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Review Article (Invited)

Concepts of a synthetic minimal cell: Information molecules, metabolic pathways, and vesicle reproduction

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How do the living systems emerge from non-living molecular assemblies? What physical and chemical principles supported the process? To address these questions, a promising strategy is to artificially reconstruct living cells in a bottom-up way. Recently, the authors developed the "synthetic minimal cell" system showing recursive growth and division cycles, where the concepts of information molecules, metabolic pathways, and cell reproduction were artificially and concisely redesigned with the vesicle-based system. We intentionally avoided using the sophisticated molecular machinery of the biological cells and tried to redesign the cells in the simplest forms. This review focuses on the similarities and differences between the biological cells and our synthetic minimal cell concerning each concept of cells. Such comparisons between natural and artificial cells will provide insights on how the molecules should be assembled to create living systems to the wide readers in the field of synthetic biology, artificial cells, and protocells research. This review article is an extended version of the Japanese article "Growth and division of vesicles coupled with information molecules," published in SEIBUTSU-BUTSURI vol. 61, p. 378-381 (2021).

Key words: artificial cell, origin of life, synthetic biology, protocell, lipid world hypothesis

Significance

Biological cells consist of the overwhelming accumulation of molecular machinery. Hence, how simply can we reproduce their essences? Such investigations are called "minimal cell" research, but the specificity of the authors' trial is avoiding the use of biological molecular machinery for the construction of cell-like simple reproduction systems, called "synthetic minimal cells." This approach is motivated by the intention to make clear the physical and chemical principles behind living systems by comparing natural and synthetic cells. This review explains the recently developed synthetic minimal cell by the authors together with several important concepts of biological cells.

Introduction

Living systems present diverse and complex images [1]. Although it is difficult to reach the general formulation of living systems [2,3], we can find several common natures among them at this stage, such as that (i) cells are the fundamental units of living systems and that (ii) cells take in materials from their surroundings and use the materials to generate copies of themselves. To investigate "what is life?" researchers are taking two major stances; first one tries to thoroughly unveil all the components constituting biological cells, and second one tries to seek several concise essences (or principles) underlying such complex systems. One of the most promising strategies for the latter is the construction

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of artificial model systems focusing on specific biological functions and structures, known as synthetic biology or artificial cell research [4-7]. In a popular approach in these fields, starting from a model compartment composed of amphiphiles (e.g., vesicles [8,9]), they try to reconstruct important biological behaviors by implementing various functional molecules and chemical reactions in the compartments.

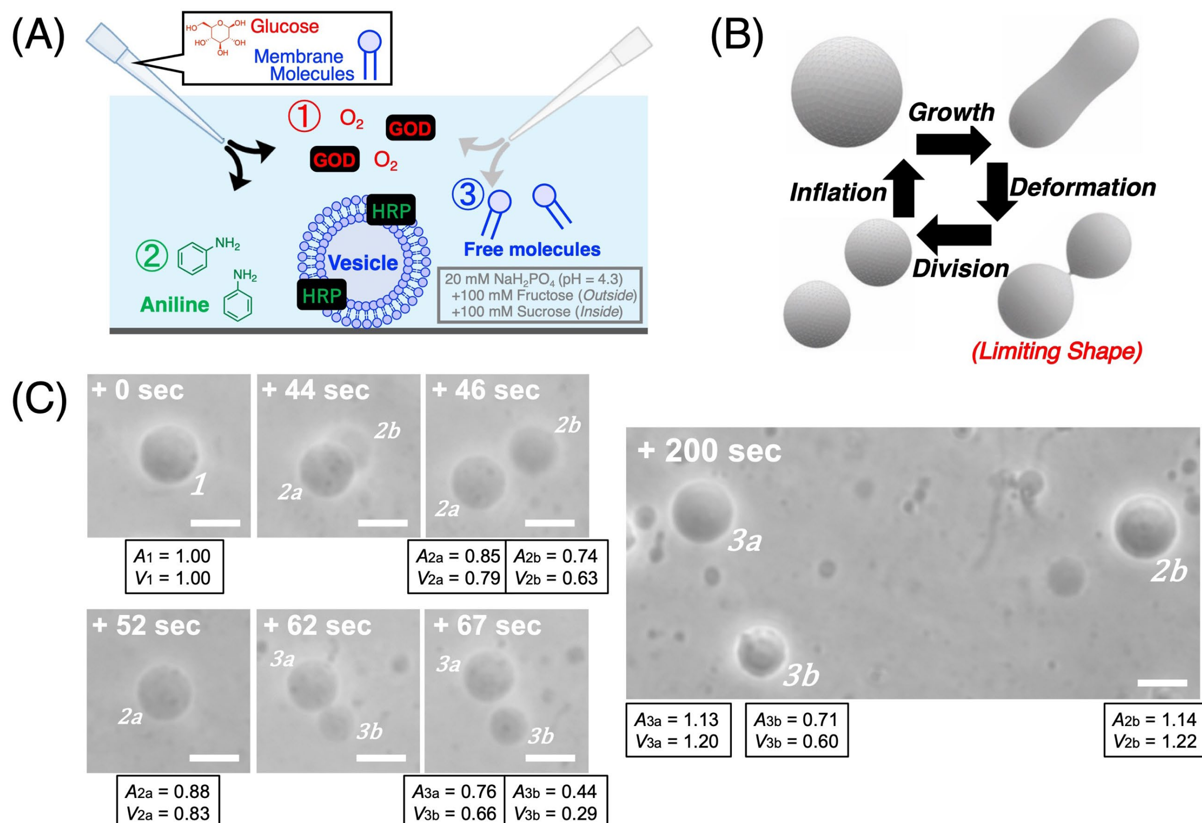

Figure 1 Our synthetic minimal cell system [14]. (A) Schematic of the experimental system. Binary bis (2-ethylhexyl) sulfosuccinate (AOT)/cholesterol (Chol) (9/1, mol) vesicles encapsulating 20 mM NaH_2PO_4 and 100 mM sucrose solution (pH = 4.3) was sunk in 20 mM NaH_2PO_4 and 100 mM fructose solution (pH = 4.3) containing 3.0 mM amphiphiles (as vesicles and free molecules), 4.0 mM aniline, 1.0 μM glucose oxidase (GOD), 0.92 μM horseradish peroxidase (HRP), and dissolved oxygen (O_2). When the solution containing 20 mM membrane molecules and 100 mM glucose were supplied as food molecules, production of H_2O_2 (red, 1), polymerization of aniline on the vesicle surface (green, 2), and subsequent uptake of vesicle-forming molecules (blue, 3) were triggered (see also Figure 3B). (B) Reproduction cycle in our synthetic minimal cell [7]. (C) Phase contrast microscopy images for the reproduction of the binary AOT/Chol (9/1, mol) vesicles (membrane growth, deformation, division, and volume growth) coupled with the artificial metabolic pathways (Figure 3B), i.e., our synthetic minimal cell [14]. The membrane growth was promoted by the artificial information molecule, PANI-ES, localized on the vesicle surface. The PANI-ES was synthesized in the enzymatic cascade reaction, called artificial metabolic pathways, in response to the external supply of ingredient molecules. The vesicles with growing membranes spontaneously deformed and divided themselves according to their membrane elastic properties. The volume growth was triggered by the asymmetric permeation of saccharides and the subsequent osmotic pressure difference. Here, the mother vesicle (#1) produced two daughter vesicles (#2a and #2b). One of the daughter vesicles (#2a) produced two granddaughter vesicles (#3a and #3b), while another daughter vesicle (#2b) was floated away. After the production of the granddaughter vesicles (#3a and #3b, ~70 sec), the external supply of ingredients ceased to wait for the volume recovery. A_i and V_i in each panel denote the surface area and volume of each vesicle normalized by the values of the initial mother vesicle (#1). Length of the scale bars: 10 μm. This figure is taken from Kurisu, M. et al. *Commun. Chem.* 6, 56 (2023); open access article.

