the lamellarity of vesicles formed in situ has been demonstrated using the thiol-Michael reaction[101]. Unilamellar vesicles were observed when equimolar amounts (5 mM) of a reduced lysolipid thiol and acrylate-functionalized lipid tail underwent a thiol-Michael addition in the presence of 2.5 mM 2-methylimidazole catalyst and water. Increasing the concentration of the catalyst results in multilamellar vesicles (FIG. 3d). Continued optimization of reactions such as this might enable control over multicompartment or hierarchical synthetic vesicle assembly.

The formation of the first fluorogenic phospholipids was facilitated by the development of an efficient, mild method for the synthesis of *ortho*-sulfiliminyl phenols. The reaction of a non-fluorescent, coumarin-functionalized lysolipid with a linear alkyl sulfenylation reagent at room temperature yields a fluorogenic phospholipid derivative that self-assembles into fluorescent vesicles[102] (FIG. 3e). Fluorogenic tools such as this could be used as a readout of artificial cell assembly in a high-throughput setting with no need for hydrophobic fluorescent dyes.

De novo formation of natural lipid compartments. In addition to the many strategies for abiotic synthesis of artificial phospholipids, we recently described a chemical system for the de novo, non-enzymatic formation of natural phospholipids from synthetic precursors[103]. Synthesis of DOPC, DOPA, dipalmitoylphosphatidylcholine (DPPC) and 1,2-didecanoyl-*sn*-glycero-3-phosphocholine (DDPC) was achieved through acylation of a lysophospholipid with a water-soluble cationic thioester (FIG. 3f). Favourable electrostatic interactions between a positively charged 2-(oleoylthio)-*N,N,N*-trimethylethan-1-aminium chloride acyl donor and the negatively charged phosphate group of the lysophospholipid facilitated the formation of DOPC.

De novo-synthesized DOPC spontaneously formed membranes in soda lake and hydrothermal vent water (pH 8.8–10) and maintained a proton gradient when transferred to citrate buffer (pH 4.6). It is plausible that similar scenarios played out in alkaline hydrothermal vents when vesicles were expelled into the more acidic water of the Hadean ocean. Prebiotic geological scenarios aside, synthetic strategies to synthesize natural phospholipids that can maintain proton gradients will be particularly useful if strategies can be implemented to harness the proton gradients to form chemical energy.

Autocatalysis. In the 1990s, the Luisi group spearheaded investigations of lipid compartment autopoiesis in micelles[104,105], reverse micelles[104,106], SCA giant vesicles[70] and phospholipid vesicles[107]. These early investigations looked beyond the ability of a compartment to grow and divide, and aimed to design compartments with the ability to catalyse their own reproduction. Since this seminal work, several examples of self-reproducing compartments have been demonstrated using various synthetic strategies.

One strategy is to use the hydrolysis of membrane precursors to enable membrane growth and reproduction through physical autocatalysis. For example, Luisi used the hydrolysis of oleic anhydride to give rise to autocatalytic vesicle growth[70]. In an analogous approach, the Sugawara group constructed a synthetic, hydrolysable lipid precursor that facilitates membrane growth in the presence of catalytic, pre-existing lipid membranes[108,109] (FIG. 4a). An alternative to physical catalysis is to couple a molecular autocatalyst to membrane formation. This reaction synthesizes membranes and produces further catalyst, which prevents dilution of the catalyst as membranes accumulate. Oligotriazoles such as tris-(lauryl triazole)amine can bind Cu(I), accelerating CuAAC reactions, including the formation of additional tris-(lauryl triazole)amine. By using a self-reproducing oligotriazole catalyst, our group established a one-pot system capable of de novo compartment formation and reproduction. In the presence of Cu(I), the membrane-embedded oligotriazole autocatalyst self-reproduces and also catalyses the synthesis of artificial phospholipids from a continual feedstock of reactive azide and alkyne SCA precursors[110] (FIG. 4b). More recently, the Fletcher lab found that increasing the hydrophobicity of the copper catalyst in CuAAC autocatalytic systems increases the reaction kinetics of autocatalysis facilitated by micellar aggregates[111,112]. The group has used several autocatalytic systems for de novo micelle formation that were predecessors to the development of vesicles capable of undergoing dissipative self-assembly (that is, forming out-of-equilibrium ordered structures, which are discussed below)[113–116].

Escaping equilibrium with dissipative self-assembly. Living matter perpetually escapes equilibrium and exists far from local energy minima by exchanging energy with the environment[117]. Dissipative self-assembly harnesses the energy of chemical reactions to maintain an out-of-equilibrium state[118]. Artificial membranes built from

Autopoiesis
From the Greek auto 'self' and poiesis 'formation': a property of a system that enables maintenance and reproduction of itself through self-regulation.

chemistries that enable dissipative self-assembly exhibit the life-like feature of escaping equilibrium. The Fletcher group has applied autocatalytic thiol chemistry to create an out-of-equilibrium micellar self-replicator that maintains its own dissipative self-assembled state[119]. The phase-separated starting materials, 1-octanethiol and 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), react slowly to make the product surfactant, 2-nitro-5-(octyldisulfanyl)benzoic acid. Micellar aggregates of the surfactant facilitate mixing of the starting materials and their own self-replication. A thiol–disulfide exchange of the surfactant with the 1-octanethiol precursor results in a by-product and an inactive waste product. Using H_2O_2 as an oxidizing chemical fuel, the by-product can be regenerated as the starting material DTNB, maintaining the system in an out-of-equilibrium state. Exchanging the one-tailed precursor 1-octanethiol with tridecane-7-thiol formed a two-tailed surfactant, 2-nitro-5-(tridecan-7-yldisulfanyl)benzoic acid. The two-tailed surfactant self-assembles into autopoietic vesicles able to maintain an out-of-equilibrium state[120] (FIG. 4c). The Fletcher group also extended their previous work with CuAAC-driven de novo membrane formation to form vesicles capable of self-reproduction, dissipative self-assembly and competition between micelle and vesicle formation[121].

