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Review

Advancing Biomimetic Functions of Synthetic Cells through Compartmentalized Cell-Free Protein Synthesis

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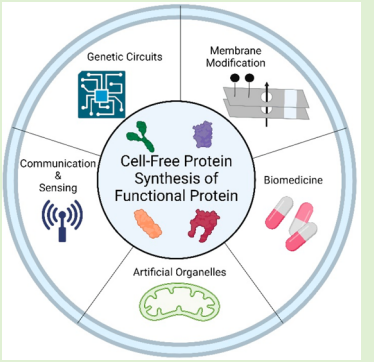
ABSTRACT: Synthetic cells are artificial constructs that mimic the structures and functions of living cells. They are attractive for studying diverse biochemical processes and elucidating the origins of life. While creating a living synthetic cell remains a grand challenge, researchers have successfully synthesized hundreds of unique synthetic cell platforms. One promising approach to developing more sophisticated synthetic cells is to integrate cell-free protein synthesis (CFPS) mechanisms into vesicle platforms. This makes it possible to create synthetic cells with complex biomimetic functions such as genetic circuits, autonomous membrane modifications, sensing and communication, and artificial organelles. This Review explores recent advances in the use of CFPS to impart advanced biomimetic structures and functions to bottom-up synthetic cell platforms. We also discuss the potential applications of synthetic cells in biomedicine as well as the future directions of synthetic cell research.



1. INTRODUCTION

Cells are fundamental units of life, exhibiting structural and chemical diversity among living organisms. However, our understanding of the complex mechanisms that sustain living cells and their biochemical processes remains an ongoing challenge. This challenge has motivated researchers to pursue interdisciplinary efforts in physics, biology, and engineering to recreate the structure and function of living cells on a synthetic platform. Synthetic cells, also known as artificial cells or minimal cells, are engineered constructs that mimic the essential components and functions of living cells. They offer a unique opportunity to control the structural complexity and biochemical functionality of cells, enabling a more precise examination of the dynamic and variable processes that sustain life. The development of synthetic cells has the potential to revolutionize our understanding of life and its origins. It could also lead to new applications in biomedicine, bioengineering, and environmental science.

The development of synthetic cells is pursued through two distinct approaches: top-down and bottom-up.[1] The top-down approach involves modifying living cells to reduce their inherent complexity,[2] while the bottom-up approach aims to recreate essential features of living cells through engineering of synthetic building blocks.[3] This Review specifically focuses on the bottom-up approach. Despite the large variability in biological cells, in terms of form and function, all living cells contain at least three essential features. These include a cell membrane, which provides compartmentalization and regulates transport of nutrients and waste; genetic information, which governs cell behavior; a gene expression mechanism, which enables the realization of the instructions encoded within the genetic information. Many synthetic cell platforms aim to mimic these features, often starting with the synthesis of a cell membrane for compartmentalization. Synthetic membranes primarily consist of bilayer structures derived from lipids, polymers, peptides, proteins, or a mixture of these building blocks. Each type of membrane has its own advantages and disadvantages with respect to the design of synthetic cells.[4−14] Other methods of compartmentalization, such as phase separation[15,16] and hydrogels,[17−19] have also been explored to develop different types of synthetic cells.

Cell-free protein synthesis (CFPS) is a powerful tool that can be used to replicate key features of the complex biological systems that regulate gene expression and lineage.[9,20] In CFPS, the essential components for transcription and translation are extracted from living cells and utilized outside the cell to express protein *in vitro*.[21] The use of CFPS in synthetic cell

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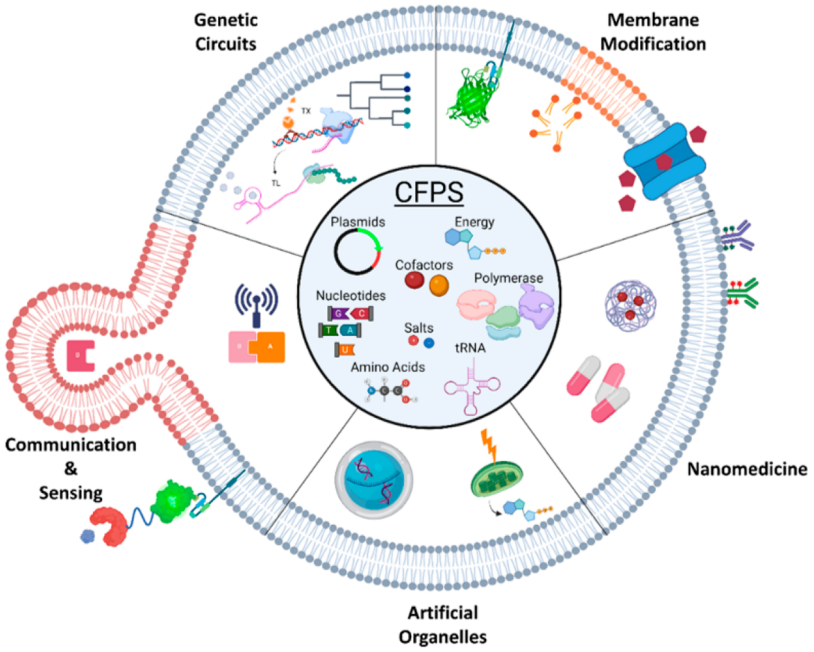

Figure 1. Schematic representation of synthetic cell functions highlighted in this Review, showcasing recent developments achieved through the cell-free protein synthesis (CFPS) of functional proteins. Compartmentalized CFPS integrates nucleotides, polymerase, tRNA, salts, amino acids, and an energy source. CFPS of diverse functional proteins facilitates the emergence of innovative synthetic cell capabilities, encompassing engineered genetic circuits, postsynthesis membrane modification, communication and sensing mechanisms, artificial organelles, and the enhancement of nanomedicines. Created with Biorender.com.

research has attracted attention due to its biomimetic nature. CFPS recapitulates the central dogma of biology in a synthetic platform, which is the process of converting genetic information into proteins. Given that functional proteins underlie virtually all biological processes within living cells, the ability to precisely program the expression of proteins through CFPS represents an appealing strategy for replicating lifelike functions in synthetic cells. Moreover, CFPS offers distinct advantages for expressing recombinant proteins,[9] such as the facile incorporation of nonstandard amino acids,[22] the expression of toxic or insoluble proteins,[23] the ability to program genetic behaviors,[24,25] and the postsynthesis modification of synthetic cells.[26,27]