One of the unique properties of living systems is their ability to reproduce. A traditional approach for self-reproducing artificial cells involves the encapsulation of DNA replication and protein expression systems in a lipid vesicle, which

aims to reconstruct the reaction pathways that synthesize vesicle-forming amphiphiles using proteins expressed in DNA [10-12]. Such vesicle-based systems will facilitate reproduction. However, understanding the emergence of living systems from non-living molecular assemblies still remains challenging because this approach begins from the central dogma of molecular biology, which is an already biological system. An alternative approach, starting from the matter side, is to synthesize the simplest reproduction systems that show essential behaviors of life in an artificial design. For example, vesicles should reproduce themselves based on their information polymers and metabolic pathways in different ways than biological ones [7,13]. Then, by looking for similarities and differences between complex biological systems and artificially designed model systems, we will deduce the physical and chemical principles constituting essential concepts of living systems.

Recently, we developed a synthetic model cell system, redesigning the following fundamental concepts in concise and artificial ways: information molecules, metabolic pathways, and a reproduction cycle [14,15]. These concepts were implemented in a vesicle, and the vesicle showed the reproduction coupled with the artificial information molecule, emeraldine salt form of polyaniline (PANI-ES). Here, we briefly introduce the experimental setup (Figure 1A). A vesicle composed of bis (2-ethylhexyl) sulfosuccinate (AOT) and cholesterol (Chol) encapsulating sucrose solution is sunk at the bottom of a chamber filled with fructose solution. The bulk solution further contains three groups of molecules for an artificial metabolism: the molecules for producing energy molecules that trigger the downstream reactions (red, 1), the molecules for synthesizing specific polyaniline (PANI-ES) that functions as the information molecule for the vesicle reproduction (green, 2), and the free membrane molecules in the bulk solution (blue, 3). By continuously supplying membrane molecules and glucose as food molecules by the micro-injection technique, the artificial metabolic pathways consisting of the above three subunits are triggered coupled with the synthesis of the artificial information polymer (PANI-ES). Simultaneously, the vesicles incorporated free membrane molecules with the help of PANI-ES localized on the vesicle surface, resulting in the reproduction cycle of vesicles (i.e., membrane growth → deformation → division → volume recovery [7]; Figure 1B) over several generations (Figure 1C) [14]. This "synthetic minimal cell" system is artificially and concisely designed compared to biological cells. Therefore, the reaction pathways and the vesicle deformation pathways are quantitatively described well by the kinetic model and the membrane elasticity model [14].

In this review, we provide an overview of the concepts behind our synthetic minimal cell compared with the counterparts in biological cells, including information molecules, metabolic pathways, membrane and volume growth of cells, and cell division. The methodology and construction strategy used to achieve such an artificial vesicle reproduction system will contribute to the broad fields of bottom-up biophysical studies and possibly to the mystery road from matter to life.

Concept of Information Molecules

Biological cells have lipid membrane compartments (e.g., cell membranes) that separate the inside and outside of the cell, defining individual cells. The membrane compartments contain internal cellular structures, such as organelles composed of lipid membranes and cytoskeletons made of protein filaments [1,16]. The chemical reactions that maintain such cellular structures are regulated by catalytic proteins (=enzymes), and the sequences of 20 amino acid species are recorded as the sequences of four base pairs in DNA via RNA (the central dogma of molecular biology). Thus, from the viewpoint of the reproduction of cellular structures, genetic information flows as DNA → RNA → enzymes (proteins) → membrane compartments in biological cells. We call DNA the "information molecule" of biological cells since DNA encodes structures of enzymes through which metabolic pathways and synthesis of cellular components are regulated.

Notably, in the translations and transcriptions of the sequence information, the synthesis of each biopolymer is strongly regulated to form a specific sequence based on the template sequence; otherwise, non-coding random sequences are obtained where no genetic information is transferred (Figure 2A). The essence of such template polymerization processes [17] lies in the formation of specific hydrogen bonding, which uniquely designates a base or an amino acid linked to the elongating site of a biopolymer [18]. Here, we can find the trade-off between entropy and enthalpy. In biological cells, the enthalpy gain (ΔH) due to the formation of hydrogen bonds compensates the entropy loss (ΔS) by selecting only one base or amino acid out of four or twenty (i.e., $\Delta H - T\Delta S \leq 0$) [18]. Consequently, the genetic information is successfully transferred among DNA, RNA, and proteins.

Artificial cells and the origin of life research have focused on (i) information molecules (DNA and RNA) that are located upstream of the information flow and govern compartmentalized chemical reactions and (ii) a membrane compartment (i.e., vesicle) that is located downstream of the information flow and defines an individual cell [4,7,19-21]. Based on the two essential components of cells, researchers in these fields have tried to adopt the following principal strategy for synthesizing cells [19]: first develop self-replicating information molecules and self-reproducing vesicular compartments, and then try to integrate the two components into a single self-reproducing cellular system (i.e., an artificial cell or protocell). Since this strategy has not been successful yet using "biological" information molecules, the authors worked on an "artificial" system to integrate information polymers and vesicles; the direct connection of an upstream information

molecule and a downstream membrane compartment by utilizing a membrane-templated polymerization reaction (Figure 2B) [22,23], where vesicle surface guides the polymerization reaction in a regioselective way. This contrasts with biological systems that utilize polymer-templated polymerization reactions, such as DNA-DNA replication and DNA-RNA and RNA-protein transcriptions. Therefore, our artificial information molecule, polyaniline emeraldine salt (PANI-ES), directly encodes and decodes the information of vesicles, enabling the extreme simplification of the essences of metabolism (see the next section).