There is likely tremendous potential for applying dissipative strategies for maintaining lipid compartments far from equilibrium[122–124]. There are various fuel-driven reaction cycles for the assembly and disassembly of other synthetic supramolecular structures[125]. Many of these chemistries could be explored and adapted to lipid compartments.

Control of vesicle self-assembly

Multicompartment assembly. As discussed above, control over unilamellar vesicle self-assembly is challenging, but achievable (FIG. 5a). An additional challenge is the development of multicompartment models, rather than isolated ones, to best mimic life's dependence on more than one compartment to carry out biological functions. The study and manufacture of vesicle aggregates, or multicompartment vesicle assemblies, aid the development of artificial cell–cell communication, bioreactors and synthetic organelles. Biological interactions can be modelled with pairs of synthetic vesicles and with comparatively massive 'artificial tissues' made from hundreds of interconnected water-in-oil drops[126,127]. Control of multicompartment vesicle assemblies is typically limited by vesicle instability and collapse; however, control over their composition and size has been attained using microfluidic and chemical methods.

Microfluidics offers tunability, which is typically unachievable with bulk lipid assembly methods. Microfluidic systems primarily employ emulsion techniques to form lipid bilayers using water-in-oil droplets to scaffold assembly[128–130]. In an elegant method of assembling multicompartments, unilamellar vesicles were formed from water-in-oil-in-water double emulsions using microfluidics[131]. Addition of an additional triblock copolymer surfactant, Pluronic F-68, increased control of the scaffold stability in the dewetting and

multicompartment assembly process, enabling the formation of oil-free monodisperse liposomes containing multiple cargo-loaded compartments (FIG. 5b,c). The two-compartment or three-compartment unilamellar vesicle aggregates functioned as cell-like bioreactors to facilitate in vitro transcription and translation. Moreover, the insertion of membrane nanopores led to intercompartmental exchange of small molecules.

Aggregates of a predetermined size can be obtained through temperature control rather than being templated by microfluidics[126,132]. Vesicle aggregate size can be controlled by covalently linking vesicles using lipid-modified biotin and streptavidin, or reversibly with C18-pNIPAm (a thermosensitive surfactant with a C_{18} tail and poly(N-isopropylacrylamide) head group)[126]. Small aggregates, such as vesicle pairs, are easily achieved under both systems because the linkers diffuse quickly towards the interaction site, inhibiting the attachment of additional liposomes — termed 'self-limiting aggregation'. Combining the biotin–streptavidin and C18-pNIPAm systems facilitated temperature-controlled, stepwise growth of vesicle aggregates to predetermined sizes[132]. The C18-pNIPAm acts to sterically hinder biotin–streptavidin interactions in a reversible, temperature-dependent manner (FIG. 5d). This control facilitates the exponential growth of streptavidin–biotin-linked vesicle aggregates with oscillations in temperature.

Hierarchical assembly. Hierarchically assembled lipid membranes (that is, one vesicle within another) mimic natural cells and their inner membranous organelles. Platzman, Spatz and colleagues have created sophisticated polymer-supported oil-in-water microfluidic fabrication methods to form complex GUVs capable of hierarchical assembly[133,134]. Microfluidics was used to encapsulate an aqueous solution containing SUVs and Mg^{2+} in an uncharged polyethylene glycol-based fluorosurfactant. Addition of a negatively charged perfluoropolyether carboxylic acid fluorosurfactant (Krytox) leads to localization of the Mg^{2+} ions at the inner droplet interface, facilitating SUV fusion at the polymer boundary and creating droplet-stabilized GUVs. The addition of perfluoro-1-octanol releases the GUVs from the polymer boundary into an aqueous phase. Altering the charge at the surfactant boundary by increasing the concentration of Mg^{2+} in the aqueous phase in conjunction with the amount of Krytox can result in the assembly of neutral, positive or negative droplet-stabilized GUVs. Interestingly, a stoichiometric excess of SUVs in the encapsulation solution yields a hierarchical system of SUVs within GUVs. The viability of picoinjection of biomolecules, such as purified transmembrane and cytoskeletal proteins, into the droplet-stabilized GUVs was also demonstrated[134].

A disadvantage of microfluidics is the limited scalability. Owing to their larger yields, bulk vesicle assembly can be an attractive alternative. The bulk assembly of biphasic water droplet-in-oil droplet-in water and oil droplet-in-water droplet-in oil systems can form hierarchical lipid membranes in non-aqueous conditions[135]. But the construction of fully aqueous