In this Review, we discuss the utilization of CFPS in the bottom-up development of synthetic cells and provide an overview of the biomimetic functions that have been achieved using CFPS. In recent years, CFPS methods have improved in terms of efficiency, the variety of available organisms used as sources, and the ability to synthesize a wider range of proteins.[28−30] This has led to a notable increase in the utilization of CFPS in the field of synthetic cells which has been periodically updated.[9,11,12,20,31−34] This Review highlights key advancements in synthetic cells facilitated by compartmentalized CFPS of functional proteins, including the development of genetic circuits, postsynthesis membrane modification, inter- and intracellular communication and sensing, creation of artificial organelles, and the design of nanomedicines (Figure 1). These noteworthy features of synthetic cells hold the potential to revolutionize biotechnology, cellular engineering, biomaterials design, and medicine by enabling the creation of customizable, responsive, and versatile biomimetic systems. For example, CFPS-derived genetic circuits offer precise control over cellular processes, emulating living functions and representing a significant stride in the field of synthetic biology. Postsynthesis membrane modification and the establishment of communication and sensing mechanisms play pivotal roles in achieving sophisticated cellular functions, such as transport, protection, or biological responses to external stimuli. Integrating CFPS to trigger phase separation inside synthetic cell lumens can provide fundamental insights into the mechanisms that govern evolution and function of natural organelles. Lastly, the development of nanomedicines based on such synthetic cells holds great promise for targeted drug delivery and therapeutics. Therefore, we introduce recent advancement of synthetic cells, highlighting these biomimetic functions achieved through compartmentalized CFPS one by one, in the following sections.

2. DEVELOPING GENETIC CIRCUITS

Gene regulation is a fundamental process that governs cellular functions and enables cells to fulfill their designated roles in multicellular organisms. Recent advances in the toolkit for programmable gene regulation have expanded the capabilities of CFPS systems for engineering synthetic cells. These advancements have enabled the synthesis of functional proteins within liposomes[35] and the design of complex mechanisms observed in living cells such as cellular communication,[24,36] combinatorial genetic circuits,[37] and multicellular assemblies.[38] Here, we introduce several state-of-the-art works demonstrating genetic circuits controlled by CFPS in synthetic cells.

Adamala and colleagues demonstrated the feasibility of combinatorial genetic circuits in a synthetic cell platform by inducing the fusion of liposomes equipped with unique CFPS systems (Figure 2a).[37] One liposome type was designed to contain a CFPS system encoding GFP but lacking a T7 RNA polymerase, while the other liposome type was designed to facilitate a CFPS reaction to express the T7 RNA polymerase.

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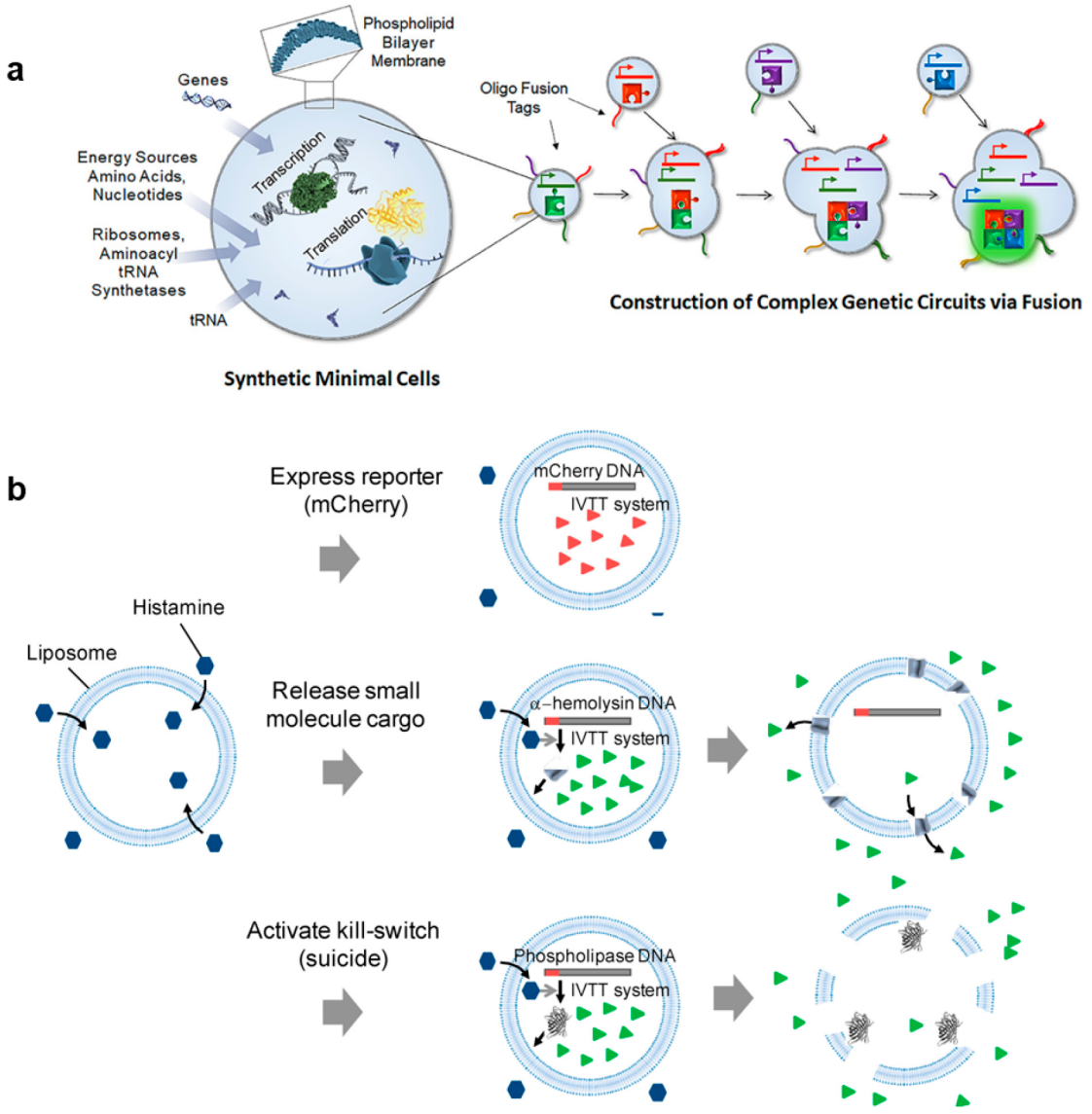

Figure 2. (a) Schematic of synthetic cells binding through DNA fusion tags to regulate gene pathways adapted with permission from ref 37. Copyright 2022 American Chemical Society (https://creativecommons.org/licenses/by/4.0/). (b) Schematic representation of synthetic cells demonstrating *de novo* expression of functional protein for small molecule cargo release and programmed cell death controlled via histamine-responsive riboswitch, adapted with from ref 40. Copyright 2019 American Chemical Society.