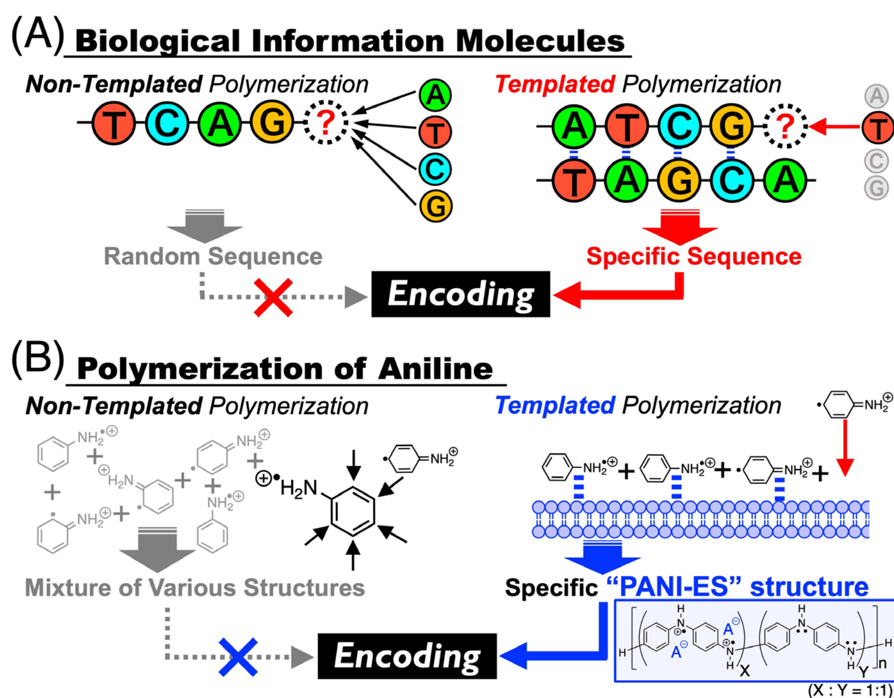

Figure 2 Concepts of biological and artificial information molecules. Template structures restrict the polymerization reactions to specific directions, encoding the information of the templates. (A) In the replication of DNA sequences, polymerization reactions that are restricted by specific hydrogen bonds result in the formation of a specific sequence, where the sequence information of the template DNA is encoded. (B) Synthesis of our artificial information molecule, PANI-ES. Polymerization of aniline radical cations without any template macromolecules leads to extensively branched polymeric compounds. In contrast, in the presence of vesicles composed of amphiphiles with sulfate/sulfonate head groups, such as AOT, the vesicle surface works as a template for guiding polymerization reaction to form regioselective product relevantly: linear polyaniline in its emeraldine salt form (PANI-ES) [14,22]. Blue "A" beside the PANI-ES structure represents a counter ion (AOT). The presence of a composite structure between PANI-ES and membrane molecules on the vesicle surface selectively promotes the incorporation of membrane molecules from bulk solution into the vesicle membrane, see also "Concept of Growth" section.

As a promising membrane-templated polymerization system that directly links information molecules and vesicles, we focused on the AOT vesicle surface-confined polymerization of aniline (Figure 2B) [22,24,25]. In aqueous media without template macromolecules, the enzymatic oxidation of aniline leads to the production of aniline radical cation, and their non-guided polymerization results in the formation of extensively branched polymeric products with various chemical structures and oxidation states. These products are hard-to-characterize aggregates, and thus, little is known about them, more than they are polymeric mixtures [26,27]. However, when aniline molecules are oxidized in the presence of macromolecules containing sulfate/sulfonate structures, the polymerization reaction becomes regioselective, and half-oxidized and half-reduced linear polyanilines with delocalized unpaired electrons (i.e., polarons) are dominant [22,24,28]. This specific polyaniline is called PANI-ES and has been extensively studied in materials science due to its electroconductive properties [29-31]. The vesicles composed of AOT, which has a sulfate structure in its hydrophilic head group, can serve as template macromolecules for PANI-ES synthesis. The formation of specific hydrogen bonds, electrostatic interactions, and steric interactions between monomers and the AOT vesicle surface [32-34] greatly influences the polymerization process, leading to the regulation of the polymerization reaction to synthesize PANI-ES as the dominant structure.

Analogous to the genetic information transfer in biopolymers, which can pay the enthalpy cost from the interaction between the monomer and the template [18], the regioselective polymerization of aniline results in the formation of the entropically unfavorable specific product, PANI-ES (i.e., linear head-to-tail linkage of aniline is dominant [14,22]). Here, the formation of PANI-ES reflects the property of the template vesicles (i.e., the sulfate/sulfonate hydrophilic head groups) in its regioselective polymer structure, which is an encoding of the information of the vesicles. Notably, such regular sequenced polyaniline (PANI-ES) gives positive feedback to the template vesicles; PANI-ES on the vesicle surface encourages the selective uptake of free membrane molecules from bulk solution to the vesicle membrane and makes the vesicle grow (see "Concept of Growth" section) [14,15]. Of course, our artificial information molecule, PANI-ES, is far inferior to the biologically sophisticated information molecules, DNA and RNA. However, in terms of encoding the information in polymers by compensating entropy loss with enthalpy gain and in terms of expressing the functions for reproducing membrane compartments, PANI-ES in our system artificially and concisely realizes the concept of information molecules for vesicles.

Concept of Metabolic Pathways

Biochemical pathways in living systems are diverse and complex. To understand the concept of metabolism, the stems and branches of metabolic pathways should be clarified. Essential metabolic pathways have been investigated in various approaches. As a pioneering theoretical approach, Gánti proposed the "chemoton" (=chemical + automaton) model [35] suggesting that the simplification of chemical pathways of living systems will result in only the three essential autocatalytic cycles; the one producing biomembranes (membrane cycle), the one producing genetic polymer (polymer cycle), and the one catabolizing ingredient molecules and producing precursor molecules for driving the above two cycles (metabolic cycle). In the approach from molecular biology, at least approximately 250 genes are currently considered necessary [36-38]. For example, the "essential" genes have been reported based on whether their loss is lethal to *S. sanguinis* [38], one of the simplest known bacteria. Such essential genes were associated with three fundamental biological functions; the energy production unit which synthesizes energy molecules from glucose taken up from the environment, the processing of the genetic information unit which relates to the replication of DNA and protein synthesis via RNA, and the maintenance of cell envelope unit which synthesizes membrane forming lipids from the byproducts of energy production. Furthermore, in a recent synthetic biology approach, a minimal set of cellular systems is being investigated. The "JCVI-syn" series of minimal cells are known as one of the trials to develop biological cells with the minimal genome. By characterizing the 452 protein-coding genes in their biological minimal cell (JCVI-syn3A), the genes were classified into three primary functional units; genetic information processing, metabolism, and cellular processes units, except 91 unclear genes [39].

Based on such trials to understand the essence of metabolism, one can draw a rough picture overviewing the three stem concepts of metabolic pathways in biological systems (Figure 3A) [14]; from the ingredient molecule glucose, cells harvest energy molecule ATP during glycolysis (orange). Energy molecules are used with nucleosides to replicate the information molecule DNA and are also used with amino acids to synthesize proteins (enzymes) based on genetic information (green). In the downstream processes, using energy molecules with byproducts of glycolysis (glycerol 3-phosphate and pyruvates), lipid molecules are synthesized with the help of enzymes. Lipid molecules are inserted into the cell membrane (blue), and the cells proliferate.

This conceptual understanding of biological metabolic pathways allowed the authors to design artificial metabolic pathways for synthetic minimal cells (Figure 3B) [14,15] using the experimental setup shown in Figure 1A. In our system, to circumvent the difficulties in trans-membrane molecular transportations, the metabolic pathways were developed on the outer surface of vesicles. In the energy production unit (orange, 1), externally supplied ingredient molecules glucose were consumed to produce energy molecules for this system, oxidant hydrogen peroxide H_2O_2, with the help of enzyme glucose oxidase (GOD). The H_2O_2 production unit was linked to the information molecule synthesis unit (green, 2) through enzymatic cascade reactions [40]. In comparison with the environmental pH (4.3 in 20 mM NaH_2PO_4), the pK_a value of the anilinium cation is 4.6 (i.e., ~67% of aniline molecules were present as cations), and the isoelectric point (pI) of the enzyme horseradish peroxidase (HRP) is 8.8 [22]. Thus, the reaction occurred near the surface of the negatively charged vesicles [22,24]. Here, aniline molecules were activated to the reactive aniline radical cations, consuming the energy molecules H_2O_2 with the help of HRP. Subsequently, in the presence of the template effect due to the interaction with the AOT vesicle surface, aniline radical cations are non-enzymatically polymerized to form a specific form of polyaniline, PANI-ES, which is characterized by the presence of specific delocalized unpaired electrons with absorption spectrum measurements, electron spin resonance measurements, and Raman spectrum measurements [14,15,22,40]. The local formation of PANI-ES on the AOT vesicle surface was confirmed by the micro-Raman mapping technique [14]. Notably, when the vesicles are composed of amphiphiles that do not have sulfate/sulfonate structures, they do not guide the polymerization of aniline [15,24]; hard-to-characterize polymeric mixtures are produced, as in polymerization in the absence of any template vesicles. In addition, the PANI-ES structure is also formed when other sulfate/sulfonate vesicles,

such as binary sodium dodecylbenzene sulfonate (SDBS)/decanoic acid vesicles and sulfatide vesicles, are used as templates [15,28]. Thus, the polyaniline reflects the characteristic properties of the template vesicles (i.e., the sulfate/sulfonate head group) as the PANI-ES structure (=encoding of membrane molecules). The interaction between the sulfate head group of AOT and PANI-ES now binds the dispersed free AOT molecules, which selectively encourages their incorporation and the subsequent membrane growth of the template vesicle (=decoding of membrane molecules); membrane growth unit (blue, 3; see also "Concept of Growth" section). The increase in the vesicle surface area provides a further reaction field for PANI-ES synthesis, i.e., mutual catalysis between the template vesicle and the artificial information molecule, PANI-ES. Consequently, exponential vesicle membrane growth was achieved [14]. The overall reaction network is concise compared to biological systems; therefore, the synthesis of PANI-ES and vesicle membrane growth were quantified and well-described by the kinetic model of artificial metabolism, considering only five primary model reactions [14].