Organelles
Specialized subcellular structures that perform specific functions for the cell.
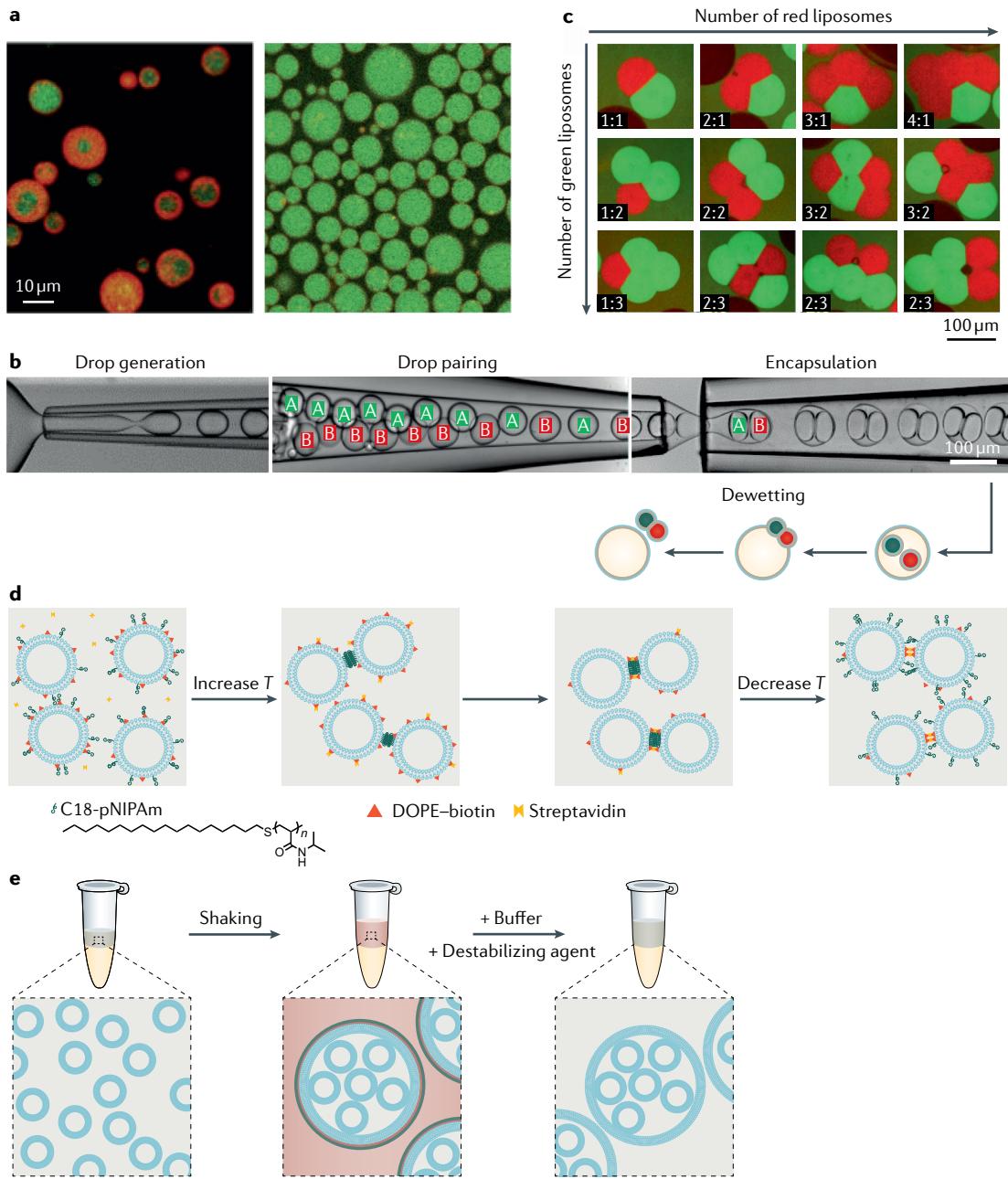
Fig. 5 | **Control of vesicle self-assembly, aggregation and hierarchical order. a** | Salt concentration affects the morphologies of oleic acid vesicles (red; membrane stained with rhodamine B) encapsulating RNA (green)[59]. The vesicles formed in 200 mM Na^{+} bicine, pH 8.43 have heterogeneous morphologies (left). By contrast, those formed in 50 mM Na^{+} bicine, pH 8.43 are unilamellar (right). **b** | Microfluidic strategy for the controlled assembly of multicompartment vesicles[130]. Photos of drop generation, drop pairing and encapsulation. Drops A (green) and B (red), which contain different aqueous cargo, are generated surrounded by oil and paired within an aqueous phase encapsulated by oil (double emulsion). During the dewetting step, the aggregated vesicles are released from the oil droplet (yellow) into aqueous solution (grey). **c** | The microfluidic strategy shown in part **b** enables control of aggregate size and composition[130]. **d** | Vesicle aggregation can be controlled through temperature (*T*)-dependent steric hindrance[131]. A thermosensitive surfactant, C18-pNIPAm, sterically hinders soluble streptavidin from binding to dioleoylphosphatidylethanolamine (DOPE)–biotin. Heat aggregates C18-pNIPAm, recruiting nearby vesicles and enabling streptavidin recruitment to biotin. The streptavidin–biotin reaction leads to irreversible binding of nearby vesicles. A decrease in temperature results in C18-pNIPAm relaxation and steric hindrance of additional vesicle–vesicle interactions. The process can be repeated for further vesicle aggregation. **e** | One-pot assembly of hierarchical vesicles[135]. An aqueous solution of small unilamellar vesicles (grey layer) sits atop fluorinated oil with polyethylene glycol-based fluorosurfactants (yellow layer). Shaking induces the formation of giant unilamellar vesicles (GUVs) from the encapsulated small unilamellar vesicles; the GUVs are stabilized by a polymer shell of fluorosurfactant (green and pink). The addition of buffer and Krytox (a destabilizing agent) facilitates GUV release into aqueous solution. Part **a** adapted with permission from REF.[59], ACS. Parts **b** and **c** adapted with permission from REF.[131], ACS. Part **d** adapted with permission from REF.[132], Royal Society of Chemistry. Part **e** adapted from REF.[136], CC BY 4.0.

