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Engineering materials for artificial cells

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ABSTRACT

The grand challenge of engineering a minimal artificial cell provides a controllable framework for studying the biochemical principles of life. Artificial cells contribute to an increased understanding of complex synthetic systems with life-like properties and provide opportunities to create autonomous cell-like materials. Recent efforts to develop life-like artificial cells by bottom-up approaches involve mimicking the behavior of lipid membranes to recapitulate fundamental cellular processes. This review describes the recent progress in engineering biomimetic artificial minimal cells and recently developed chemical strategies to drive *de novo* membrane formation from simple synthetic precursors. In the end, we briefly point out the challenges and possible future directions in the development of artificial cells.

1. Introduction

Cell theory states that cells are the basic structural and fundamental unit of life [1]. There exists a general understanding of the requirements for cellular processes to occur in a biological cell, such as the need for a semipermeable membrane, nucleic acids to encode genetic information, and metabolic and energy production [2]. However, despite this knowledge, and rapidly growing research in the biological sciences, we still do not presently know how life began or how to define life. What makes certain matter alive, and how can non-living matter transform into living? As Addy Pross has asked, how can chemistry become biology [3]? To approach this fundamental problem, chemists, bioengineers, and materials scientists have been trying to develop artificial cells, which can be considered life-like materials. Researchers are getting better at constructing life-like artificial cells due to advances in chemistry, biochemistry, materials science, and analytical techniques. The ultimate goal of building a functional artificial cell *de novo* would lead to a much deeper understanding of biology and likely shed light on the physicochemical mechanisms that led to the origin of life on Earth [4,5].

We believe the definition of artificial cells should be expansive and encapsulate different terms like "synthetic cells," "protocells," or "minimal cell" [6]. Here, we will use the word "artificial cells," which is loosely defined as cell-like materials that possess one or many functions of a biological cell. The Chemoton model, introduced by Hungarian theoretical biologist Tibor Gánti in 1971, is one of several life theories that are often used to describe the requirements for minimal cellular life [7]. The model's basic assumptions are that life should fundamentally have three properties: (i) a self-reproducing chemical motor (metabolic cycle), (ii) a chemical information system, and (iii) a chemical boundary system such as lipids. Needless to say, developing synthetic materials that satisfy the chemoton model's criteria is an ambitious goal. However, artificial cells do not need to be considered "alive" but instead are aimed at mimicking life functions. Like many other scientific fields, technological advancements and scientific exploration have enabled progress in materials that mimic life. Fig. 1 summarizes some selected milestones in artificial cell development [8-15].

In this review, we describe some of the latest (i.e., over the last seven years) advancements in developing materials as artificial cells, including artificial cell construction approaches and cell-mimetic compartments. We focus on the generation of "living" materials by using chemical reactions composed of small chemical precursors, mimicking metabolic processes. Finally, we briefly discuss the challenges, opportunities, and future directions of artificial cells. As the field is large and this review is not meant to be exhaustive, many excellent contributions will be omitted, but we direct the reader to some recent, more expansive reviews on the subject [16-21].

2. Features of artificial cells

Artificial cells are biological cell imitators. Ideally, artificial cells should have similar structure and characteristics to biological cells [22]. Every cell requires a membrane to facilitate the biological reaction and,

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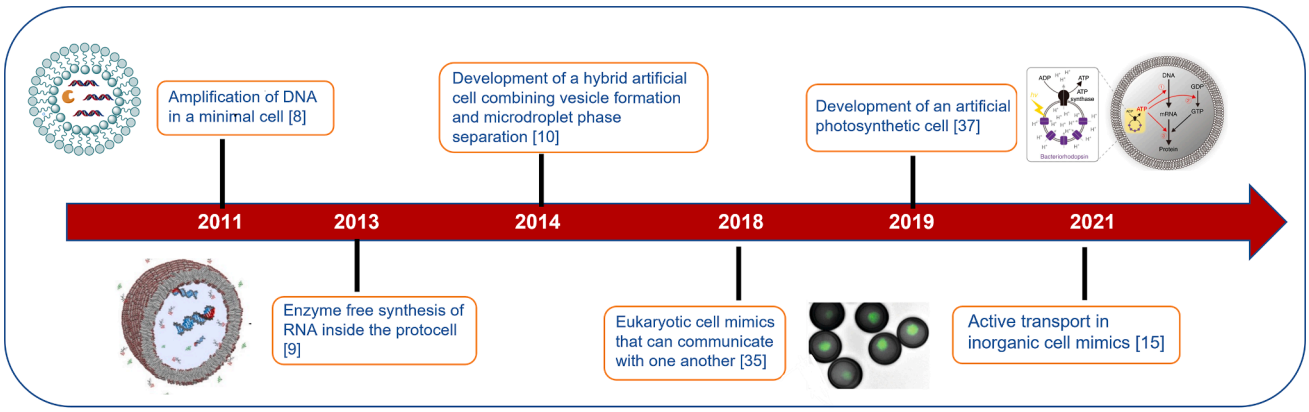

Fig. 1. Some recent key advances in the development of artificial cells [8, 9, 10, 11, 12, 13, 14, 15, 35, 37]. Image (bottom left) reproduced with permission from REF[9]. Copyright © 2013, American Association for the Advancement of Science, Springer Nature Limited. Image (bottom right) adapted from REF[35], CC BY 4.0. Springer Nature Limited. Image (top right) adapted from REF[37], CC BY 4.0.