Complementary single-stranded DNA fusion tags are attached to the liposome surfaces. These DNA tags bind to each other in solution, facilitating the fusion and mixing of the liposomes and, as a result, leading to the cell-free expression of GFP. Furthermore, this work demonstrated the successful construction of complex combinatorial genetic circuits, incorporating gene pathways with up to ten genes. This was achieved by creating additional synthetic cell species using a combination of transcriptional switches including RNA polymerases, recombinases, and a bioluminescent reporter protein. Interestingly, the precise order of the synthetic cell fusion events dictated the final gene expression outcomes. This work has advanced the development of synthetic minimal cells and introduced a platform capable of simulating mating, complex gene regulation, and control over synthetic cell lineage in a synthetic *in vitro* platform.

Synthetic riboswitches, gene switches similar to the traditional transcription switches used in bacteria, have been used to develop complex genetic circuits and demonstrate sensing and response to stimuli in synthetic cells. Riboswitches work by binding to a compatible RNA aptamer, which triggers conformational changes that expose the ribosome binding site and allow translation of the expression mRNA.[39] Riboswitches have also been used to create biomimetic functions in synthetic cells, such as programmed cell death (i.e., apoptosis-like response) and communication between two species of synthetic cells or between synthetic cells and live bacteria. For example, Yokobayashi et al. developed a synthetic cell encapsulating a CFPS system controlled by a histamine responsive riboswitch to induce expression of several target proteins, including a fluorescent reporter protein (mCherry), a pore-forming protein for triggered release of small cargo (α hemolysin), and a lipid-destroying enzyme (phospholipase C) for programmed cell death (Figure 2b).[40] Boyden and colleagues have shown a synthetic minimal cell platform, referred to as "synells", that leverages the flexibility of

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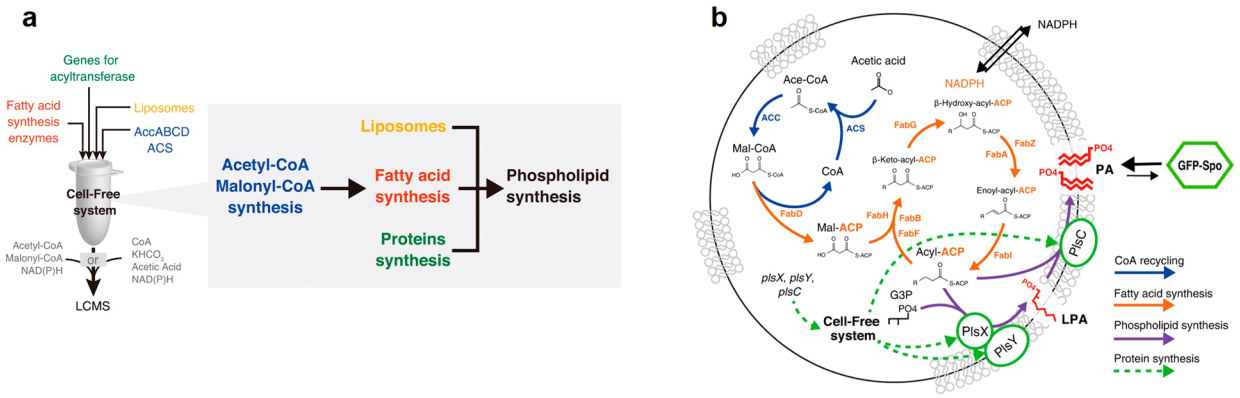

Figure 3. Schematic of cell-free and lipid synthesis processes operating in tandem (a) and schematic representing cell-free and lipid synthesis processes and subsequent lipid integration in the vesicle membrane and fluorescent tagging of newly synthesized lipids in the vesicle membrane (b). Adapted with permission under a Creative Commons CC-BY 4.0 from ref 67. Copyright 2022 Nature Communications Biology (https://creativecommons.org/licenses/by/4.0/).

transcriptional and translational switches to design more complex genetic circuits and reaction cascades within a synthetic cell.[41] They designed four distinct families of genetic circuits using transcriptional and translational switches and release mechanisms as follows. The first genetic circuit takes place within one liposome. The second genetic circuit features two separate circuits independently operating in distinct liposome species. The third genetic circuit allows two species of liposomes to communicate with each other. Lastly, the fourth genetic circuit demonstrates two separate genetic circuits running in tandem through fusion of two distinct liposome species without unintended cross talk. These synells are an example of a highly modular synthetic cell platform that utilizes a myriad of synthetic biological systems in conjunction with one another with high specificity and without cross talk or a need for environmental compatibility. This work implies that the use of transcription and translational switches enables a greater degree of modification and modularity to CFPS reactions and thus awards more freedom to engineer biomimetic processes within synthetic cells.