hierarchical vesicle-in-vesicle assemblies still poses a challenge. Platzman, Spatz and colleagues adapted their microfluidic liposome assembly strategy towards a one-pot, large-scale encapsulation method[136]. The method uses water droplet-in-oil techniques to create fully aqueous hierarchical vesicles. An aqueous solution containing SUVs is layered on top of an oil–surfactant mixture. Vigorous vortexing assists the formation of water-in-oil droplets and subsequent fusion of the SUVs at their periphery to form droplet-stabilized GUVs. The droplet-destabilizing agent, perfluoro-1-octanol, is added to release the GUVs from the surfactant shell and oil phase into the aqueous layer (FIG. 5e). Reconstitution of membrane proteins and cholesterol was demonstrated in this system, as well as the encapsulation of pyranine, mRNA, polystyrene beads, HeLa mitochondria, *Escherichia coli* and GUVs. Hierarchical GUV assembly may prove useful for the study of synthetic organelle functions in artificial cells.

Installing cellular machinery

The chemistries reviewed above demonstrate that protocells and synthetic membranes can exhibit life-like phenomena: synthesis, self-assembly and response to external conditions. More complex cellular behaviours (such as reproduction, differentiation or subcellular specialization) arise when other subsystems are introduced into a compartmentalized system. Strategic installation of macromolecular machinery into lipid compartments has revealed the active role of the lipid membrane in regulating biochemistry. Through integrating biological components with synthetic membranes, complex attributes such as cell-to-cell communication and morphogen-mediated differentiation can be achieved. In the following, we examine the interplay between synthetic membranes and biological material, and discuss recent progress in mimicking the intricacies of natural cellular behaviour with minimal synthetic models.

A compartmentalized central dogma

RNA and DNA localization, amplification and catalysis in compartments. It is speculated that the minimal criteria for a living cell are self-replicating, information-containing molecules and the ability to concentrate and compartmentalize these molecules[137]. It follows that nucleotide–lipid membrane interplay is an important area of investigation in origins-of-life research and artificial cell development. At first glance, it may appear that lipids have a mostly passive role encapsulating biomolecules. However, lipid membranes can actively assist self-replication of information-containing molecules and vice versa[138]. The Deamer group demonstrated that prebiotically relevant dehydration–hydration cycles can lead to the encapsulation of biomolecules such as polymerases, which could synthesize RNA inside liposomes using exogenously available nucleotides[139,140]. Additionally, dehydration–hydration cycles facilitate the non-enzymatic formation of RNA-like polymers from mononucleotides in the presence of phospholipids; the lipid molecules organize and concentrate mononucleotides within the dried lipid matrix, promoting subsequent condensation reactions[141].

There have been intensive efforts to study the self-replication of RNA within lipid membranes. Citrate-chelated Mg^{2+} and elevated temperatures facilitate non-enzymatic primer extension of RNA in fatty acid vesicles. Mg^{2+} increases the permeability of the fatty acid vesicles to negatively charged oligonucleotides as large as tetramers without disrupting the structural integrity of the membrane[142]. Mixed fatty acid and acylglycerol vesicles, which are more tolerant of cations, can facilitate encapsulated RNA catalysis with exogenous addition of Mg^{2+}. Membrane-encapsulated catalysis was demonstrated with the self-cleavage of the hammerhead ribozyme inside fatty acid vesicles supplied with Mg^{2+} (REF.[143]). Compared with vesicles composed of SCAs, phospholipid vesicles are less permeable to the ions necessary to sustain RNA replication. Physically disrupting phospholipid vesicles facilitates ion entry and enables RNA replication and distribution to newly formed vesicles. A DOPC-encapsulated Qβ replicase system, which can replicate RNA without exogenously added template RNA, can undergo ten freeze–thaw and RNA replication cycles, mimicking cell division and reproduction[144]. Although an exciting step towards artificial cell replication, the biological relevance falls short, as nucleic acids must be continually added and membrane fission is physically induced by the shear forces of freeze–thaw. There is scope to optimize these chemical and physical methods of compartmentalized replication to propagate artificial cells capable of Darwinian evolution.

The Sugawara lab has studied how DNA replication can be coupled to lipid vesicle reproduction[145,146]. Changing the pH or adding lipid precursors can result in vesicle division and passaging of encapsulated genetic information to next-generation vesicles. A synergistic relationship between lipids and encapsulated DNA was demonstrated by showing that the lipid-centric phenomenon of division can be directly driven by nucleic acid replication[147,148]. Protocellular division was driven by DNA replication in a length-dependent, sequence-independent manner for several cycles. The electrostatic interaction between DNA and a cationic lipid catalyst forms a supramolecular, 'lipo-deoxyribozyme', catalyst. This catalyst hydrolyses a synthetic lipid precursor at an imine bond, and the lipid product accumulates at regions of the membrane near the lipo-deoxyribozyme, leading to budding, division and DNA inheritance within liposomes. Interestingly, in competitive proliferation experiments between long (3,200 bp), medium (1,164 bp) and short (374 bp) DNA chain lengths, medium-length sequences were the most successful with regard to inheritance in subsequent liposome generations. Additional experimentation will be needed to better understand the effects of lipid precursor availability and DNA chain length on vesicle reproduction.