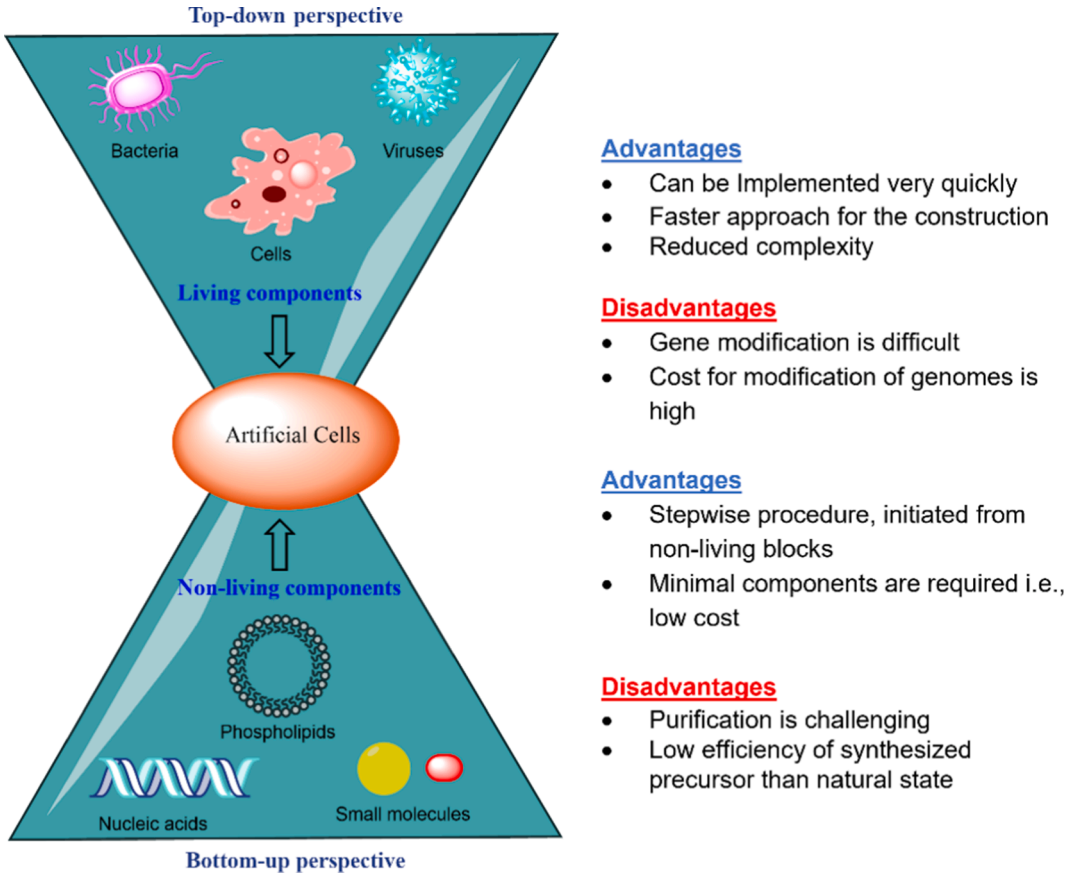

Fig. 2. Comparing the two approaches followed in synthetic biology research to design and construct artificial cells. In the top-down approach, artificial cells are created by stripping or replacing the genomes of living systems such as cells, etc., and reducing complexity. In contrast, in the bottom-up approach, artificial cells are constructed by assembling biological or non-biological molecules that can replicate the essential properties of natural cells [26].

thus, separate its content from the outside [23]. The membrane of an artificial cell can be made up of lipid membranes, simple polymers, or cross-linked proteins [24]. At the same time, the membrane can be prepared to allow the selective passage of different types of molecules. One can make artificial cell membranes by using different synthetic and biological precursors and there is great potential for physical alterations and extensions that affect the membrane permeability.

3. Criteria and construction of artificial cells

This section provides a brief overview of the essential criteria for making an artificial cell and summarizes the approaches for constructing artificial cells that reconstitute basic life processes. For decades, scientists have been working to create an artificial cell that imitates the characteristics of living cells. To make a more cell-like system, first, we consider the main components of biological cells. An ideal scaffold for an artificial cell should possess the essential properties of living cells. Some essential features of biological cells are [25,26]: (i) It contains a