Based on our understanding of the concepts of stem metabolic pathways, we artificially shortened and redesigned metabolism to directly link the upstream information molecule to the downstream vesicle. Although biological metabolism uses ATP as an energy molecule, our system utilizes H_2O_2 as an energy molecule, which is an oxidant driving the downstream reaction steps. Furthermore, while biological metabolisms separate the biopolymers for storing genetic information (DNA) from the biopolymers for catalyzing chemical reactions (proteins), our artificial information molecule (PANI-ES) stores the compositional information of template vesicles and simultaneously shows catalytic activity to encourage membrane growth of the template vesicle, which helped simplify the artificial metabolic pathways. However, a considerable drawback occurred in our artificial metabolic pathways for redesigning biological pathways: vesicle-forming molecules were not synthesized. Biological cells synthesize membrane molecules, such as phospholipids, using complicated reaction pathways with the help of various catalytic proteins. The obtained membrane molecules are then incorporated into the cell membrane, resulting in the growth of the cell membrane (Figure 3A). Reluctantly, in our artificial metabolism, the catalytic activity of PANI-ES was limited for incorporating membrane molecules. Instead, we supplied the membrane molecules as finished products. Coupled with other concepts of growth and division (see below), our artificial metabolic pathways resulted in the reproduction of vesicles, i.e., a synthetic minimal cell (Figure 1C) [14].

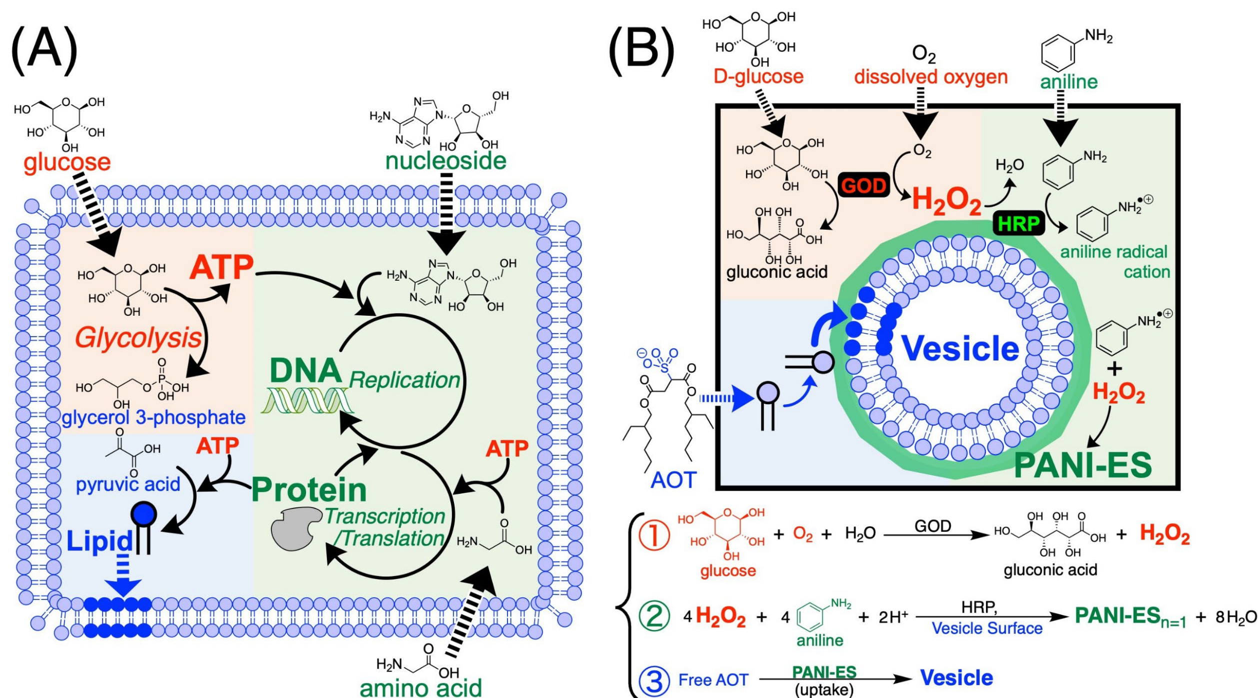

Figure 3 Concepts of biological and artificial metabolic pathways [14]. (A) Rough view of biological metabolism emphasizing three stem units by color; energy production (orange), processing of genetic information (green), and synthesis of membrane molecules (blue). This figure is taken from Kurisu, M. et al. *Commun. Chem.* 6, 56 (2023); open access article. The size of characters was enlarged. (B) Rough view of our artificial metabolic pathways consisting of three units; energy production (orange, 1), synthesis of information polymer (green, 2), and membrane growth (blue, 3). The dashed arrows represent the reactants (ingredients) supplied close to the vesicle. For experimental setup, see Figure 1A.

Concept of Growth

Living systems are self-reproducing compartment systems. Therefore, the above-mentioned concepts of information molecules and metabolic pathways must result in the growth and division of the compartment. Here, we classified the concept of growth into two sub-concepts: growth of the vesicle membrane and growth of the encapsulated volume.

How do the compartmental membranes grow in biological systems? The growth of cell membranes, consisting mainly of phospholipids, is primarily realized by two processes [1]: fatty acids synthesis in the cytosol and phospholipid synthesis in biomembranes. Glycolysis is the essential metabolic pathway in the cellular systems, which produces energy molecules ATP by catabolizing food molecules glucose (Figure 3A; orange). Notably, glucose also works as a carbon resource for synthesizing membrane molecules. Glycerol 3-phosphates and pyruvates are obtained as byproducts of glycolysis. Pyruvates are converted to acetyl-CoA and, subsequently, to long-chain fatty acids in the cytosol. Long-chain fatty acids are hydrophobic molecules that instantly aggregate in aqueous media. Therefore, native long-chain fatty acids cannot be transported to biomembranes, which are the sites of phospholipid synthesis using fatty acids. To circumvent this problem, the hydrophobic chains of fatty acids in biological cells are masked by fatty acid-binding proteins, which decrease molecular hydrophobicity and disperse the fatty acids in the cytosol (Figure 4A) [1,41]. After being transported to the endoplasmic reticulum membrane, fatty acids are activated by CoA in the presence of CoA transferase, followed by the addition of two fatty acids to glycerol 3-phosphate using acyl transferase. The phosphatidic acids obtained are sufficiently hydrophobic to remain in the lipid bilayer and cannot be extracted by fatty acid-binding proteins. The chemical modification of the hydrophilic head group of phosphatidic acids leads to the formation of various phospholipids [1]. Biochemical details and enzymology in phospholipid synthesis are different between eukaryotic cells and *E. coli*, and also between *E. coli* and other bacterial cells [16,42]. Nevertheless, we can find several common strategies among them; fatty acid synthesis is completed in cytosol, their hydrophobic parts are masked for the transportation to biomembranes, and finally the conversion to phospholipids occurs on the biomembranes.