Focus has also turned towards the active role of lipids in oligonucleotide function. As discussed above, the self-replication of anionic polynucleotides has been correlated with self-reproduction of cationic vesicles[145]. Additionally, lipid compartments can 'chaperone' the folding of catalytic RNA molecules[145,149]. The Szostak
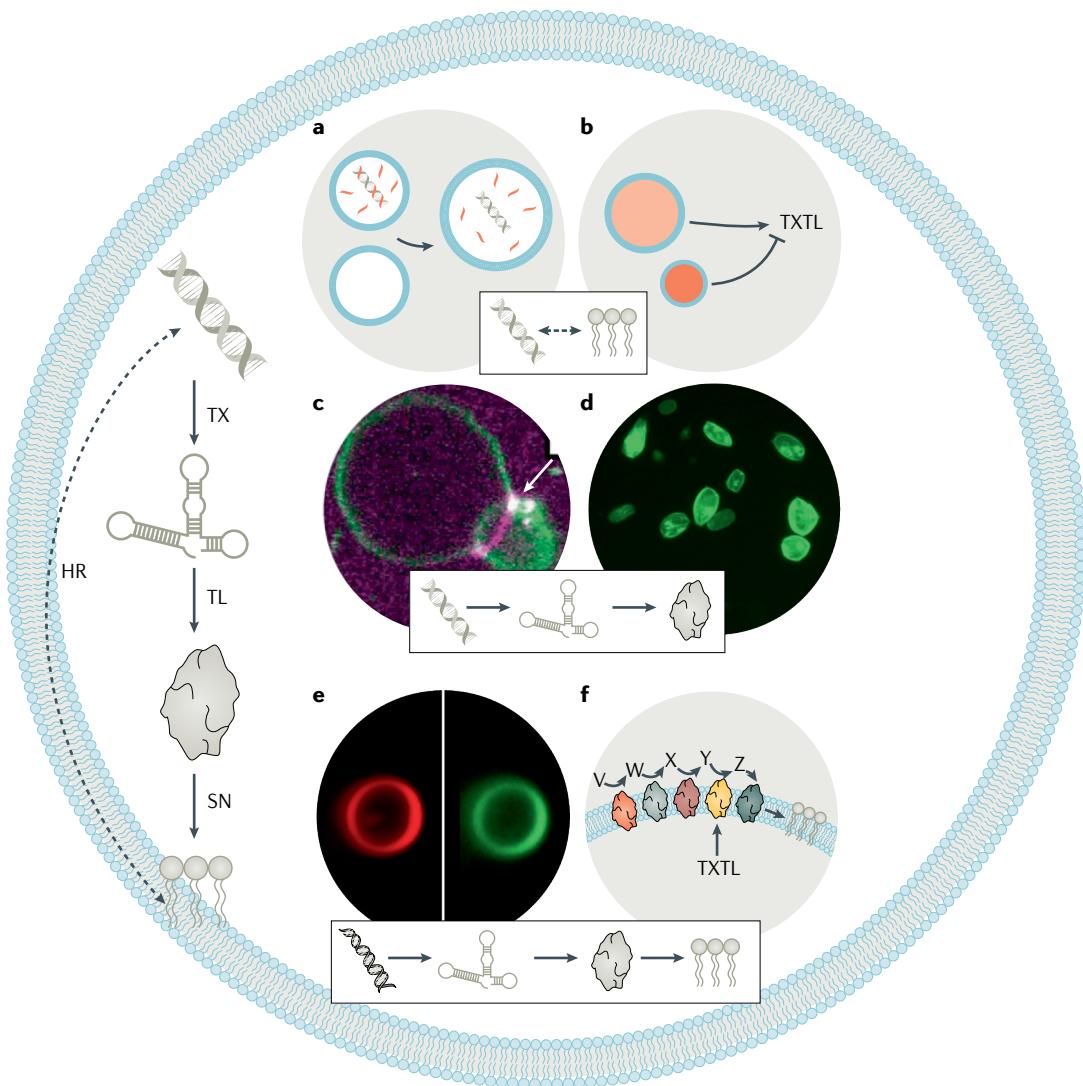
Fig. 6 | **The compartmentalized central dogma of biology.** The central dogma of biology conceptualizes the flow of genetic information to protein in cells. The schematic on the left shows DNA being transcribed (TX) to RNA, which is then translated (TL) to protein that synthesizes (SN) compartment-forming lipids. Lipid compartments and information-containing molecules homeostatically regulate (HR) one another (dashed line). The boxed insets on the right indicate the portion of the central dogma experimentally addressed in the images. **a** | Ribozyme (grey) activity is regulated by the RNA (red) concentration within compartments[149], with a high concentration inhibiting ribozyme activity. When a vesicle containing RNA-inhibited ribozyme fuses with an empty vesicle, the growth dilutes the vesicle contents, enabling ribozyme activity. **b** | A high concentration of vesicle cargo (orange, unconcentrated; red, concentrated) can inhibit transcription and translation[156]. **c** | In vitro TXTL of ZapA and FtsZ leads to the assembly of a proto-ring filament bundle (indicated by the white arrow on the confocal fluorescence image) within liposomes (green)[153]. **d** | After mreB (a membrane-associating protein) is expressed within liposomes and recruited to the membrane, membrane crowding results in deformation of the lipid vesicles[154], as seen in the fluorescence microscopy image. **e** | Confocal fluorescence microscopy images showing that FadD10 (green) recruits to the de novo-formed membrane (red) after expression with in vitro TXTL[159]. **f** | Liposome-encapsulated expression of phospholipid-synthesizing enzymes[160,161]. V, W, X, Y and Z represent intermediates in the phospholipid synthesis. Part **a** adapted from REF.[150], Springer Nature Limited. Part **c** adapted from REF.[154], CC BY 4.0. Part **d** adapted with permission from REF.[155], National Academy of Sciences. Part **e** adapted from REF.[160], CC BY 4.0.

group has shown that the growth of lipid membranes can modulate ribozyme activity[150]. High concentrations of hammerhead ribozyme and complementary oligonucleotides were encapsulated within mixed lipid vesicles consisting of myristoleic acid, glycerol monomyristoleate and DOPA, resulting in inhibition of ribozyme activity by the oligonucleotides. When these vesicles were swelled with myristoleic acid–glycerol monomyristoleate

feeder vesicles, the internal solution was diluted and ribozyme activity was activated (FIG. 6a). This model suggests that primitive physical homeostatic mechanisms could have been directly activated by changes in lipid compartment morphology.