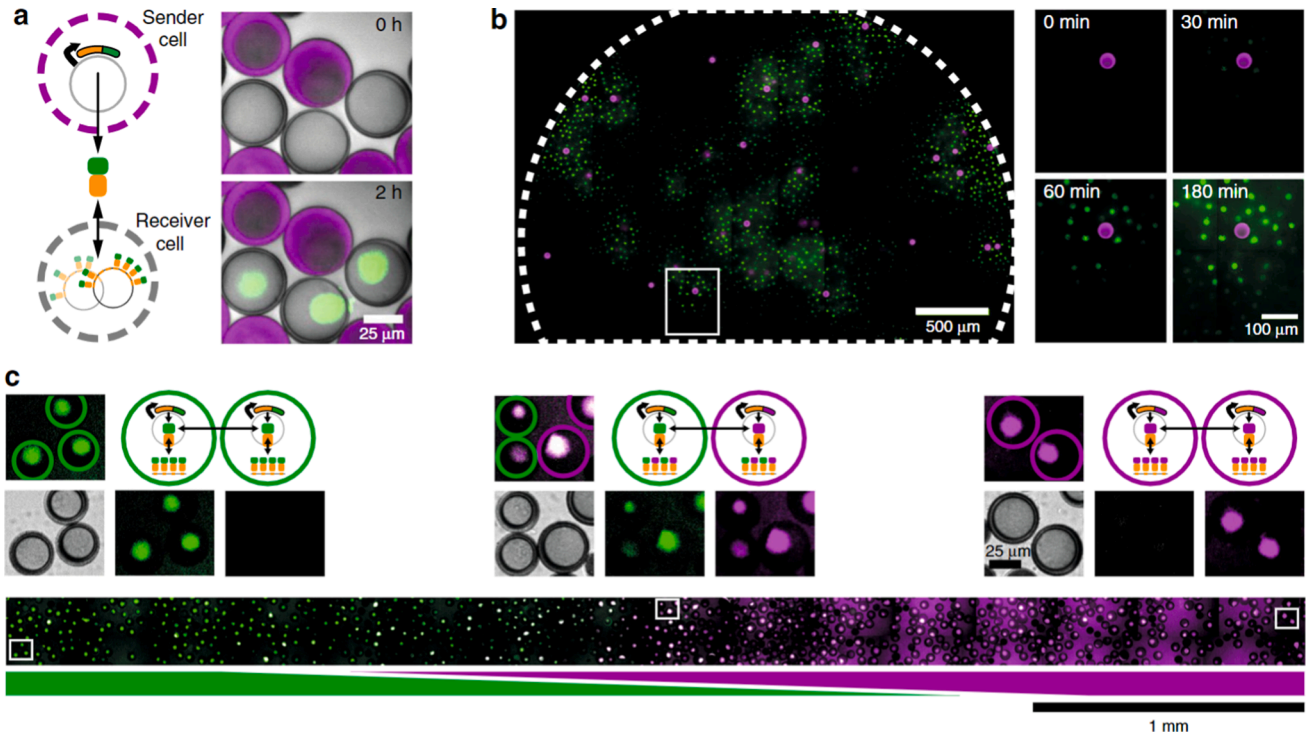

Fig. 3. Exchange of protein between cell-mimics. (**a**) Demonstration of diffusive TetR-sfGFP exchange protein products between neighboring sender cell and receiver cell. After mixing, only the nuclei of the receiver cell-mimics increased in fluorescence intensity as seen in the timelapse images (sender membranes, magenta; tetR-sfGFP, green). (**b**) TetR-sfGFP (green) spread in a large excess of receiver cells and scattered sender cells (magenta) after three hours of expression. (**c**) Heterogenous mixing of two types of cell-mimics in a reaction chamber producing and binding different color reporter proteins. tetR-sfGFP / tetO (green) and tetR-mCherry / tetO cell-mimics (magenta) were diffused in a channel so that they mix in the center but remain separated at the sides. The bottom image shows the fluorescence circulation of sfGFP and mCherry channels after five hours. The middle part of the image shows the merging of these two channels which ends in a white signal. Enlarge images from highlighted areas along the channel are shown above. Combined images with cell-mimic are displayed by colored, dashed circles (top), and brightfield, sfGFP, and mCherry signals are shown individually (below). Springer Nature Limited. Part **a**, **b** and **c** were adapted from REF[35], CC BY 4.0.

semipermeable membrane that allows the exchange of selective material such as nutrients and waste products (compartmentalization), (ii) contains a series of metabolic pathways for providing energy to the cell, making them self-sufficient, (iii) carries a chemical information system such as DNA or RNA (iv) is capable of undergoing growth and division cycles.

Recent developments in artificial cells indicate that it has become possible to reconstitute various biological systems *in vitro*, which exhibit lifelike features by assembling defined molecules [27]. For constructing an artificial cell, scientists have traditionally followed one of two approaches: a top-down approach and a bottom-up approach (Fig. 2) [26,28,29]. Both the top-down and bottom-up approaches seek to understand the minimal requirements for life.

3.1. Top-down approach

The top-down approach seeks to build a minimal cell from an already living system by genetic engineering of a preexisting natural organism (Fig. 2) [30]. The approach removes all cellular parts that are not essential for the cell's survival [31] and adds components for the desired functions. Top-down genome minimization requires the removal of nonessential contiguous genome stretches successively. In 2016, a team led by Craig Venter made a significant breakthrough through the generation of a minimal cellular genome using a top-down approach [32]. Using insights from transposon mutagenesis of a free-living bacterium, Mycoplasma mycoides (JCV-syn1.0), a minimized functional genome that includes 473 genes (530 kb) was generated. Notably, the genome contained 149 genes of unknown function, highlighting a drawback of top-down approaches in that the complexity is still high.

Knocking out genes one at a time is cumbersome. The challenge of developing artificial cells by replacing the biological cell's original genes with synthetic ones is enormous. As this review focuses on synthesizing novel materials as artificial cells, we will instead focus on bottom-up approaches.

3.2. Bottom-up approach

In contrast, the bottom-up approach constructs artificial cells from simple building blocks that can be either derived from living organisms or completely synthetic (Fig. 2). The theory behind the bottom-up strategy is that researchers can only truly understand how living materials operate if a cell can be made from scratch. Constructing a cell from the bottom-up approach will give us a deeper understanding of how biological processes interact with one another and may help define the minimal requirements for the "origin of life". The construction of a living cell from non-living materials will likely require three basic things: (i) Cell membranes, (ii) information-carrying molecules such as RNA/DNA (iii) a metabolism system [2,27]. The recent work discussed here highlights that bottom-up artificial cell synthesis is an active and growing field with contributions from diverse disciplines like synthetic chemistry and biochemistry.