The regulation of genetic circuits using designed CFPS in synthetic cells is highly desirable in the pursuit of achieving more life-like functions. The research highlighted herein demonstrates that intricate control over gene circuits has advanced the field of synthetic cells, bringing us closer to mimicking complex biological systems, such as epigenetics and cell life cycles. Despite the advances in gene regulation and programmability in CFPS using synthetic cells, our knowledge of the fundamental parameters that affect CFPS in confinement is still incomplete. For example, the effect of molecular crowding on gene expression and regulation in minimal artificial cells is not fully understood, especially with respect to size and concentration of crowding agents.[42,43] The continued development of more sophisticated genetic circuitry, coupled with an improved understanding of the dynamics of compartmentalized gene expression, will propel the development of synthetic cells. Therefore, researchers continue to improve our theoretical understanding of CFPS in synthetic cells through modeling of protein expression processes.[44,45]

3. ENABLING MEMBRANE MODIFICATION

Researchers have made significant progress in achieving biomimicry in synthetic cell membranes by altering the building blocks of the membrane with block copolymers, peptides, proteins, nanoparticles, and polyelectrolytes. These modifications have enabled synthetic cells with more functionalities and tunability.[46−51] Recent comprehensive reviews have covered the strategies employed in modifying synthetic cell membranes.[4−6,52−54] Reconstitution of CFPS-derived membrane proteins in a synthetic membrane is one strategy utilized to further engineer synthetic cell membranes. This method enables the incorporation of proteins that are difficult to obtain due to their inherent cellular toxicity. In certain cases, it also provides enhanced control over the synthesis of recombinant membrane proteins.[26,55]

CFPS has been shown to be beneficial for integrating membrane proteins into various types of synthetic membranes, including polymersomes[56,57] and liposomes.[58−61] For example, cell-free synthesis of membrane proteins integrated into a lipid bilayer has provided a better understanding of the complex structure and functions of human membrane proteins in a simplified biomimetic environment.[62] One study confirmed the successful synthesis of 95% of a library of 250 human channel proteins via CFPS. Mayeux et al. have reconstituted CFPS-derived membrane proteins in synthetic membranes and demonstrated the biomedical applications of this approach, such as antibiotic-free vaccination against *Psuedomonas aeruginosa* through reconstitution of the outer membrane protein OprF in liposomes.[63] CFPS derived membrane protein integration is a particularly distinguished approach as it enables integration of membrane proteins that self-assemble at the membrane, which are difficult to express and purify in bacteria.

In addition to integrating membrane proteins, researchers have explored novel strategies to achieve membrane modification, including *in vitro* lipid and fatty acid synthesis using CFPS.[64−67] The combination of CFPS and lipid synthesis has enabled efficient synthesis and integration of lipid molecules into the membrane of artificial cells while preventing crowding due to intermediate accumulation. By incorporating a CFPS system into lipid synthesis reactions, *in vitro* transcription and translation of acyltransferase can be achieved to convert fatty acids into phospholipids and integrate them into the existing membrane (Figure 3). This unique strategy of postsynthesis membrane modification using lipid building blocks shows promise in addressing the grand challenge of growth and replication of lipid-based synthetic cells.[67]

Indeed, living cell membranes exhibit remarkable diversity and perform a variety of essential functions. They are highly

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dynamic and vary not only in lateral and leaflet asymmetry but also from cell to cell. To replicate the structure and function of cell membranes, state-of-the-art techniques employing CFPS have been used to achieve postsynthesis membrane modification in synthetic cells. As demonstrated in the examples above, CFPS is expected to continue supporting research aimed at developing a synthetic membrane with life-like capabilities, thereby enhancing accuracy of synthetic cell biomimicry in structure and function.

4. FACILITATING SENSING AND COMMUNICATION

Sensing and communication are essential functions for cell survival, enabling them to respond to stimuli from neighboring cells and their environment. However, replicating the nuanced sensory mechanisms and communication networks of living cells in synthetic platforms is a significant challenge due to their complexity and diversity. Nonetheless, with continuous efforts and advances in science and technology, researchers have succeeded in demonstrating various aspects of cellular communication in synthetic cells.[68] For instance, Meier and co-workers demonstrated a rudimentary biomimetic sensory mechanism in polymersomes by reconstituting the outer membrane channel protein F in a lipid/polymer hybrid membrane. This work allowed for control over enzymatic reactions by altering the permeability of the membrane using the stimuli responsive channel protein.[69]

Researchers have developed more complex methods to create sensing and communication in artificial cells by incorporating sensory proteins and CFPS mechanisms. Mansy et al. designed an artificial cell system based on CFPS to translate a chemical signal undetectable by *E. coli* into a chemical signal to which *E. coli* naturally responds.[70] Liposome-based synthetic cells have also been engineered to communicate with bacterial cells via cell-free expression of quorum sensing signal molecules that are directly recognized by neighboring bacteria.[71] Researchers have continued to leverage CFPS mechanisms to express desired proteins in synthetic cells which respond to *in vitro* stimuli such as light,[72−74] biomolecules,[37,40,41,75−78] and osmotic conditions.[27]

Moreover, CFPS is utilized to introduce novel communication capabilities between synthetic cells and between synthetic and living cells. Recently, Adir et al. developed a synthetic cell platform that uses optogenetic proteins for self-activation, membrane modification, and communication with living cells.[79] They expressed *Gaussia* luciferase (Gluc) in liposomes using CFPS to introduce a bioluminescent signal and enable intercellular communication between synthetic cells and fungal cells using light (Figure 4a). Briefly, the self-activated biomimetic functions in synthetic cells were created by encoding a recombinant protein using a bacterial transcription factor (EL222) induced by light. They recombinantly fused improved light-inducible dimer (iLID), which dimerizes with single-stranded DNA-binding protein (sspB) upon exposure to blue light, with an associated Gluc and Histidine affinity tag to localize cell-free derived proteins to the membrane. In the presence of light, iLID binds with sspB-tagged red fluorescent protein (RFP) in solution, recruiting RFP to the membrane surface. This self-activating optogenetic process demonstrates synthetic signal-mediated transcription and membrane recruitment. The demonstration of self-activating biomimetic functionality in this work contributes to the complexity for state-of-the-art synthetic cells by revealing parameters that influence fundamental biological processes, such as transcription, translation, and the preservation of genetic material within the cell.[80]