Studies such as these reveal that lipid compartments and oligonucleotides are sufficient to induce emergent phenomena typically associated with life. The lipid

membrane actively regulates and is regulated by the central dogma of biology. DNA is transcribed to RNA, which is translated to protein. Proteins synthesize compartment-forming lipids, which homeostatically regulate functions of the membrane interior (FIG. 6).

Cell-free protein expression in compartments. In principle, an artificial cell could perform protein synthesis within lipid vesicles using self-encoded, self-synthesized ribosomes. So far, the adaptation of minimal, cell-free DNA replication and protein expression systems within liposomes has introduced elements of the central dogma into artificial cells[151,152]. The Danelon group installed one of the most established cell-free, in vitro transcription and translation (TXTL) systems, the PURE (protein synthesis using recombinant elements) system, in liposomes in an attempt to reconstruct cell division biochemically[153]. In phospholipid vesicles encapsulating the PURE system, cytoskeletal proto-ring-forming proteins (FtsA and FtsZ) were synthesized de novo, subsequently inducing membrane curvature. Co-expression with ZapA, a stabilizer of FtsZ filaments, promotes the formation of longer FtsZ filament bundles[154] (FIG. 6c). Further work involving the introduction of additional FtsZ-associated proteins synthesized using the PURE system will be necessary to induce vesicle fission with proto-ring proteins.

Other cell-free expression systems are compatible with liposome encapsulation and exhibit unique artificial cell applications. Using a different cell-free TXTL system, myTXTL, it was demonstrated that molecular crowding on the surface of liposomes (composed of polyethylene glycol-linked DOPC or biotin-linked DOPC) causes membrane deformation when a membrane-associating protein, mreB, self-assembles at the membrane[155,156] (FIG. 6d). Free cell lysates isolated from *E. coli* have also been encapsulated and concentrated in phospholipid vesicles using microfluidics[157]. When added to hypertonic solutions, the vesicles osmotically shrink to yield lysate concentrations of up to 390 mg ml^{-1}. Liposome-size-dependent molecular crowding induces a switch from reaction to diffusion control of the transcription and translation kinetics (FIG. 6b). This result further emphasizes the homeostatic control that the physical structure of lipid compartments can impose on internal biochemical pathways.

Lipid biosynthesis. To reproduce, a compartmentalized system must synthesize additional compartments. Several groups have reconstituted lipid biosynthesis proteins into vesicles. When supplied with lipid precursors, the proteoliposomes are capable of phospholipid synthesis and membrane expansion[69,105,158,159]. Combining the flexibility and tunability of in vitro TXTL with proteoliposome lipid synthesis and growth could reconstitute the entire central dogma in artificial cells. Our group has developed a method to form synthetic phospholipid membranes de novo using a single soluble enzyme synthesized using a minimal recombinant TXTL system[160]. FadD10 is a soluble enzyme that catalyses the generation of fatty acyl adenylates, which react with a synthetic amine-functionalized lipid to form membranes. FadD10

cell-free protein expression using the PURExpress TXTL system results in chemoenzymatic membrane formation and subsequent localization of FadD10 to the membrane to facilitate growth (FIG. 6e). Increasingly complex lipid synthesis pathways have been reconstituted using in vitro TXTL systems. In ambitious in vitro expression efforts, co-production of multiple enzymes necessary for natural phospholipid synthesis using the PURE system resulted in biosynthesis of new phospholipid membranes[161,162] (FIG. 6f). These studies suggest that work should be undertaken to complete the central dogma in artificial cells by linking genotype with membrane formation and growth.

Life-like phenotypes

Biochemically induced membrane replication. Cell boundaries pose an inherent challenge to the central features of life: growth and reproduction. A closed compartment held shut by the hydrophobic effect will not easily overcome the energy barriers required to divide. Researchers have been pursuing chemistries that could drive compartment division. Fluctuations in pH can cause large-scale changes in SCA membrane curvature and morphology, depending on the head group protonation state[163]. Manipulating both pH and osmotic concentration gradients results in vesicle division, as recently demonstrated by encapsulating urease in POPC–oleic acid GUVs. Processing of urea by urease yields ammonia and carbonic acid, which shifts the pH, altering membrane stability and resulting in vesicle fission[164] (FIG. 7a).

Direct manipulation of membranes by proteins can cause membrane deformation and division. In a purely synthetic GUV system comprised of sodium bis-(2-ethylhexyl) sulfosuccinate (also known as AOT) amphiphiles, enzymatic polymerization of aniline catalysed by horseradish peroxidase and H_2O_2 led to the elongation of the GUVs into rods[165]. The addition of cholesterol to this system induces membrane asymmetry, leading to GUV growth and eventual division. Cell-penetrating peptides are known to induce membrane curvature and vesicle deformation[166]. The addition of polyarginine peptides to phosphatidylcholine vesicles can also induce vesicle division[167]. If coupled with in vitro TXTL methods, it might be possible to programme artificial replication by expressing proteins and peptides known to induce membrane division. Further discussion of biochemically induced membrane division and replication can be found elsewhere[168–171].