Protocells have been proposed as an transitional intermediate between nonliving materials and the evolution of life, and generally consist of self-organized lipid structures. Scientists have constructed giant unilamellar vesicle (GUV) model protocells made up of newly designed lipids and macromolecules and we highlight some outstanding examples. The Kurihara group developed a self-proliferative GV-based model protocell that can self-propagate for three generations [33]. Their model created membranes composed of a confined number of phospholipids that encapsulate DNA and deoxyribonucleoside triphosphates

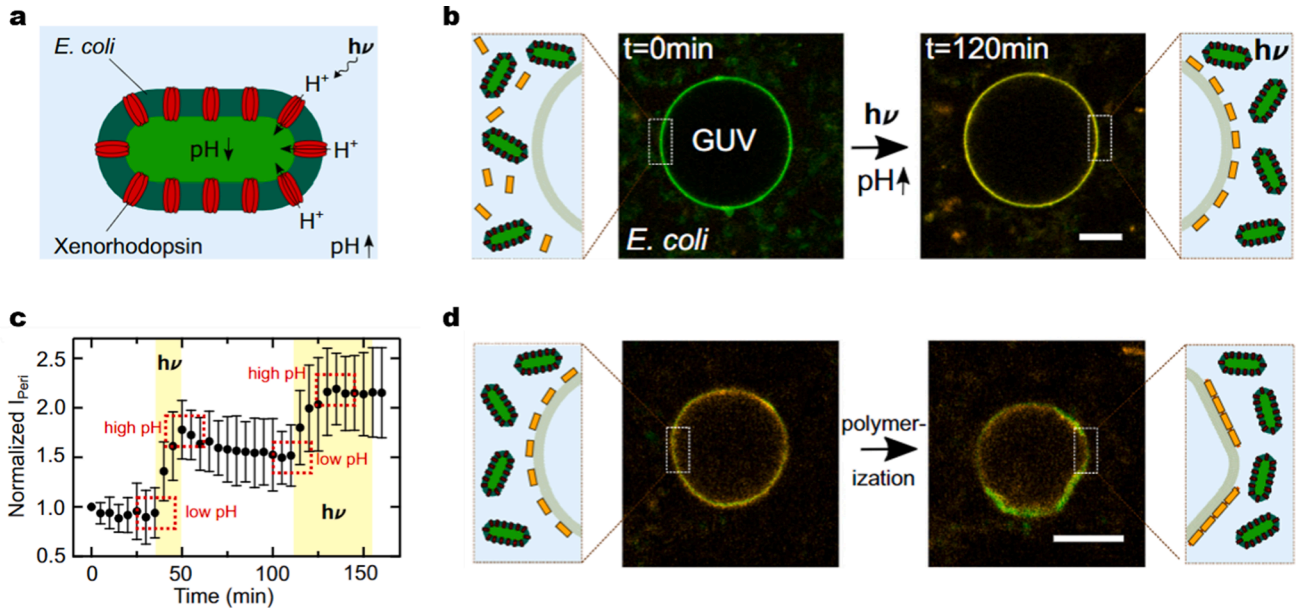

Fig. 4. Light-responsive *E. coli* triggers DNA origami attachment and deformation of giant unilamellar lipid vesicles, abbreviated as GUVs. (**a**) Schematic representation of an E. *coli* expressing xenorhodopsin, a light-driven proton pump (red), allows the pH-reversible changes. Upon illumination, Xenorhodopsin increases the pH in the extracellular environment. (**b**) DNA origami attachment. Confocal images of a GUV (λex = 488 nm, green) functionalized with cholesterol-tagged DNA immersed in a solution of *E. coli*. Upon light illumination, the pH-sensitive DNA origami (λex = 561 nm, orange) attached to the GUVs due to an increase in the pH (**c**) Quantification of the amount of DNA origami attachment. Normalized fluorescence intensity Iperi(mean ± s.d.,n = 11) of the triplex-forming DNA at the GUV boundary was measured over time. The time period of illumination is indicated in yellow color; light illumination causes an increase in the pH and therefore triggers DNA origami attachment. (d) DNA origami polymerization. Confocal images of a GUV (λex = 488 nm, green) after light-mediated DNA origami (λex = 561 nm, orange) attachment to the membrane and insertion of staple strands induce blunt-end stacking right), leads to the deformation of the GUV membrane within two hours (step ii). Scale bar: 10 μm. Springer Nature Limited. Part **a**, **b** and **c** were adapted from REF[41], CC BY 4.0.

(dNTP), which could be entirely divided symmetrically after adding a catalyst to the cells. Our lab recently discovered that oleoyl β-D-1-thiogalactopyranose (OTG) could spontaneously form highly stable micrometer-sized vesicles [34].