In terms of the uptake of membrane molecules, our growing vesicles decrease the molecular hydrophilicity of AOT by masking its polar head group (not hydrocarbon chains) in the vicinity of the vesicle surface [14,15]. A plausible mechanism is as follows (Figure 4B): In the absence of PANI-ES, the dispersed AOT molecules in the bulk solution are in equilibrium with the bilayer-forming state with the critical vesiculation concentration of ~1.5 mM in 20 mM NaH_2PO_4 solution (pH = 4.3) [15]. PANI-ES synthesized on the vesicle surface attracts the sulfate head group of AOT, which binds the free AOT molecules in the bulk solution onto the vesicle surface-bound PANI-ES. In this bound state, the negatively charged polar head groups of the AOT molecules are masked from the surrounding aqueous media, resulting in a decrease in molecular hydrophilicity. This encourages the uptake of AOT molecules into the vesicle bilayer, i.e., vesicle membrane growth.

Importantly, PANI-ES seems to selectively promote vesicle growth through the specific interaction between AOT and PANI-ES; only amphiphiles with sulfate/sulfonate head groups, such as AOT and SDBS, have been confirmed to be incorporated [15]. In our system, polyaniline reflects the properties of the template vesicle (sulfate/sulfonate head group) in its structure (PANI-ES), i.e., encoding of vesicle composition. As decoding, PANI-ES encourages membrane growth of the vesicle with the same molecular species (i.e., sulfate/sulfonate) encoded in PANI-ES. This mutual catalytic relationship between the information molecule and the template vesicle is the heart of our synthetic minimal cell system.

The membrane growth mechanisms of biological and our artificial system share a fundamental concept: the hydrophilicity of the membrane molecules is controlled near the vesicle surface for their incorporation into membranes. In biological cells, the molecular hydrophilicity of fatty acids is regulated by the masking and unmasking of hydrophilic acyl chains. Our artificial system also regulates molecular hydrophilicity but takes a different approach: the polar head groups of the native membrane molecules are masked on the vesicle surface. In biological cells, fatty acid molecules are synthesized in the cytosol before incorporation and then converted to more sophisticated membrane molecules (phospholipids) after incorporation. We did not address such complex processes; instead, we focused only on the incorporation process, which is the least essential process for membrane growth.

To reproduce cellular systems, we need to increase the encapsulated volume in addition to the membrane surface. Biological cells encapsulate high densities of both membrane-permeable and impermeable molecules. The osmolarity inside their membrane compartments is approximately 300 mOsm/L [43]. Biological cells increase their volumes by passive water influx due to the osmotic pressure difference [44] while maintaining their internal molecular densities (Figure 4C).

To artificially and concisely redesign this volume growth concept, we adopted an asymmetric permeation strategy [45,46]. We contained the disaccharide sucrose inside the vesicle at the same concentration as the monosaccharide fructose in the external aqueous media (Figure 4D) [14]. Monosaccharides pass through the AOT bilayers more easily than disaccharides. Therefore, such a setup results in asymmetric permeation and an almost one-way diffusion of saccharides from the outside to the inside of the vesicle. Consequently, even though the outside and inside solutions are initially isotonic, the total osmolarity of the encapsulated solution becomes slightly higher than that of the environment over time,

generating a long-term passive water influx. This vesicle swelling is not accompanied by chemical reactions. Our approach utilizes the mixing entropy as the driving force; the molecular diffusion between the inside and outside of the vesicle membrane increases entropy of the system from the initial state where fructose and sucrose are completely separated by the membrane. The free energy stored in the vesicle ($T\Delta S$) is converted to the work pushing the vesicle membrane by an osmotic pressure difference ($p\Delta V$). As the mixing of the two saccharide species proceeded, the volume growth became slower since the increase in the mixing entropy per time became suppressed. Eventually, the synthetic minimal cells stopped increasing in volume. Suppose we can contain an anabolism pathway that synthesizes disaccharides from the monosaccharides diffused to the inside of the vesicle. In this case, we will be able to fully redesign the same volume growth concept as the biological one.

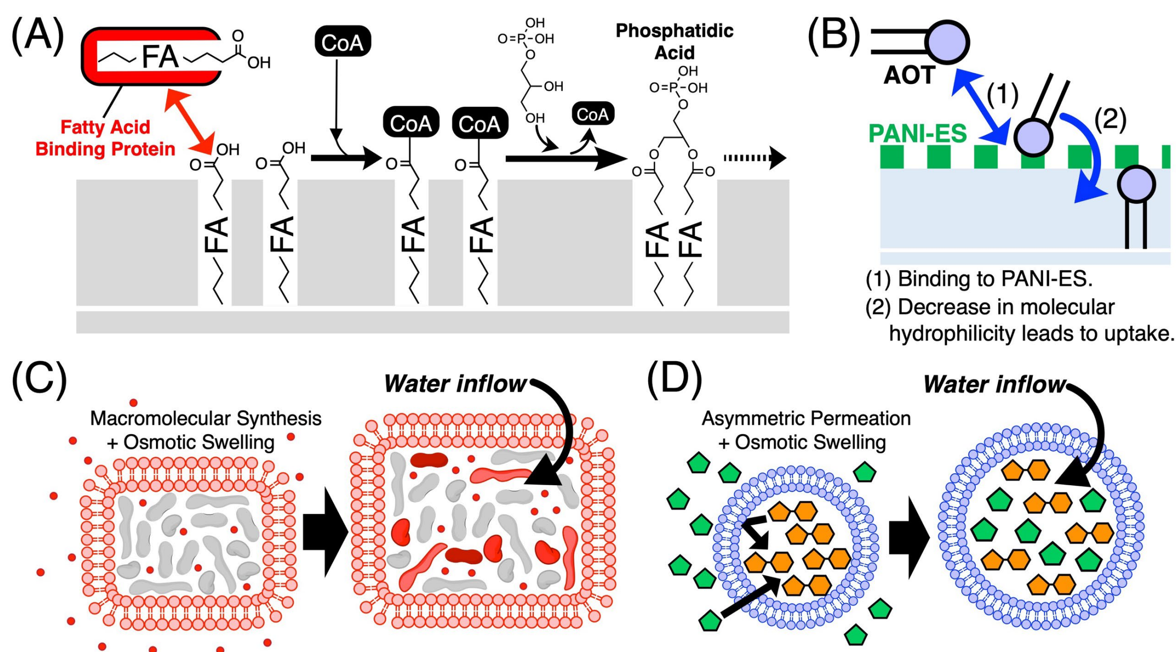

Figure 4 Concepts of membrane growth and volume growth in biological cells and in our synthetic minimal cell. (A) Schematic of phospholipids biosynthesis from fatty acids at the endoplasmic reticulum [1]. (B) Schematic of the plausible mechanism for AOT molecules to be incorporated into the vesicle membrane covered by PANI-ES. (C) Schematic of volume growth of biological cells. Grey objects inside the membrane stand for membrane-impermeable biological macromolecules. Red particles and red objects stand for ingredient molecules and membrane-impermeable macromolecules newly synthesized using the ingredients. (D) Schematic of volume growth of our synthetic minimal cell. Green pentagons represent monosaccharide fructose, and orange pairs of a hexagon and a pentagon represent disaccharide sucrose. The initial concentrations of saccharides are 100 mM in 20 mM NaH_2PO_4 solution (pH = 4.3) in our synthetic minimal cell. In contrast to biological cells, monosaccharides incorporated from the environment into the inside of a vesicle are not converted to macromolecules.