Artificial organelles. Like its living counterpart, a fully functioning artificial cell might require specialized subcompartments akin to organelles. As with biological organelles, synthetic organelles may need to exchange molecular cargo. Multicompartment vesicles can be equipped with unique PURE in vitro TXTL mixtures that express different proteins in each compartment[172]. When one compartment expresses a substrate required in another, the addition of α-haemolysin peptide pores enables the compartments to undergo spatially segregated enzymatic cascades, resulting in a specific product

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In vitro transcription and translation
(TXTL). A cell-free system of minimal biochemical components necessary to synthesize a protein from a DNA template.

Proteoliposomes
Liposomes in which proteins have been incorporated in the membrane.

a Biochemically triggered membrane replication

$t = 3.0\,s$ $t = 8.0\,s$ $t = 12.0\,s$ $t = 16.5\,s$ $t = 17.5\,s$ $t = 64.5\,s$

100 µm

b Cell-to-cell communication

+ Ionophore

c Artificial organelles

50 x (µm)   50 y (µm)   z (µm)   60   0 0

d Spatiotemporal control of protocell signalling

50 min

e Morphogen-mediated protocell differentiation

y x   500 µm
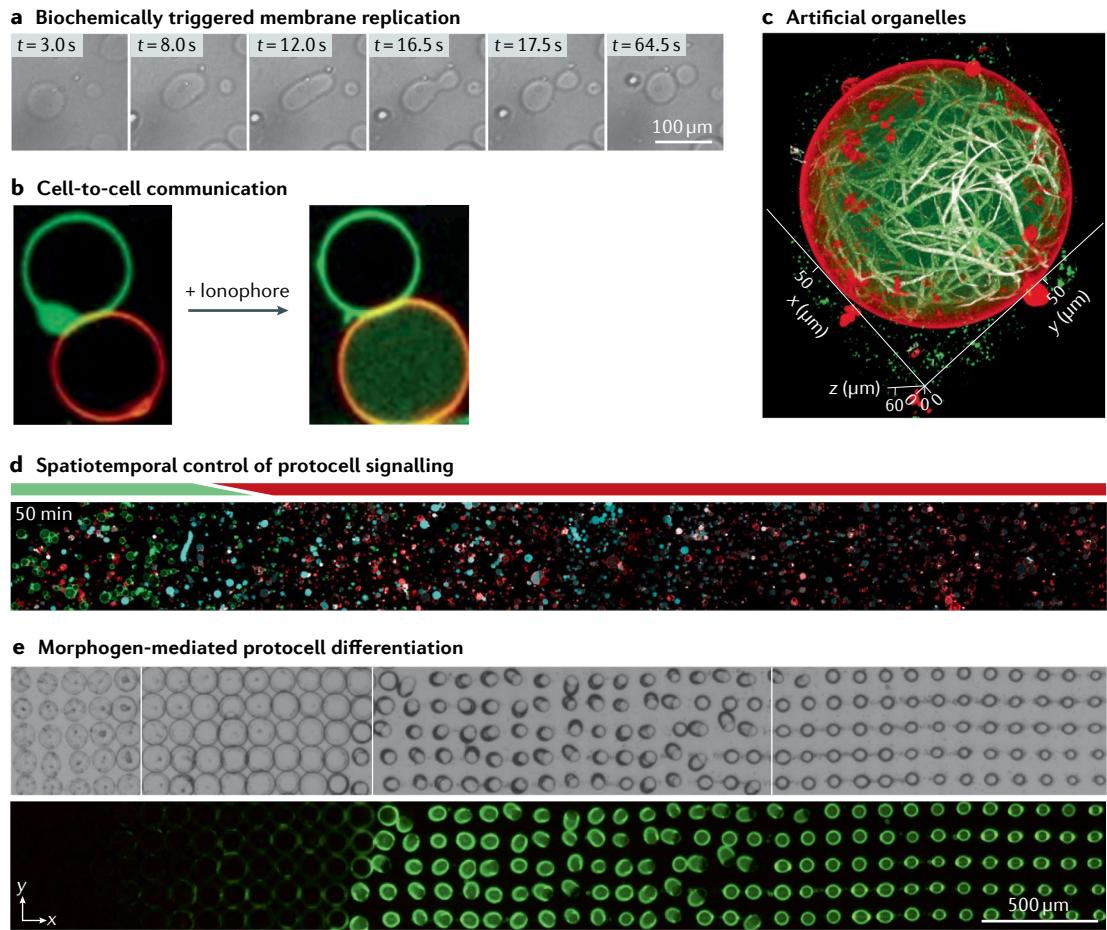
Fig. 7 | **Life-like phenotypes of artificial cells. a** | Time (*t*) series showing the pH-dependent division of single-chain amphiphile vesicles driven by the urease processing of urea[163]. **b** | A vesicle pair exchanges information upon ionophore addition, resulting in an ion-dependent fluorescent readout[172]. **c** | A giant unilamellar vesicle (red) encapsulating an artificial photosynthetic system of small unilamellar vesicles (green)[176]. Upon stimulation with red light, ATP production results in actin polymerization (white) within the giant unilamellar vesicles, as seen in the 3D reconstruction of the system from confocal microscopy images. **d** | Microscopy image showing that, after 50 min, synthetic transmitter cells (green) send a signal to receiver cells (red), resulting in a fluorescent readout (blue)[182]. **e** | Addition of a morphogen to an array of lipid coacervates results in diffusion-mediated differentiation into different vesicle morphologies[183], as shown in the optical (top) and corresponding fluorescence microscopy (bottom) images. Part **a** adapted with permission from REF.[164], Royal Society of Chemistry. Part **b** adapted from REF.[173], CC BY 3.0. Part **c** adapted from REF.[177], Springer Nature Limited. Part **d** adapted from REF.[183], CC BY 4.0. Part **e** adapted from REF.[184], CC BY 4.0.

that would be unattainable if the compartments were not connected[173,174] (FIG. 7b).