When OTG-vesicles were supplied with genetic material, they could successfully amplify DNA/RNA with a large membrane pore to let dNTPs (the building blocks for DNA) into the cell. We also devised a porous membrane artificial cell-mimic containing a nucleus-like DNA-hydrogel compartment capable of gene expression and communication through diffusive protein signals (Fig. 3) [35,36]. The artificial cell expresses a gene for green fluorescent protein (GFP) by simply adding the necessary components to the medium surrounding the cells. Several other groups have also created a limited version of synthetic life by using the bottom-up approach [37-40]. However, as discussed in Fig. 2, both the top-down and bottom-up approaches have pros and cons. Recently, Göpfrich and coworkers tried to merge the advantages of the two approaches to engineer a more complex artificial cell pathway by using bacterial light-driven proton pumps and DNA origami [41]. The authors genetically engineered modified light-harvesting bacteria (*Escherichia coli*) to overexpress the light-driven proton pump XeR (Xenorhodopsin) (Fig. 4a). Upon illumination of *E. coli* with white light, a proton gradient is induced across the membrane leading to acidification of the lumen (Fig. 4a). *E. coli* acts as a light-activated synthetic organelle to change the pH of internal and external cell compartments. Furthermore, the use of the proton gradient strategy was extended to DNA technology. A DNA strand sequence was first synthesized, which was then tagged with cholesterol, resulting in attachment to the edge of the cell compartment, observed microscopically. Later, the authors combined the DNA origami-based systems with genetically modified *E. coli* in a two-step process (Fig. 4b and 4d). In the first step, DNA origami monomers were attached to GUVs. In the second step, the DNA origami was polymerized on the GUV membrane, which changed the compartment mechanics and morphology. Here, *E. coli* mediates the attachment of DNA origami which was verified by tracking the normalized fluorescence intensity over time (Fig. 4c). As discussed, significant advancements have been made in creating analogs from synthetic and natural components using the bottom-up approach.

All living cells require a semipermeable boundary that allows only specific molecules or ions to pass through them, such as nutrients, waste, etc. A biological cell carefully controls these processes by using cell boundaries, usually in the form of lipid membranes with embedded proteins. Every living cell depends on lipid membranes to facilitate biological reactions and maintain a far-from-equilibrium state. Although biological cell membranes are very complex and hard to mimic, artificial cell membranes will be expected to possess some of the critical features of natural membranes.

Artificial membranes are built up to mimic the behavior of living mammalian cell membranes. Lipid membranes mostly consist of phospholipids, which are molecules that possess two hydrophobic "tails" derived from fatty acids, and a hydrophilic "head" containing a phosphate group. The key precursors for synthesizing phospholipids are monoacylglycerols and diacylglycerols [42,43]. Membrane-bound acyltransferases enzymatically generate phospholipids in the Kennedy lipid synthesis pathway [44]. Many scientists have speculated that the first living cells were composed of fatty acids [45,46]. Due to the restrictions of prebiotic chemistry, many protocell membranes utilize single-chain amphiphiles instead of phospholipids [47]. When unnatural lipid precursors are hydrated, they can assemble into biomimetic cell membranes. Chemically, it is possible to synthesize precursors with properties that are tuned by external stimuli such as temperature, concentration, and buffer solutions. This section discusses the synthesis and morphology of unnatural lipid compartments from simple synthetic precursors.

3.3. *De novo formation of phospholipid membrane*

Advances in synthetic chemistry and biology have enabled the *de novo* generation of synthetic phospholipid membranes [24]. Our group