Concept of Division

Cell division is one of the most dynamic events in cells [47,48]. Most prokaryotes use a protein known as FtsZ for their cell division, recruiting at least ten other proteins. After most of the chromosome has been duplicated and segregated into the cell poles, FtsZ localizes to the cell midpoint and assembles into the Z ring (Figure 5A) using spatiotemporal regulation mechanisms. Then, with the help of other membrane-associated proteins, the contraction of the Z ring exerts a pinching force on the membrane, which synthesizes the division septum. The further contraction of the Z ring and the invagination of the septal wall result in the production of two separate daughter cells. Thus, the division of (bacterial) cells is driven by a sophisticated ATP-consuming mechanism. Recently, several bottom-up reconstruction approaches successfully reproduced the formation of contractile rings inside lipid compartments [49,50]. Nevertheless, only slight deformation of the compartments was observed using this division strategy. If we quit adhering to the sophisticated protein machinery, what other more uncomplicated strategies will there be?

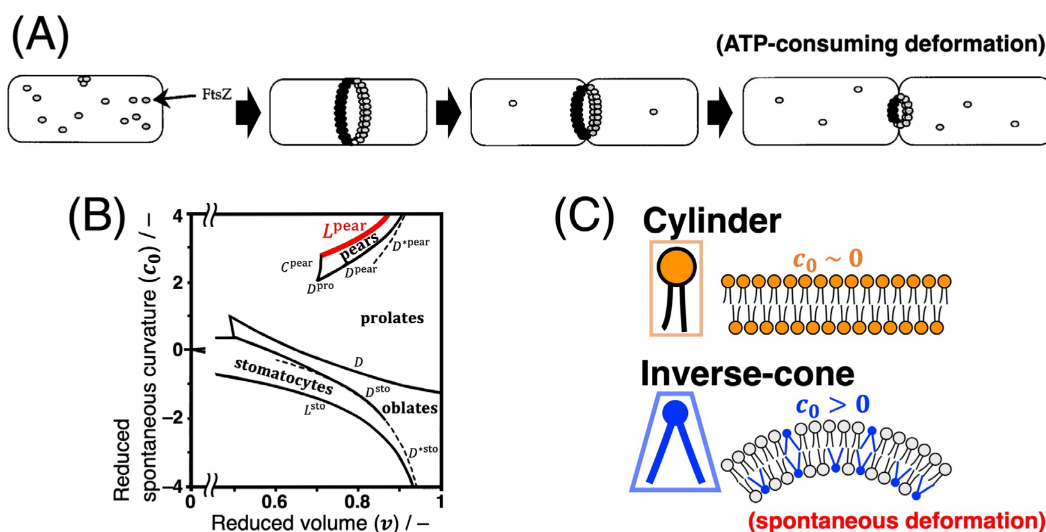

Figure 5 Concepts of cell division in biological cells and our synthetic minimal cell. (A) Behavior of FtsZ during the cell division cycle. During division, the Z ring decreases in diameter at the leading edge of the septum. (Reprinted with permission from Lutkenhaus, J. & Addinall, S.G. *Annu. Rev. Biochem.* 66, 93–116 (1997); Copyright 1997 Annual Review Inc.). (B) Phase diagram of stable vesicle shape based on the simulation study using the spontaneous curvature model [52]. Limiting shape vesicles appear on the red L^{pear} branch. The left end of the branch locates at $(v, c_0) = (1/\sqrt{2}, 2\sqrt{2})$, where the two vesicles with the same size are connected by the neck. Going upper right along the L^{pear} branch, the size difference between the two vesicles is enlarged. (C) Schematics of shapes of membrane molecules and preferable spontaneous curvature (c_0) of the membranes.

We focused on a reproduction cycle of vesicles (Figure 1B) [7] based on the membrane elasticity theory of vesicles. This cycle consists of three stable vesicle shapes: a spherical shape, a prolate shape formed by the membrane growth of a spherical vesicle, and a limiting shape vesicle to which a prolate-shaped vesicle deforms. Subsequently, destabilization and breaking of the narrow neck of the limiting-shaped vesicle completes the division to form two spherical vesicles. Each vesicle shape is stable and is theoretically given by considering a vesicle shape that has minimal free energy against the two parameters characterizing the vesicle: reduced volume (v) and reduced spontaneous curvature (c_0) (Figure 5B) [51,52]. The reduced volume characterizes the volume-to-surface area ratio of the vesicle and defines a completely spherical vesicle when $v = 1$. As the vesicle membrane grows against vesicle volume (i.e., as the vesicle becomes flabby), the reduced volume decreases. The reduced spontaneous curvature corresponds to the natural length of springs, defining the membrane with $c_0 = 0$ as one preferring a flat state. Here, our discussion is based on the spontaneous curvature model of vesicles where the timescale of interlayer flip-flop motion of bilayer-forming molecules is much faster than the timescale of the morphological changes of vesicles. For more details, see Ref [7,9,51,52]. In the phase diagram of stable vesicle shapes concerning v and c_0, the stable formation of the limiting-shaped vesicle is located on the red bold L^{pear} branch. To reach this branch, an initial spherical vesicle should grow faster in surface area than in volume (i.e., decrease in v from 1.0) and should have the spontaneous curvature $c_0 \gtrsim 3$ (above the value at the left end of the branch). Then, the growth of spherical vesicles will result in spontaneous deformation into the limiting-shaped vesicles without any external force.

Several approaches are known for imposing spontaneous curvature on the lipid bilayer [7]. We adopted one of the simplest approaches, introducing inverse-cone-shaped membrane molecules into a vesicle membrane composed of cylindrical molecules (Figure 5C) [53-55]. In the curved bilayer of a vesicle, inverse-cone-shaped molecules tend to stay in the inner leaflet due to molecular flip-flop motions and the geometrical preference deriving from molecular shapes [56], which may generate enough spontaneous curvature to form a limiting shape. In our case, cholesterol (Chol) was introduced into the AOT vesicle as an inverse-cone-shaped molecule [14,15]. Then, the vesicle couples with artificial metabolic pathways (Figure 3B) and grows in surface area by incorporating additional membrane molecules through PANI-ES, which is a faster process than the volume growth using osmotic pressure differences (Figure 4D). Consequently, the binary AOT/Chol (9/1, molar ratio) vesicle successfully grew and deformed to the limiting shape, whereas the unary AOT vesicle grew in surface area but did not deform to the limiting shape due to the lack of enough spontaneous curvature [14,15].

The neck of the limiting shape is normally stable [57], i.e., the free-energy barrier to break the neck and form two spherical vesicles cannot be overcome using thermal energy. However, there are a few approaches for destabilizing the neck structure. The Gaussian curvature of vesicle membranes and the Gaussian bending rigidities of membrane molecules

make a difference [54,58,59]. The limiting-shaped vesicle consists of two parts with opposite signs of Gaussian curvature: the spherical parts with moderately positive Gaussian curvature and the narrow neck part with a significantly large negative Gaussian curvature. In a binary vesicle, if the molecular preferences against Gaussian curvature (i.e., Gaussian bending rigidities) are sufficiently different between two amphiphiles, one species of membrane molecule prefers to stay in spherical regions and is expelled from the neck region [54,59]. This molecular segregation in the neck region produces an interface between the neck and the spherical regions, which may reduce the energy barrier and destabilize the neck, causing spontaneous vesicle division. This spontaneous division process was reproduced in coarse-grained molecular dynamics simulation studies [59,60]. Fortunately, this scenario was valid for the binary AOT/Chol (9/1) vesicle, which recursively showed spontaneous divisions a few seconds after the limiting shapes were formed (Figure 1C) [14]. By coupling the vesicle deformation to the limiting shape based on the membrane elasticity model (Figure 5B) with the vesicle division based on the destabilization of the neck, our synthetic minimal cells spontaneously divided themselves in a simple way that did not require mechanical forces from complex molecular machinery.