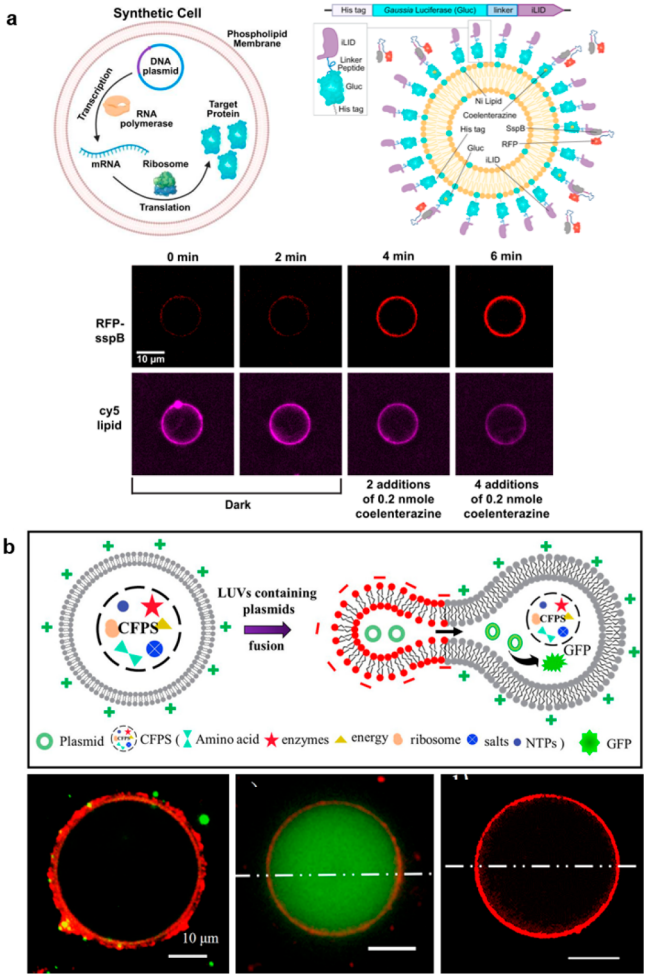

Figure 4. (a) Schematic representation of light emitting synthetic cells (top left) and self-activating synthetic cells with membrane recruitment (top right). Fluorescence micrographs (bottom) showing recruitment of sspB-RFP to the vesicle membrane by iLID upon exposure to bioluminescent light stimulated by coelenterazine. Adapted with permission under a Creative Commons CC-BY 4.0 from ref 79. Copyright 2022 Nature Communications (https://creativecommons.org/licenses/by/4.0/). (b) Schematic representation of molecular transport of DNA across giant unilamellar vesicle (GUV) membranes through fusion of oppositely charged large unilamellar vesicles (LUVs). Fluorescence micrographs of LUVs sequestering to the GUV membrane (left), CFPS derived GFP after delivery of DNA to the GUV and 37 °C incubation for 4 h (middle), and control sample in which LUVs without DNA fuse with the GUV membrane and demonstrate no CFPS of GFP within the lumen (right). Adapted from ref 36. Copyright 2022 American Chemical Society. All scale bars are 10 μm.

Vesicle fusion is considered not only in the replication and differentiation of synthetic cells but also in communication and transport processes. In 2022, Han et al. demonstrated a synthetic cell that utilizes vesicle fusion combined with CFPS to model a simplified metabolic transport process across a lipid membrane. In this study, an incomplete CFPS reaction lacking DNA was encapsulated inside a giant unilamellar vesicle (GUV). Additionally, DNA encoding green fluorescent protein (GFP) was encapsulated within another GUV, whose membrane had an opposite charge. Fusion of these GUVs

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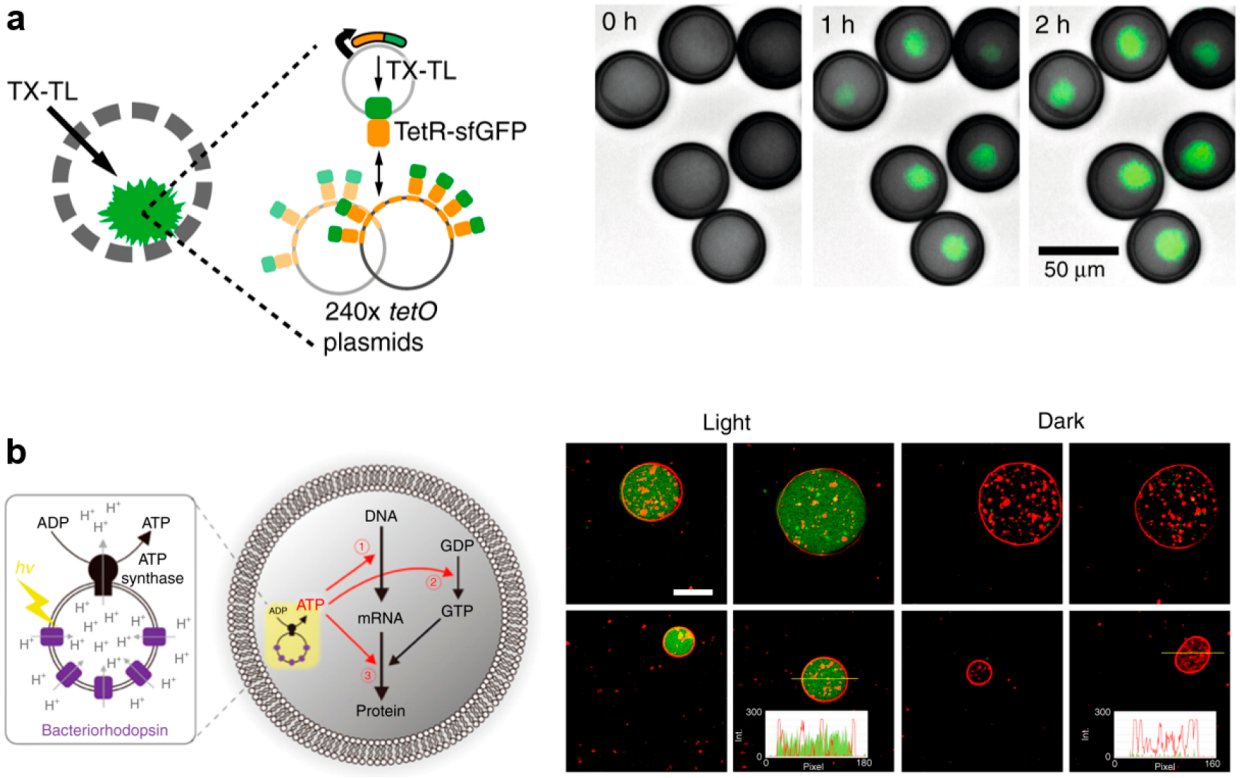

Figure 5. (a) Schematic and fluorescent micrographs of clay-DNA hydrogel-based artificial nuclei in polymersomes showing localization of transcription and translation to the DNA hydrogel. Reprinted with permission under a Creative Commons CC-BY 4.0 from ref 87. Copyright 2018 Nature Communications (https://creativecommons.org/licenses/by/4.0/). (b) Schematic of photosynthetic artificial organelle with reconstituted bacteriorhodopsin and F type ATP synthase and fluorescence micrographs of cell-free synthesis of GFP driven by light inside of synthetic cells encapsulating CFPS components excluding ATP and photosynthetic artificial organelles. Adapted with permission under a Creative Commons CC-BY 4.0 from ref 90. Copyright 2019 Nature Communications (https://creativecommons.org/licenses/by/4.0/).

resulted in the successful delivery of DNA and expression of the model GFP through CFPS (Figure 4b).[36] This study leveraged electrostatic interactions to achieve a vesicle transport process in a synthetic cell that simplifies intercellular communication and metabolism in living cells.