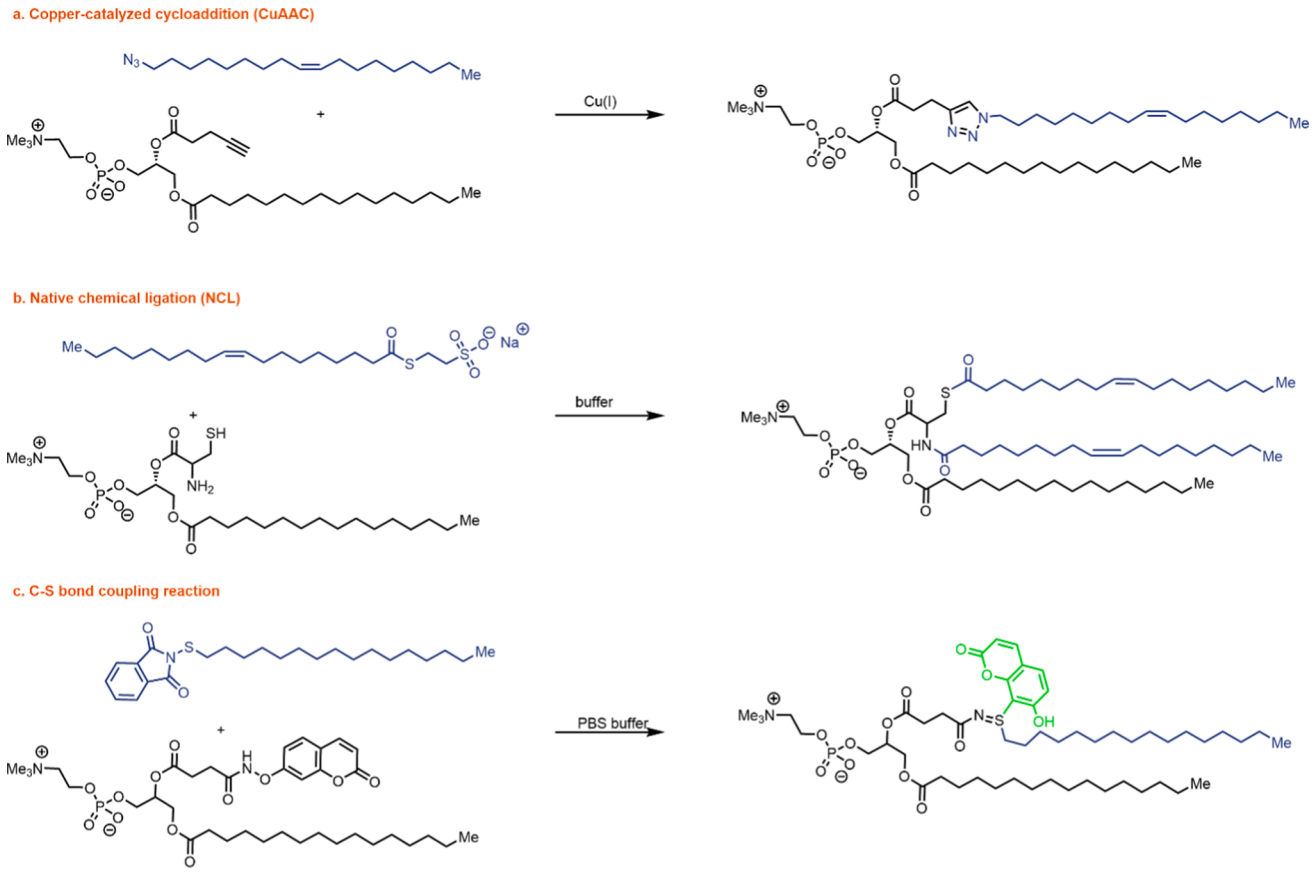

Fig. 5. De novo formation of phospholipids membranes. (**a**) CuAAC biomimetic coupling reaction forming triazole-involved phospholipid. (**b**) Synthetic phospholipid membrane formation by using the NCL approach. (**c**) Metal-free C–H sulfenylation reaction forming fluorogenic phospholipid membranes.

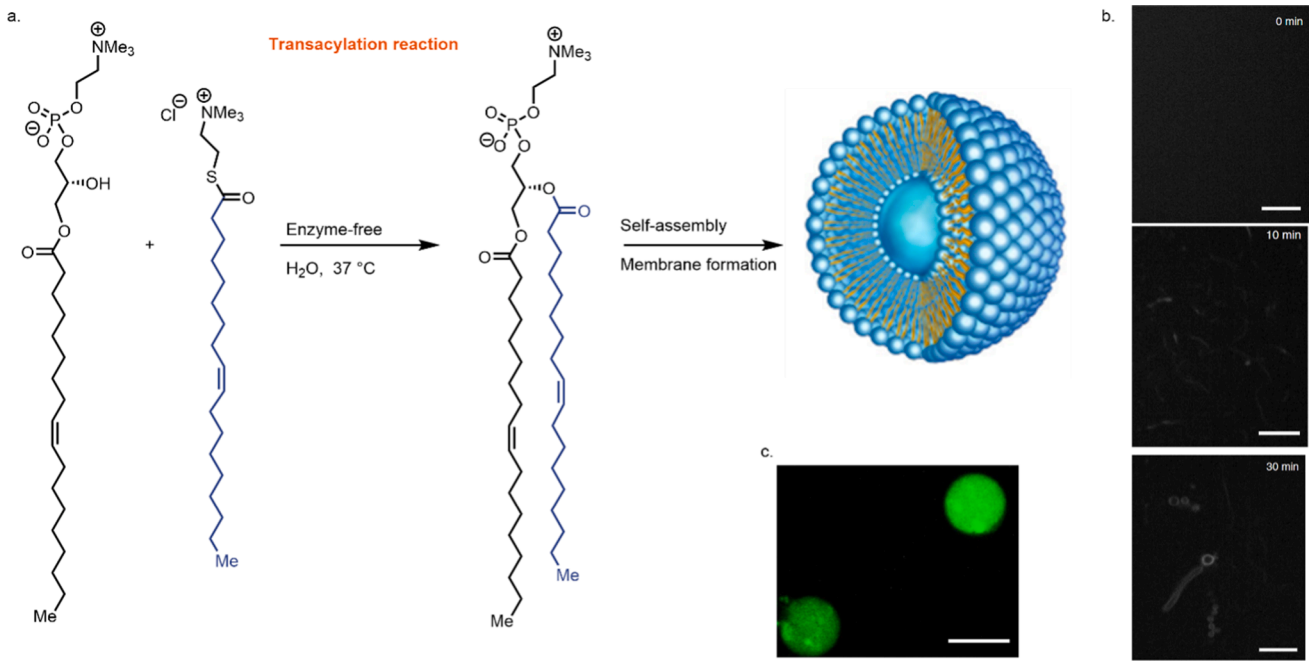

Fig. 6. Non-enzymatic synthesis of phospholipid membrane in water. (**a**) De novo synthesis of diacylphospholipids leads to an *in situ* self-assembled membrane formation. **Evolution of phospholipid membranes**: The reaction was carried out by mixing 1-oleoyl-2-hydroxy-*sn*-glycerol-3-phosphocholine (0.5 mm) and oleoylation reagent (0.75 mm) at 37 °C. (**b**) Fluorescence images are shown: Na_2CO_3/$NaHCO_3$ buffer was used as a solvent; samples were taken from the reaction mixture at different time points and were stained by using 0.1 mol% Nile red dye. Scale bars, 10 μm. (**c**) A fluorescence image of membrane-bound vesicles was observed after mixing of reactants in mono Lake water of pH = 10 containing the pH indicator dye HPTS, two hours after the vesicle media was changed to citrate buffer (pH = 4.6). Scale bar, 10 μm. Springer Nature Limited. Part **a**, **b** and **c** were adapted from REF[54], CC BY 4.0.