What We Learn from This Synthetic Approach

The reproduction of our synthetic minimal cell can be compared with the division mode of L-form bacteria [61-63], which is considered a model of the proliferation of primitive cells. Unlike ordinary bacteria, L-form bacteria do not have cell walls by which bacterial morphology is determined. In addition, the cell division of L-form bacteria is not mediated by Z-rings. Therefore, the division of L-form bacteria may be driven simply by the excess lipid synthesis rate compared to the volume growth rate, which decreases the volume-to-surface area (V/A) ratio [61] as seen in the deformation strategy in our synthetic minimal cell. Moreover, the synthesis of certain branched-chain fatty acids is critical for the L-form bacterial division [61,64], which agrees with the importance of the inverse-cone-shaped lipids (i.e., cholesterol) in reproducing our synthetic cells. Although not the in-situ lipid synthesis but the uptake of externally supplied membrane molecules triggers the reproduction in our system, we can find several similarities between the synthetic cell and L-form bacteria. In terms of membrane compartments, the essence connecting non-living molecular assemblies and the emergence of living systems might be the coupling between only two species of lipid molecules, i.e., the lipids with the cylindrical shape (as a main component) and the inverse-cone shape (as a component triggering deformation and division).

Despite the above possible essence for the emergence of a cell-like reproduction system, the authors recognized several difficulties in designing (or improving) the synthetic minimal cell. For example, maintaining a steady reproduction cycle of vesicles requires sophisticated regulations of metabolic pathways. We performed the reproduction of our synthetic minimal cell (Figure 1C) 50 times [14]. The vesicles experienced the first division in 45 cases, the second division in 22 cases, the third division in 14 cases, and only a few cases showed the fourth division. Moreover, while the vesicles were typically divided into two vesicles with almost the same sizes during the first and the second division (as shown in Figure 1C), most vesicles in the fourth division shifted their division mode; a vesicle was divided into larger one and smaller one, and the smaller vesicles no longer swelled enough to the size of their parent vesicles. Such asymmetric divisions probably come from the increasing uptake rate of vesicle-forming molecules to the outer leaflet of the vesicle bilayer [14]. Under the constant supply of ingredient molecules (Figure 1A), the catalyst for incorporating certain amphiphiles, PANI-ES, was synthesized during the vesicle reproduction and would gradually increase their concentration on the vesicle surface. This would provide an increased uptake rate of amphiphiles from the environment to the outer leaflet of the bilayer. In earlier generations of our minimal cells (e.g., the first and second division), amphiphiles would be quickly transferred from the outer leaflet to the inner leaflet through fast flip-flop motions, which equilibrated the bilayer structure against the expansion of the outer leaflet. However, in later generations (e.g., the third or more division), the influence of the increasing uptake rate would no longer be equilibrated enough by flip-flop motions, leading to excess spontaneous curvature on the vesicle membrane. Then, in the phase diagram of stable vesicle shapes (Figure 5B), the growing vesicles (i.e., reducing v from 1.0) would reach the limiting shape (L^{pear}) branch in higher c_0 region compared to the earlier generations, which means that such limiting shapes are more "asymmetric" in the size of two spherical regions. Furthermore, too much accumulation of PANI-ES over the vesicle membrane will contrarily hinder the uptake of amphiphiles. If we carefully regulated the molecular supplies as in sophisticated reaction-regulating mechanisms of biological cells, the synthetic minimal cells would possibly sustain the symmetric division. Although the authors did not address this issue due to the technical difficulties, the analysis above can be given since the physical background of our model experimental system is clearer than complex biological cells. This is the great advantage of the bottom-up synthetic approaches in discovering the essence bridging the gap between non-living and living systems.

Moreover, our synthetic minimal cells possibly provide an insight into the very early stage of abiogenesis, especially the origins of genetic information. The genetic information in contemporary biological cells is transferred from DNA to proteins (enzymes) through RNA, i.e., the central dogma of molecular biology, and finally to membrane compartments (Figure 6). While the base sequences in the DNA (genotype) are faced with natural selection whether the functions and structures they express (phenotype) are suitable or not, the inverted information flow is impossible since proteins cannot

determine the base sequences. In contrast to such a unidirectional information flow which differentiates the roles of an information carrier and a compartment, our synthetic minimal cell system consists of a bidirectional information flow; the composition of template vesicles determines the composition of polyaniline (PANI-ES), and PANI-ES selectively encourages the growth of the template vesicle by incorporating specific amphiphiles (i.e., the composition of template vesicles is also affected by PANI-ES) [14,15]. Thus, the information carriers of the system are both the template vesicle and the information polymer (PANI-ES), and a mutual catalytic relationship between them drives the system. We have called PANI-ES an "information polymer" for comparing biological and artificial metabolisms (Figure 3A and 3B). However, in this bidirectional information flow, the template vesicle is more genotypic since the composition of a template vesicle is the origin of information in this experimental setup (Figure 1A), and the information polymer of vesicles (PANI-ES) that triggers the reproduction of vesicles is more phenotypic [14], while the difference of their roles is not so clear as the central dogma. The bidirectional information flows and the genotypic template vesicles suggest that our synthetic minimal cell system may help discuss abiogenesis according to the "lipid world" scenario, as mentioned below.

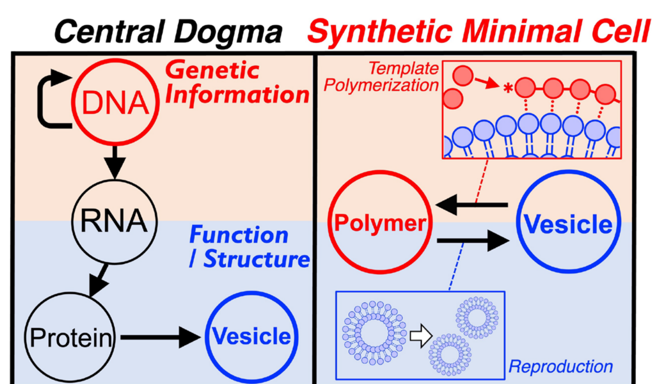

Figure 6 Comparison between the central dogma and our synthetic minimal cell in the view of genotype (orange)/phenotype (blue) and directions of information flows. In our synthetic minimal cells, the vesicle and the polymer cooperatively store the compositional information of the system by forming a bidirectional information flow. In the central dogma of molecular biology, the sequence (i.e., genetic) information is stored only in DNA and unidirectionally transferred to the membrane compartments through RNA and proteins.

Comments on the Lipid World Hypothesis

Concerning the origins of genetic information, the "RNA world" scenario [65] is the majority opinion, claiming that the origin of genetic information is the "sequence" information, which is carried primarily by the self-replicating and evolving sequence(s) of RNA. As its alternative or ancestor, the "lipid world" scenario [66,67] claims that primitive genetic information originates from the "compositional" information of self-assembled heterogeneous molecular assemblies coupled with catalytic reaction networks. In other words, the former and the latter scenarios can be classified into "replicator-first" and "metabolism-first" scenarios, respectively. The originally proposed lipid world scenario focuses mainly on the micelle-catalyzed reactions leading to the diversifications of micellar compositions. Here we suppose that such autocatalytic micellar systems account for only a portion of a multicomponent chemical network (i.e., communicate with other subsystems in a large catalytic network), bearing in mind more general autocatalytic networks [68].