Stimuli response is often regarded as a fundamental characteristic of living organisms, emphasizing the crucial role of sensing and communication of living cells. The research highlighted in this context underscores the potential of CFPS as an attractive tool for engineering sensing and communication functions in synthetic cells. Considering that numerous biological sensing and response mechanisms rely on functional proteins, CFPS is expected to serve as a promising avenue in ongoing research efforts. Moreover, the continued advancements of sensory and communication mechanisms within synthetic cells would facilitate sophisticated functions in diagnostics and metabolic engineering.

5. CREATING ARTIFICIAL ORGANELLES

A key step in developing complex synthetic cells is to create nested multicompartment structures, which can mimic the structure of eukaryotic cells containing distinct organelles and the nucleus.[81] Modular and multicompartmentalized vesicles can segregate incompatible chemistries and increase the functionality of the resulting synthetic cell. Researchers are interested in creating artificial organelles to introduce new functions within living cells[82] as well as to make synthetic analogues of specific organelles that can be used outside of cells.[81,83−85] Recent approaches in bottom-up construction of synthetic cells have explored compartmentalization through the utilization of CFPS, liquid−liquid phase separation (LLPS),[15] engineered hydrogels,[18,19] or microchannel devices.[86]

Niederholtmeyer et al. created semisynthetic cells containing artificial nuclei using a clay-DNA hydrogel particle[87] after reports showed the increased efficiency of cell-free expression within clay microgels.[88] Cell-free transcription and translation components were then added to the polymersomes, where they diffused into the artificial nucleus and expressed a model GFP (Figure 5a). Similarly, Simmel and Aufinger designed artificial organelles, which allow for spatial organization of transcription and translation and lead to efficient protein expression, by utilizing DNA-immobilized hydrogel.[89] The authors partitioned DNA encoding the target protein to transcription organelles and single-stranded DNA encoded to capture transcribed RNA in translation organelles, respectively. This modular design allowed transcription and translation to be partitioned to separate artificial organelles, which could operate simultaneously and in tandem with one another within one artificial cell. This work also enables programming of biochemical reaction networks through regulation of the length and sequence of DNA immobilized on the hydrogel network.

CFPS has also been used to develop artificial photosynthetic organelles in liposomes.[90] Kuruma et al.[90] designed a synthetic cell in which CFPS reactions are fueled with light-generated adenosine triphosphate (ATP). They encapsulated a CFPS system within protein/lipid hybrid vesicles bearing reconstituted bacteriorhodopsin (bR) and ATP synthase. Upon

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exposure to light, the proton pump activity of bR fills the artificial photosynthetic organelle with protons. Then, ATP synthase phosphorylates ADP in the synthetic cell lumen to produce ATP, which is used for the CFPS of model GFP (Figure 5b). This work demonstrated a system to self-generate components for energy production by encoding bR and ATP synthase in the CFPS reaction. The novel creation of a synthetic cell that utilizes light to generate enough energy for transcription and translation of functional proteins will serve as a significant step toward the development of fully autonomous synthetic cells. Furthermore, self-constituting artificial organelles may contribute to elucidating the methods that primitive cells evolved to develop organelles and support metabolism before the advent of electron chain transfer gradients.

While canonical organelles such as mitochondria and ribosomes are compartmentalized by lipid membranes, advances in biology have revealed the importance of membraneless organelles. The theory that membraneless organelles are formed and maintained in eukaryotic cells through LLPS has developed relatively recently and is now widely accepted.[91,92] Since this realization, researchers have pursued engineering artificial membraneless organelles.[93−97] One approach toward the development of membraneless artificial organelles involves harnessing LLPS and CFPS systems together. In 2014, a basic model of an artificial organelle was developed through imbibing multiphase aqueous droplets with a cell-free protein expression system.[98] This study demonstrated the partitioning of cell-free transcription and translation of a model fluorescent protein within an aqueous two phase droplet consisting of dextran and polyethylene glycol (PEG) in a water-in-oil emulsion.[98] Since then, researchers have developed more complex artificial cells using CFPS to further elucidate the role and genesis of LLPS in cells and create membraneless organelles with controlled physical properties and biological functions. For example, Maeda et al. expressed two fluorescent proteins, deGFP and mCherry, inside of water-in-oil droplets with PEG crowding agents via cell-free transcription and translation (Figure 6).[99]

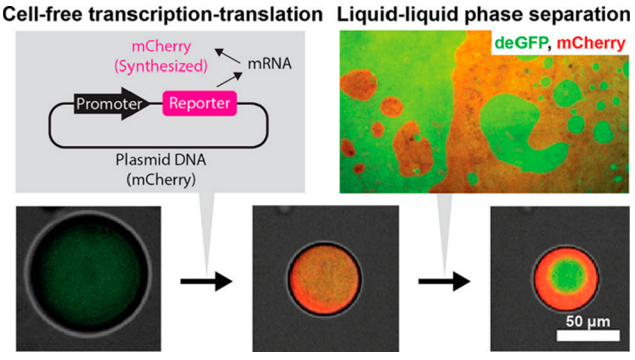

Figure 6. Fluorescence micrographs of water-in-oil droplets with PEG crowding agent, indicating a cell-free transcription and translation mechanism. Reprinted from ref 99. Copyright 2021 American Chemical Society.