Hierarchical vesicle assemblies comprising an outer GUV and encapsulated SUVs have mimicked simple signalling cascades of natural cells. GUVs containing two types of small vesicle (artificial organelles) loaded with glucose oxidase or horseradish peroxidase and an oxidation-sensitive fluorophore, Amplex Red, participate in a reaction cascade when melittin pores allow glucose to travel freely across the lipid membranes. The cascade results in H_2O_2 production in one organelle, which oxidizes Amplex Red in the other's interior to produce a fluorescent signal[175]. Similarly, chemical signalling between GUVs containing α-haemolysin and encapsulated SUVs containing reconstituted secretory phospholipase A2 (sPLA$_2$) resulted in an influx of Ca²⁺ ions into the GUV interiors, activation of sPLA$_2$ and release of the fluorescent SUV contents[176].

Photoinducible control of artificial organelle signalling was demonstrated with photosynthetic SUVs containing photoconverters (proteorhodopsin and photosystem II) and ATP synthase encapsulated within GUVs embedded with calcium and magnesium ionophores[177]. Actin polymerization driven by ATP production within GUVs could be spatiotemporally controlled by red-light stimulation and green-light inhibition (FIG. 7c).

Artificial cell signalling. The ability of cells to relay information to one another is important for viability and reproduction. We briefly explore recent developments in lipid vesicle signalling and direct interested readers to REFS[178–180] for discussion of alternative compartmentalized signalling systems. Direct cell-to-cell communication via a chemical relay is a simple form of cell signalling. In one example of direct communication between synthetic vesicles, photoactivatable proteins

were immobilized on two GUV populations to selectively bind them together. Once bound, the two different artificial GUVs — one containing Ca^{2+} ions (transmitter) and the other Rhod2 dye (receiver) — were induced to fluoresce by addition of an ionophore[173] (FIG. 7b). Gene-mediated communication has also been demonstrated. The communication was driven by transmitter vesicles that contain a genetic switch controlled by the intake of a lactone inducer to produce and release glucose. Receiver vesicles containing glucose oxidase and horseradish peroxidase produced a fluorescence response through H_2O_2 production and oxidation of Amplex Red[181].

Indirect, diffusion-based chemical cell-to-cell communication has been exhibited by large populations of artificial cells. Our group demonstrated the distribution of tasks, quorum sensing and cellular differentiation based on the local environment of eukaryotic cell mimics that contain nuclei-like DNA hydrogel subcompartments[182]. The artificial cell nucleus of each cell mimic localized the genetic information for protein synthesis, and the polymer-based membranes allowed free diffusion of protein signals between communicating cells. In lipid compartment systems, others have reconstituted enzymatic cascades between two populations of artificial cells able to distribute (transmitter cells) or recognize and process (receiver cells) chemical signals[183]. This artificial intracellular communication resulted in a spatiotemporally resolved fluorescent signal (FIG. 7d). Such communication has been linked to more complex phenotypes. For example, the Mann group devised synthetic cascades that led to morphogen-mediated differentiation of coacervate droplets into different vesicle morphologies[184] (FIG. 7e). Further development of direct and indirect cell-to-cell communication might incorporate inducible genetic circuits in large populations of protocells. To advance artificial cell development, self-maintenance needs to be integrated with cellular communication[179].

Conclusion

Constructing an artificial cell from the bottom up relies on systematic development of chemistry to mimic the biological processes and behaviours that define life. Scientists are in pursuit of ordered, functional structures that can self-assemble and embody life-like phenotypes. Compartmentalization is a critical feature of life, and the synthesis of life-like compartments will be a requirement for the construction of an artificial cell. The primary method by which extant cells compartmentalize themselves from the environment, and internally in the case of eukaryotic cells, is through the use of lipid membranes. We have presented three interconnected approaches to constructing artificial cell membranes. Equipped with recent innovations in biomimetic and biochemical tools, scientists are now able to parse the minimal chemical and protein requirements for specific cellular processes. First, investigation of biology's chemical origins has revealed that lipid membranes were likely active in the emergence of life. Second, in mirroring the progression of life from chemistry to biology, creative chemical strategies have been applied to achieve de novo synthesis and self-assembly of lipid membranes. Finally, natural biomolecules have been incorporated into synthetic lipid compartments to isolate and mimic specific behaviours of natural cells. Together, these approaches demonstrate that the cooperation of membranes with other biomolecules could have supported the emergence of life on Earth and is promising for the development of artificial life. However, the next phase of synthetic membrane research will require the creation of structures that mimic living membrane function even more closely. Areas that are ripe for study include the generation of lipid membranes that persist far from equilibrium and require the input of chemical fuel, the scalable formation and maintenance of asymmetrical lipid membranes and better ways to integrate multiple integral membrane proteins to control transport and signalling. Furthermore, future efforts towards a synthetic cell will require even closer collaboration between groups that are generating new membrane-like compartments and researchers who are reconstituting phenomena such as metabolism and replication. Future integration of these synthetic approaches will recapitulate more complex cooperative processes that brought matter to life, highlighting the path that abiotic molecules may have taken to cross the border from chemistry to biology.

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