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Reconstituting Natural Cell Elements in Synthetic Cells

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Building a live cell from non-living building blocks would be a fundamental breakthrough in biological sciences, and it would enable engineering new lineages of life, not directly descendant of the Last Universal Common Ancestor. Fully engineered synthetic cells will have architectures that can be radically different from the natural cells, yet most life processes reconstituted in synthetic cells so far are built from natural and biosimilar building blocks. Most natural processes have already been reconstituted in synthetic cell chassis. This paper summarizes recent advancements in using non-living building blocks to reconstitute some of the most crucial features of living systems in a fully engineerable chassis of a synthetic cell.

1. Introduction

In the last few years, we have observed rapid progress of in vitro technologies mimicking capacities and properties of live natural cells.[1] While no live cell has yet been created completely from non-living components, much work has been done to design synthetic mimics of singular characteristics of life.[2–4] The time is right to combine all those systems into a functional live synthetic minimal cell.[5,6]

Most synthetic cell engineering focuses on building a cell that, in principle, mimics the blueprint of a natural cell: has a lipid membrane, follows the central dogma of DNA, RNA, and protein, metabolism and energy production.[7] To build a synthetic cell from non-living, but naturally derived elements, in vitro models of all those essential elements needs to be engineered. Here we provide an overview of recent progress in engineering essential components of a synthetic cell-based on specific natural live cell systems.

The field of synthetic cell engineering adopts one of the two complementary approaches: bottom-up or top-down.[8] The bottom-up approach includes reconstructing all cellular elements from simple non-living building blocks,[9] while the top-down approach involves simplifying the natural living cells until the smallest possible living organism is obtained.[10] In this review, we discuss technologies used to reconstitute basic life processes in synthetic cells using non-living components—summarizing advancements of the bottom-up approach to building life.

Although the end goal of synthetic cell research, building a self-sustaining cell from scratch that satisfies the multiple requirements of life, is evident, the overall purpose or end goal is not so cohesive. Synthetic cell research impacts a wide variety of scientific disciplines with each one advancing the technology for different purposes. For example, synthetic cells have grand importance in the field of basic biology in that they can be used now as a sort of middle-ground between in vivo and in vitro work. They allow for biological study of an isolated pathway/enzyme/etc. while still maintaining some biological relevancy of live cell systems. Ultimately, designing a self-replicating and sustainable synthetic cell would give major insights into fundamental basic questions such as "What is required to be alive?" and more generally into bare minimal systems study. On the opposite end of the spectrum, biomedicine is using liposomal technologies right now as drug delivery platforms. Liposomes have major advantages over cellular delivery systems in that they have reduced immunogenicity, meaning they do not trigger a strong immune response because they lack cell-surface receptors, and more generally anything that is not intentionally encapsulated within them. Liposomes used in medical applications are also often PEGylated, which also improves immunogenicity. The end goal of making a synthetic cell would allow for much more freedom in both drug delivery and on-site drug synthesis. A single synthetic cell population can act as a programmable lab that can synthesize a multitude of drugs/compounds on demand, whether that be once it locates a target tissue in the body or in inaccessible areas where lab work is infeasible. A unique aspect of the synthetic cell community is that many disciplines have both short-term uses for liposomal bioreactors as well as a vested interest in advancing the technology towards the end goal of a live synthetic cell. This hybrid approach is unique and useful in that it allows creativity in developing technologies for synthetic cells that can be used to address problems now, while also working to expand the toolkit that will one day assemble a fully alive artificial cell. Other examples of this include astrobiology, where synthetic cells are used to push the boundaries of life where live cells are not versatile enough (i.e., ribosome evolution), while an end goal of an artificial cell will address key questions like what biological processes will be required on non-terrestrial worlds as well as potential ways protocells began to form on a primordial Earth. This field is unique in that all these different disciplines can focus on independent areas of cellular life, while coming together to begin building something artificial that can be, perhaps, considered alive.

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2. Membrane

One of the essential elements of life is a formal barrier between the organism and the outside environment. First observed by Robert Hooke, this rule of life has remained true to this day. Therefore, one of the first requirements to consider in the design of a synthetic cell is that it must have a physical membrane to encapsulate the machinery within. Because most of the machinery used in synthetic biology is directly from natural systems, it should come as no surprise that a membrane composed of biologically relevant phospholipids is a prime choice for membrane composition in a synthetic cell. Throughout this review, the terms liposome and vesicle are used interchangeably and are defined as lipid bilayers that contain a lumen (**Figure 1**).