They demonstrated the formation of membraneless mCherry-rich and deGFP-rich compartments within an aqueous droplet by inducing LLPS with water evaporation, which increases the relative concentration of solutes. This work revealed the importance of protein species, presence of cargo, and presence of crowding agents in the formation of membraneless organelles.[99]

More recently, artificial membraneless organelles that sequester RNA to regulate protein expression have been designed through the cell-free expression of a phase-separating protein.[100] Robinson et al.[100] designed a synthetic cell that expresses RGG-GFP-RGG protein. This protein forms coacervate droplets in an aqueous environment and suppresses secondary protein expression by sequestering the RNA needed for translation. In this regard, integrating CFPS to trigger phase separation inside the vesicle lumens enables compartmentalization in synthetic cells, which facilitates the creation of more complex biological structures and functions. Ultimately, the use of synthetic cells with engineered membraneless organelles will deepen our understanding of the mechanisms that govern the evolution and function of natural organelles. Further development of artificial organelles and cells holds promise for its biomedical implications, particularly validating existing models for medical treatments,[101] as discussed in the following section.

6. ADVANCING BIOMEDICAL TECHNOLOGIES AND APPLICATIONS

The development of synthetic cells is also being pursued to advance current biomedical technologies and applications.[102,103] The unique programmability, adaptability, and sensory capabilities of synthetic cell platforms has enabled the development of sophisticated "smart" vesicle delivery vehicles,[104−107] adaptive protein therapeutics,[73] and treatments that control the behavior of cells *in vivo*.[108,109]

With the advances in protein-based therapies, CFPS has become an attractive tool for adaptive nanomedicine treatments. For example, CFPS was employed in rudimentary synthetic protocells to synthesize *de novo* anticancer proteins inside of tumors.[73] Schroeder et al.[73] encapsulated a CFPS reaction within POPC liposomes that are permeable to nutrients essential for the synthesis of protein therapeutics. The rudimentary synthetic cells were shown to effectively express the anticancer toxin−protein, *Pseudomonas* exotoxin (PE), in the presence of mammalian cancer cells. The secretion of PE led to 80% cancer cell death. Interestingly, *in vivo* treatment of PE-expressing synthetic cells to 4T1 tumors showed higher levels of apoptotic markers than treatment with purified PE. This result suggests that cell-free derived PE may enhance its treatment efficacy by being encapsulated within synthetic cell platforms that can offer improved protein stability and a sustained release profile.

The repertoire of nanomedicines has also expanded with the development of synthetic cells that can synthesize and deliver therapeutic molecules in response to environmental stimuli. For instance, Schroeder et al. reported another biomedical capability of a synthetic cell platform, which utilizes CFPS to autonomously express human basic fibroblast growth factor (bFGF) to induce angiogenesis or the growth of new blood vessels.[109] Similarly, the liposomal synthetic cells were designed using a CFPS solution to express a brain-derived neurotrophic factor (BDNF) that promotes differentiation of neurons.[110] The cell-free expression system was designed to express a pore forming protein, perfringolysin O (PFO), in the presence of an inducing molecule. Upon sensing of the inducing molecule, the synthetic cell produces PFO, which enables transport of the cell-free derived BDNF across the synthetic membrane to the surrounding neuronal cells (Figure

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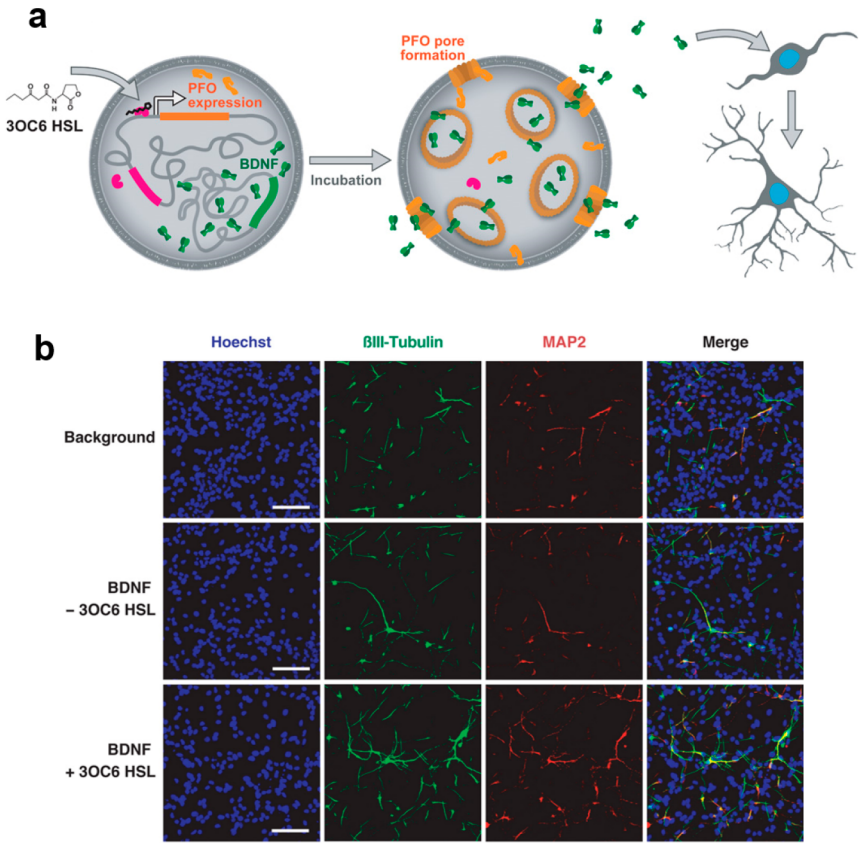

Figure 7. (a) Schematic representation of BDNF secreting synthetic cells for promotion of neuronal differentiation and (b) fluorescence micrographs of neurons demonstrating clear signs of differentiation as evidence by immunostaining of molecules which indicate neuronal differentiation βIII-tubulin and microtubule-associated protein 2 (MAP2) (scale bars, 50 μm). Adapted with permission under a Creative Commons CC-BY 4.0 from ref 110. Copyright 2020 Science Advances (https://creativecommons.org/licenses/by/4.0/).