Based on the synthetic minimal cell studies, we consider a scenario that connects the primitive compositional information world governed by simpler heterogeneous molecular assemblies and the present sequence information world governed by sophisticated biopolymers (e.g., the central dogma). On primitive Earth, prebiotic small molecules such as fatty acids, amino acids, and nucleobases are supposed to have been condensed in aqueous conditions, called primordial soup or prebiotic soup [7,65-67,69]. The amphiphilic molecules can self-assemble to form micelles and vesicles, providing various interfaces and hydrophobic environments in the aqueous media depending on species of amphiphiles. Since such molecular assemblies can show simple catalytic activities [20,23,28,67], they might guide various prebiotic reactions in the primordial soup. The lipid world scenario describes the early chemical evolution in this stage; the multicomponent catalytic networks involving catalytically active assemblies of amphiphiles (especially micelles) might have emerged [67]. Such networks could even evolve through flexible modifications of micellar catalytic activities [66,67]; the catalytic amphiphilic assemblies can flexibly change their composition due to the dynamic entry/exit behaviors of various monomers and the adsorption/desorption of other catalytically active molecules. This could result in significant complexities in chemical inventory in the primordial soup at the first stage, and then among various possible networks, specific catalytic networks with narrower compositions and capabilities of mutations would compose the homeostatic growth stage (i.e., protocellular evolution) [66,67].

After shifting from the micelle-based systems to the vesicle-based systems by the changes of pH or ion concentrations in the primordial soup, some of the homeostatic catalytic networks would survive while sustaining their compositional information. Here, the catalytic networks having specific compositional information on amphiphiles (i.e., coupling of

molecular shapes of amphiphiles like model vesicles; AOT/Chol = 9/1 [14,15], DPPC/DLPE = 8/2 [54], and mixed long-chain/short-chain fatty acids [70]) could spontaneously achieve division in the primordial soup and then could flourish compared to the other networks which did not have compositional information for vesicle division. In this stage, as long as the primordial soup stored plentiful resources of vesicle-forming amphiphiles, the network did not have to develop sophisticated catalytic systems to synthesize the amphiphiles from scratch; instead, developing simpler polymeric catalysts for incorporating specific free amphiphiles from the environment (like PANI-ES for AOT) was more economical. Our synthetic minimal cell is a model experimental system of this era. Although compositional information systems might store less information than sequence information systems, simple reproduction systems can emerge even in the former world by forming multicomponent catalytic networks and by conveying compositional information cooperatively among each component like our synthetic minimal cell (Figure 6). In this era, the information flows would still be multi-direction.

As the catalytic networks flourished and the specific free amphiphiles ran out in the primordial soup, the systems needed to elaborate their catalytic networks to synthesize more stable vesicle-forming amphiphiles such as phospholipids; otherwise, unstable amphiphiles (such as fatty acids) would be easily pulled out into the environment, or the networks were outcompeted by the other networks that could synthesize stable amphiphiles. This might be the beginning of the sequence information era because the synthesis of vesicle-forming amphiphiles is chemically sophisticated and thus requires enzymes (i.e., the products in the sequence information world). The sequence information would begin to be elaborated among polymeric catalysts consisting of multicomponent networks in the form of specific sequences of amino acids or possibly RNA, resulting in the capabilities of implementing more sophisticated reactions into their catalytic networks. On the other hand, it was impossible in principle for vesicles to develop their compositional information into sequence information. In this stage, the vesicles, which once cooperatively stored compositional information of the network, lost their roles as the information carrier and shifted to more phenotypic positions (blue region in Figure 6). In contrast, the polymeric catalysts, which previously played relatively phenotypic roles against self-assembled amphiphiles in the multicomponent networks, built up the sequence information and shifted to more genotypic positions (orange region in Figure 6). Such specialization in primitive information flows would finally result in the emergence of the central dogma, where the genetic (i.e., sequence) information is stored only in DNA and conveyed by RNA and proteins unidirectionally to the membrane compartments.

Summary and Outlook

This review outlined several essential concepts for our synthesis of minimal cell systems. Biological cells are equipped with sophisticated information molecules, DNA. Paying the enthalpy cost by forming specific hydrogen bonds between base pairs, DNA prevents the formation of entropically favorable random sequences and generates regular sequences that can encode genetic information. Inspired by this templated polymerization process, we utilized the polymerization of aniline templated by vesicle surfaces, dominantly yielding specific regular polyaniline, PANI-ES. Biological metabolic pathways are encapsulated inside cell membranes; however, the artificial metabolic pathways of our synthetic minimal cells were developed on the vesicle surface to circumvent the problem of molecular transportation. We considered the three essential units for biological metabolisms, which were concisely and artificially redesigned. The synthetic minimal cell utilized H_2O_2 as the energy molecule instead of ATP. PANI-ES functioned as an information polymer and as a catalyst for promoting membrane growth. For membrane growth, we focused on the least essential process, i.e., uptake of amphiphiles from the external solution by regulating molecular hydrophilicity. The membrane molecules were neither synthesized in solution nor further chemically converted on the bilayer before or after incorporation. The vesicle volume increased with the osmotic water inflow. Biological cells maintain their internal hypertonic conditions through the synthesis and/or transportation of osmolytes. In the synthetic minimal cell, asymmetric membrane permeation between mono- and di-saccharides sustains vesicle swelling. Cell division in biological cells is a complex process regulated by molecular machinery. We considered the promising deformation pathway of a vesicle based on the membrane elasticity model of vesicles, and inverse-cone-shaped amphiphiles (Chol) were introduced into the cylindrical amphiphile (AOT) bilayer. The spontaneous curvature and growth of the membrane resulted in the spontaneous deformation to a limiting-shaped vesicle. The coupling of the membrane Gaussian curvatures and the difference in molecular preferences against the Gaussian curvatures spontaneously broke the neck structure.

Overall, the concepts of information molecules, metabolic pathways, membrane and volume growth, and cell division were artificially and concisely redesigned, and they were integrated into a binary AOT/Chol vesicle-based system. The continuous molecular inflow and outflow by the micro-injection technique sustained a nonequilibrium state around the vesicle, which triggered artificial metabolic pathways on the external surface of the vesicle, resulting in the reproduction cycle. This is the synthetic minimal cell system constructed by the authors.

The next goal is to implement the concept of evolution. We significantly simplified biological cells into an artificial model system consisting of only two components: the information molecule and the membrane compartment. In the present synthetic minimal cell, we used only one type of artificial information molecule (PANI-ES) and one type of vesicle

(binary AOT/Chol vesicles). A promising approach to evolution will be first preparing several variations of such polymer-vesicle couplings, i.e., species, and then designing a model experimental system where several species of synthetic minimal cells can compete.

By artificially redesigning several essential concepts of cells into concise model systems, and by comparing such natural and model systems, we will be able to make clear physical and chemical principles behind living systems. In addition to such bottom-up construction approaches, the search for the origin of life in extraterrestrial environments (called "astrobiology" [71,72]) has been attracting much attention in recent years, which may elucidate not only the detailed prebiotic conditions on our planet but possibly universal requirements for abiogenesis in our universe. Needless to say, these relatively new trials are supported by the overwhelming accumulation of biological facts contributed by life sciences. Explorations in various approaches working together, we will find a road from matter to life.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

M.K. and M.I. wrote the manuscript.

Data Availability

The evidence data generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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