2.1. Lipid Synthesis

Growth is a hallmark of living organisms, down to their most basic unit, the cell. This process is not only replication of the inner cellular machinery, but also the cellular membrane in the form of lipid synthesis. It is also a necessity in an artificial cellular system as after enough successful divisions, the daughter cells would become too small to successfully divide. Several different strategies have been proposed to either synthesize or recruit phospholipids (**Table 1**). The most "true-to-life" of these being the expression of phospholipid synthesis machinery in synthetic cell systems that can synthesize phospholipids from precursors.[11] It has been demonstrated that the enzymes, GPAT and LPAAT, can be expressed via the PURE system and in turn synthesize lysophosphatidic acid and phosphatidic acid; although, the lipid product yield is low due to the two enzymes requiring different redox reaction conditions.[12] Similar results were achieved with the encapsulation of FAS-B (a fatty acid synthase) Type I enzyme within a POPC membrane. This enzyme synthesized fatty acids that were then incorporated into the phospholipid membrane.[13] Similarly, another recent success in synthesizing lipids inside liposomes came from expressing eight acyl transfer and headgroup modifying enzymes from *Escherichia coli* in liposomes. Using acyl-CoA and glycerol-3-phosphate as precursors, 1,2-diacyl-sn-glycerol 3-phosphate was synthesized in a single pot reaction, which was then used to make DPPG and DPPE.[14] Although in this case, synthesis was limited due to the magnesium ion concentration limiting the solubility of long-acyl chain CoA substrates. This work was expanded upon by encoding the

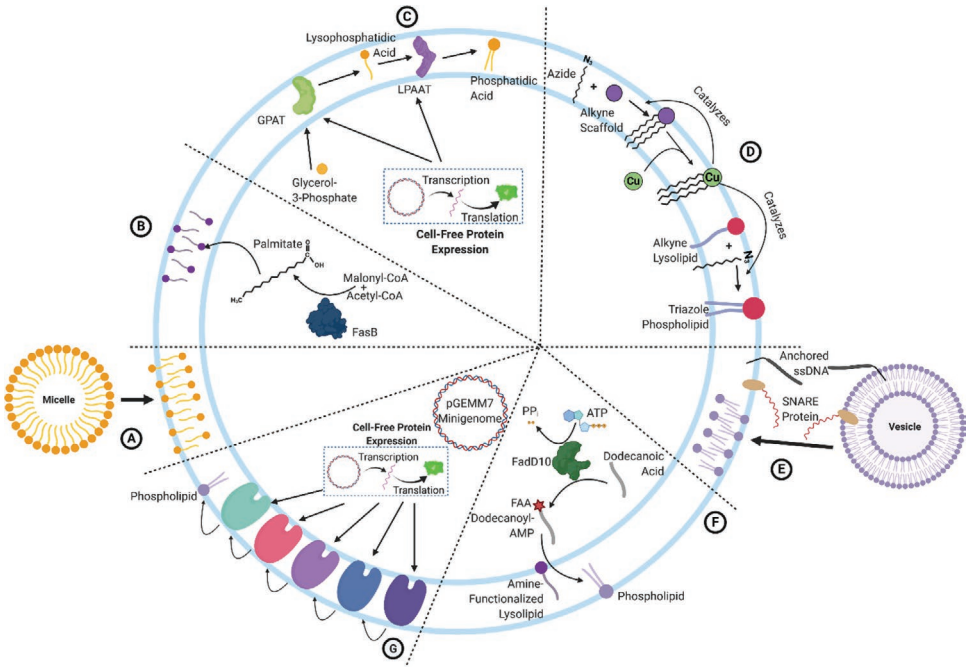

Figure 1. Various methods for growing synthetic cell membranes. A) Micelles will spontaneously fuse with liposomal membranes and incorporate the lipids within the liposomal membrane. B) FasB is encapsulated within liposomes and is used to convert Maolonyl-CoA and Acetyl-CoA to the Palmitate, which then inserts itself into the liposomal membrane. C) Encapsulated cell-free protein expression machinery is used to express GPAT and LPAAT. These two membrane proteins convert glycerol-3-phosphate to the intermediate lysophospatidic acid and the final phospholipid product phosphatidic acid D) A complex composed of azide carbon chains, an alkyne scaffold, and a copper ion catalyzes its own initial step in formation of the alkyne scaffold recruiting the azides. This self-catalyzing complex also catalyzes the formation of a triazole phospholipid from alkyne lysolipid and the same azide precursor. E) A vesicle composed of phospholipids can be programmed to fuse with a larger liposome using SNARE protein complexes or single-stranded complementary oligonucleotides to induce fusion. Post-fusion, the phospholipids are successfully incorporated in the liposomal membrane in addition to any cargo that is in the lumen of the vesicle. F) The soluble enzyme FadD10 uses ATP to prime a reactive lipid precursor (dodecanoic acid) for reaction with an amine-functionalized lysolipid to form a complete phospholipid. G) Plasmid pGEMM7 encodes seven *E. coli* lipid synthesis enzymes of the Kennedy Pathway and are all expressed within liposomes via the PURE cell-free expression system. The enzymes are incorporated within the cell membrane and can synthesize phosphatidylglycerol and phosphatidylethanolamine using Acyl-CoA and glycerol-3-phosphate as precursors. Created with BioRender.com.

Table 1. Examples of different methods for lipid synthesis for use in liposomal or micelle systems.