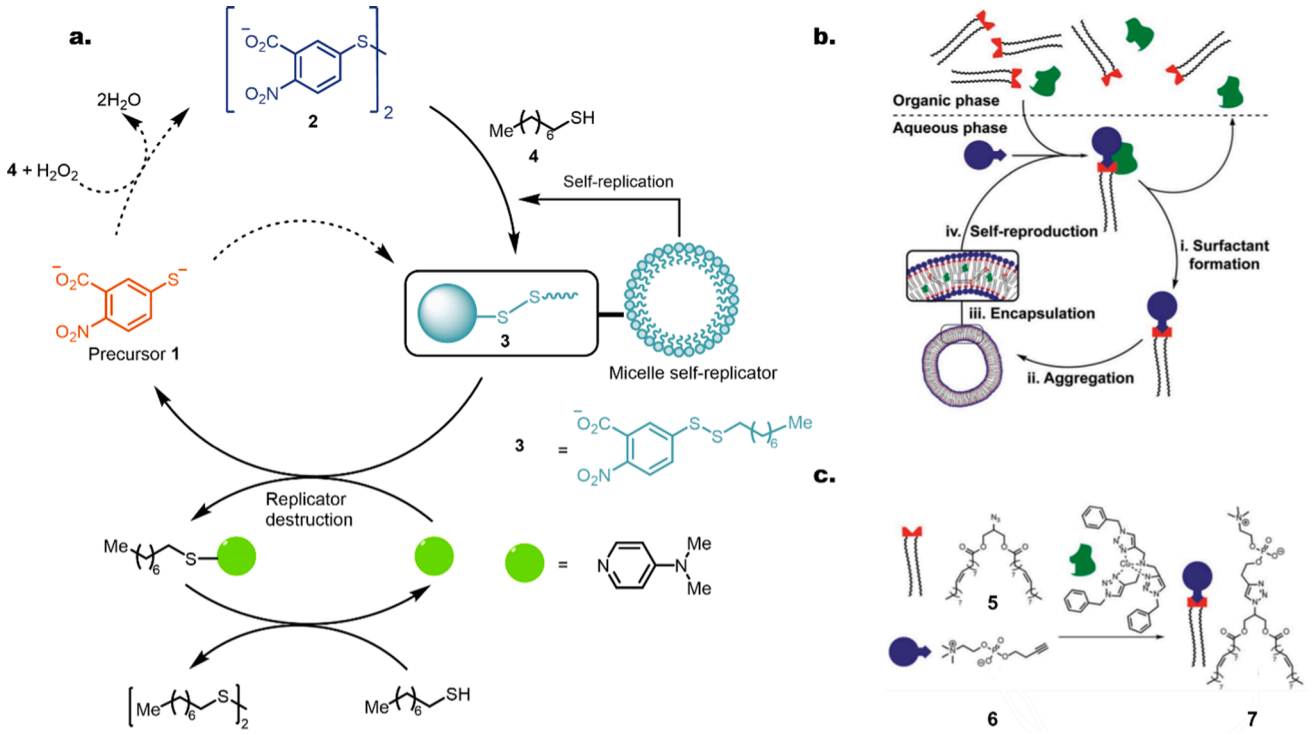

Fig. 7. The substrate cycle and reaction scheme for the autocatalytic formation of Micelles. (**a**) A simple chemical fuel, H_2O_2 (an oxidizing agent), regenerates the starting materials from waste byproducts, maintaining a simple metabolic cycle. Cutoff of the chemical fuel supply, H_2O_2, shifts the system towards thermodynamic equilibrium (dashed lines). (b) Simplified diagram of the mechanism of self-reproducing model. (c) Structural representations of the autocatalytic cycle. Part **b** and **c** were reproduced from REF[57] with permission from the Royal Society of Chemistry.

has been developing non-enzymatic bioorthogonal reactions to mimic how biological cell membranes synthesize lipids. In 2012, we took advantage of classical copper-catalyzed azide-alkyne cycloaddition (CuAAC) reaction to chemically synthesize phospholipids efficiently [48]. In the presence of a copper(I) catalyst, a simple charged alkyne-functionalized lysolipid and an oleyl azide couple to form a triazole-containing phospholipid analog. (Fig. 5a). Remarkably, the CuAAC biomimetic coupling reaction can also drive the *de novo* self-assembly of phospholipid membranes. Later, it was shown that a specific Cu(I) catalyst can catalyze its own replication so that the phospholipid membrane systems can grow indefinitely when supplied with azide and alkyne precursors [49].

Enomoto et al. added spatiotemporal control to phospholipid synthesis using an intramolecular photoinduced electron transfer process to generate copper(I) species from a photosensitizer dyad [50]. The active Cu(I) species triggers the CuAAC reaction and promotes biomimetic phospholipid membrane formation [50]. The use of redox-active copper ions causes oxidative damage to the biomolecules, such as cytotoxicity. Therefore, there is a huge demand for developing alternative strategies for forming artificial cell membranes.