7a). *In vitro* experiments on neuronal stem cells treated with synthetic cells showed clear signs of differentiation, as observed by neuronal cell counting and staining for mature neuronal markers (Figure 7b). This synthetic cell model introduces a simple yet elegant solution to coupling sensing and therapeutic drug delivery. The rudimentary synthetic cells, with lipid membranes optimized to improve protein expression and bFGF-encoded CFPS reactions, promoted angiogenesis in both *in vitro* and *in vivo* experiments.

More recently, Cash et al. developed a synthetic cell platform to model parasitic infection in simplified living cells.[111] They created both a synthetic cell and a synthetic parasite encapsulated in liposomes with different DNA cargos. These synthetic cells offered a unique opportunity to study infection-related processes, such as the host's response to resist infection and the metabolic burden, due to their simplicity. These examples of synthetic cells used in nanomedicine provided a basis for the development of next-generation biomedical technologies and demonstrated the potential to model lethal infections, host resistance, and immunization. This innovative approach not only enhances our understanding of synthetic cells but also offers a foundation for the research of fundamental interactions in diseased cells.

Current research on the use of immunotherapeutic[63] and other biologically relevant proteins to intervene in biological processes, such as immune response, cancer, and wound healing, is ongoing. Compartmentalized CFPS has the potential to allow for localized, adaptive treatment of therapeutic proteins with high efficiency in synthetic cell platforms. Hence, synthetic cells with CFPS-based therapeutics could be a solution to a broad range of biomedical applications. The systems highlighted in this section demonstrate the capacity of CFPS-integrated synthetic cells to enable creation of advanced biomedicines with a wide range of applications, already proven effective both *in vitro* and *in vivo* (Figure 8). As the design of synthetic cells and CFPS continues to advance in terms of their diversity and complexity, it becomes easier to

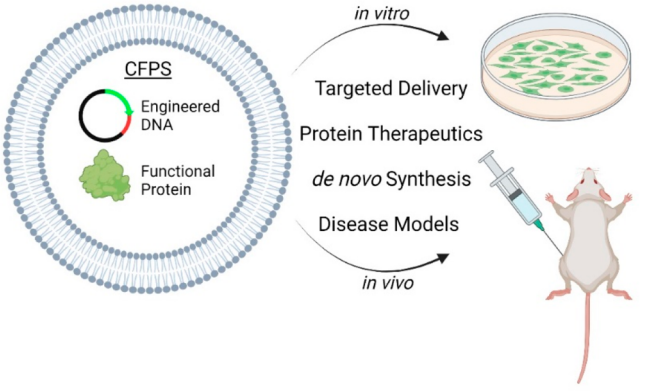

Figure 8. Schematic illustration of synthetic cells employing compartmentalized CFPS for the development of advanced biomedicines for a range of applications, including targeted drug delivery, *de novo* synthesis of protein therapeutics, and disease modeling. Figure created with Biorender.com.

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imagine future development of synthetic cell platforms that enable safe and straightforward investigation of disease fundamentals, as well as detection and treatment of living cells in their native environment.

7. SUMMARY AND OUTLOOK

CFPS has emerged as a powerful tool for the rational design of complex synthetic cells. While CFPS mechanisms are derived from biological cells, they are considered to be synthetic processes since they are not readily found in nature. However, its biocompatibility and biomimicry make it well-suited for the development of synthetic cells. Furthermore, recombinant technologies have greatly expanded the range of achievable protein-based functions through CFPS, allowing precise synthesis and control. This has enabled the exploration of functional proteins in synthetic environments, including those requiring post-translational modifications or site-specific assembly. These technologies have heavily informed the design and implementation of synthetic cell platforms in recent years, enabling significantly more sophisticated synthetic cellular features.

As the capabilities of CFPS continues to grow,[112] the development and rational design of canonical transcription factors and novel riboswitches have significantly enhanced the capabilities of synthetic cells. These diverse switching mechanisms have provided an unprecedented control of CFPS processes in synthetic environments, enabling the design of complex genetic circuits, sensing, and stimuli response. Coupled with intelligent design of compartmentalizing materials such as membranes, droplets, and hydrogels, state-of-the-art CFPS mechanisms have demonstrated impressive functionalities, allowing synthetic cells to interface with artificial stimuli, living cells, and other synthetic cell species.

Biological cells are entirely self-regulating, self-constituting, and self-replicating. These features are difficult to achieve in synthetic cells, because they are continuous processes that operate in conjunction with a wide range of biological mechanisms. However, recent developments, such as the autonomous production of energy sources, membrane building blocks, and artificial organelles, provide promising steps toward realizing these challenges in synthetic cells, as highlighted in this Review. The continued efforts to diversify and replicate cellular features will continue to drive the production of synthetic cells in the years to come. In turn, our fundamental knowledge and the available tools for advanced biomedicines will continue to grow.

An essential challenge that remains in this field is the integration of multiple biomimetic functionalities into a single synthetic cell. Multicompartment synthetic cell design, which takes inspiration from eukaryotic cells in segregating incompatible processes within synthetic cells, holds promise for enabling the coexistence of various functionalities and preventing interference. Therefore, technologies that improve fabrication, spatial control, and repeatability of artificial components will undoubtably contribute to the development of synthetic cells. The advancements discussed in this Review have the potential to synergistically contribute to the complexity of synthetic cells. For example, complex genetic circuits using expression switches can enable a response to multiple chemical signals, informing the autonomous production of artificial organelles or activating positive or negative feedback loops. Vesicle transport mechanisms can be employed to further discretize incompatible communication or enable the development of more complex artificial organelles, such as the nucleus. The nature of the CFPS synthetic cell design has also proven valuable in the development of novel nanomedicines. The goals of sensing, stimuli response, and active biomolecule synthesis overlap in the fields of synthetic cell development and nanomedicine. Therefore, the ongoing efforts in synthetic cell design are sure to drive the development of novel nanomedicines which improve upon the abilities of smart delivery vehicles and protein therapeutics.

In conclusion, the development of synthetic cells with increasing complexity continues to provide valuable insights into the origins of life and evolution of cellular compartments but also sheds light on important parameters related to known biological processes. The rapid innovation of synthetic cell capabilities, increasing in complexity, serves as evidence that the CFPS is a powerful tool in synthetic cell development. While the timeline of synthetic cell development cannot be predicted, CFPS is expected to continue contributing to the complexity and advancement of new and improved biomimetic functions in synthetic cells.

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Notes

The authors declare no competing financial interest.

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