The native chemical ligation (NCL), a two-step process, is one of the most popular methods for synthesizing peptides and proteins [51]. We demonstrated that NCL can be used to form membranes by coupling cysteine-functionalized lysolipids and charged thioesters in buffer to synthesize phospholipids (Fig. 5b) [52]. Upon ligation, the *de novo* formation and growth of the phospholipid membrane occurs. Later, a mild and metal-free C–H sulfenylation reaction employing an internally oxidizing O–N bond led to the formation of the first fluorogenic phospholipid membranes [53] (Fig. 5c). The reaction of non-fluorescent coumarin-functionalized lysolipid with a linear alkyl sulfenylation reagent at pH 7.4 gave a fluorogenic phospholipid that self-assembled into fluorescent vesicles *in situ*.

The previously mentioned examples utilize non-canonical phospholipids that are analogous but different from phospholipids found in living organisms. Recently, our group developed a chemoselective reaction for the *de novo* formation of natural phospholipid membranes under aqueous conditions in the absence of enzymes [54] (Fig. 6). During the reaction synthesis, the spontaneous formation of membrane-bound vesicles occurs. The transacylation reaction of lysophospholipids with acyl donors afforded natural diacylphospholipids in water in high yields (>80%) (Fig. 6a).

We used time-lapse fluorescence microscopy to observe the *de novo* formation of cell-like lipid membranes (Fig. 6b and 6c). No vesicle formation was observed when lysophospholipid or the oleyl thioester were incubated alone in Na_2CO_3/$NaHCO_3$ buffer solution. However, after mixing both, spherical vesicles were observed after 30 min. This approach results in the formation of phospholipid membranes without preexisting cell membranes and could be applied to generate cell-like lipid membranes.

An exciting new strategy for building up artificial cell membranes is dissipative self-assembly (DSA). Dissipative self-assembly requires the continuous uptake of energy (e.g. chemical fuels) and dissipates it into the environment to maintain an out-of-equilibrium assembled state [55]. Recently, Fletcher and coworkers developed a strategy that uses two non-assembling segments combined in the activation step to give an amphiphilic product that can self-assemble [56]. The oxidation of thiol derivative **1** drives the reaction cycle into their corresponding disulfide **2** at the expense of hydrogen peroxide (an oxidizing agent) as simple chemical fuel (Fig. 7a) [56]. The soluble precursor **2** reacts with 1-octanethiol **4** to afford a disulfide surfactant **3** (Fig. 7a). The amphiphilic surfactant then self assembles into micelles once the product concentration is higher than 0.2 mM. The deactivation reaction comprises a second thiol-disulfide exchange between 1-octanethiol, and the product **3** deactivates into the precursor **1** and forms octyl disulfide as waste. Overall, one molecule of H_2O_2 and two molecules of 1-octanethiol are combined to form the corresponding disulfide and water products.

Recently, the Fletcher group reported that CuAAC driven *de novo* membrane formation can form self-reproducing phospholipid vesicles

while maintaining a dissipative population of vesicles (Fig. 7b) [57]. The protocell system is generated from a phase-separated medium and organic, and aqueous building blocks operate via autocatalysis [60]. Hydrophobic azide **5** represents the organic phase that reacts slowly with hydrophilic alkyne modified phosphocholine **6** via a CuAAC reaction (step i, Fig. 7b). Above the critical micelle concentration (CMC) of the surfactant, self-assembly occurs, forming spherical vesicles (step ii). These vesicles behave as phase-transfer catalysts by taking azide **5** and facilitating the intermolecular interaction with modified alkyne precursor **6**. As a result, the catalyst accelerates the formation of triazole phospholipid **7**, closing the final autocatalytic step (step iv). In general, combining a minimal amount of building blocks generates a self-reproducing protocell model that shows several emergent life-like properties.

Artificial cells can have broad applications in many fields, from biotechnology to bio- and nanomedicine, such as drug delivery or medical imaging [58-60]. Each of these applications deserves a substantial evaluation, which is beyond the scope of this review.

4. Conclusion and future perspectives

Scientists from diverse fields are trying to design artificial cells using bottom-up approaches to answer one of the ultimate scientific questions in chemical biology: What is life? In this review, we have highlighted the remarkable progress that has been made in the rational design and construction of artificial cells and membranes in the last decade. The novelty of the framework needed to achieve the ambitious goal of building artificial life endeavors is an unprecedented challenge that requires, in our opinion, a better understanding of the interface between living and non-living materials. Researchers are approaching this problem by synthesizing artificial cells and developing coupling reactions that drive the self-assembly, growth, and reproduction of lipid vesicle assemblies. Creating living materials that are chemically different from extant life will be fascinating and lead to a myriad of applications in biology and medicine.

Looking forward to future perspectives, one must ensure that formed artificial cells have an effective metabolism to sustain biomimetic processes performed within the compartment and are able to replicate genetic information. In addition, implementing communication skills in non-living cells comparable to living cells would also open exciting avenues to collective behavior inspired by single-celled and multicellular organisms [61]. Undoubtedly, the creation of artificial cells would provide an exciting platform to study the evolution of life on Earth and answer fundamental questions in biology. Research on artificial cell-like materials helps bridge the gap between non-living and living materials and contributes to developing a unifying theory for the emergence of biology within a physical universe. Eventually, innovative design and tuning should provide a robust and well-integrated synthetic cell cycle that will be a major step towards an artificial cell that grows and divides autonomously.

Declaration of Competing Interest

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

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