Substrate	Product	Catalyst	Comments
Sn-glycerol-3-phsphate, acyl-CoA	Diacyl-phosphatidic acid (PA) (universal precursor of glycerophospholipids in bacteria)	Glycerol-3-phosphate acyltransferase (GPAT) lysophospholipid acyltransferase (LPAAT)	The yield was low due to the two expressed enzymes requiring different redox reaction conditions Enzymes were synthesized via PURE system within liposomes[12]
Malonyl-CoA, acyl-CoA	Palmitic Acid	FAS-B Type I (fatty acid synthase)	Fatty acid, not phospholipid[13]
acyl-CoA, glycerol-3-phosphate	1,2-diacyl-sn- glycerol 3-phosphate *then* → DPPG and DPPE	GPAT/LPAT *Then also* *For* DPPG: CdsA, PgsA, PgpA/C, *Or for* DPPE: CdsA, PssA, Psd	Single pot in liposomes[14]
For Catalyst: Alkyne scaffold, Azide carbon chain Cu ion *For Phospholipid*: Carbon chain azide, Alkyne lysolipid,	Triazole phospholipid catalyst	Produced in-situ	Non-biological reaction[16]
Fatty acids, Mg^{2+}, ATP, an amine-functionalized lipid fragment	Phospholipids	Fatty acyl adenylates via FadD10 Phospholipids via spontaneous chemistry	Useful technology for non-membrane bound phospholipid synthesis machinery[19]

Kennedy phospholipid synthesizing pathway within a termed "mini-genome" and expressing them via the PURE system. These seven genes were able to process acyl-CoA and glycerol-3-phosphate into phosphatidylethanolamine and phosphatidylglycerol, two of the four phospholipids that compose cellular membranes in nature.[15] The important breakthrough of combining phospholipid synthesis with cell-free protein expression cannot be overstated since this allowed these bioreactors the potential to synthesize new biological machinery after protein degradation or dilution from divisions. Additionally, encoding the machinery within a "mini-genome" allows for a much more true-to-life means of gene regulation and sets the stage for coupling more complex networks together without overwhelming the relatively limited production capabilities of the cell-free protein expression systems.

Other alternative approaches include development of a chemical catalyst that is able to attach a long carbon chain azide to an alkyne lysolipid via click chemistry. The interesting aspect is the reaction products create more catalyst, which is vital in an indefinitely replicating system so that the enzyme does not get diluted out over subsequent generations. After two cycles of removing 90% of the membrane and allowing time for the system to rebuild, there are no detectable phospholipids from the first generation which demonstrates the viability of this system in creating and maintaining a membrane.[16] Another system that has the potential to supply liposomes with new membrane components utilizes fusion with smaller vesicles mediated through either anchored SNARE proteins[17] or single-stranded DNA.[18] This technology has implications in "feeding" the artificial cell with membrane components as well as other encapsulated nutrients.

Another interesting approach is that of developing a means to synthesize a membrane de novo. Encapsulated within liposomes, FadD10 is able to convert fatty acids, Mg^{2+}, and ATP into fatty acid adenylates (FAAs). These FAAs can interact with an amine-functionalized lipid fragment to produce phospholipids that can aggregate and form a liposome de novo.[19] This technology is impressive and addresses the issue of how membranes arose pre-biotically. Additionally, because membrane-bound proteins are often difficult to synthesize within liposomes, this technology has potential to be a stepping stone for synthetic cells on the way to conventional membrane-bound phospholipid machinery.

It is worth mentioning the difference in product formed via liposomes formed out of fatty acids compared to phospholipids. Fatty acid membranes are generally more dynamic, and much less stable when exposed to divalent cations (like Mg^{2+}) at biotic concentrations and less dynamic than phospholipid membranes; however, they are relatively more permeable to small molecules and ions. This is theoretically an important feature in a pre-membrane protein world, where environmental exchange would need to be done solely through the membrane, without the aid of channels.[20] Of course, virtually all biological building blocks we have today rely on a phospholipid membrane. There is a potential middle ground between these two approaches—a hybrid model. It is shown that a membrane composed of a blend of oleic acid (a fatty acid) and POPC (a phospholipid) is both stable to higher concentrations of divalent cation, while still remaining permeable to small charged molecules and ions, a perfect combination of features for a primordial cell incapable of synthesizing trans-membrane proteins or channels.[21] Of course, this system has implications outside of origin of life research because just as primordial cells did not the ability to make membrane proteins, so to are current synthetic cells faced with the same obstacle. Despite the relative robustness of fatty

acid membrane systems, they are generally much less compatible with other modules that would be necessary for a true synthetic cell. In addition to the previously described instability in the presence of cations, they also inhibit polymerase activity and would require an environment of a non-polar solvent/oil, unlike how most life exists today.[22,23] These features allow fatty acid-based synthetic cells to be useful in developing early protocell models, but less than ideal compatibility-wise for joining multiple synthetic cell technologies together to make an artificial cell.

2.2. Membrane Division

In conjunction with growth via membrane synthesis, eventually, the synthetic cell will reach an unstable surface area to volume ratio. At this point, a mechanism for cellular division will be necessary and this synthetic system would successfully display another aspect of life: self-replication. There are many different proposed strategies for synthetic cellular division, ranging from extrusion through primordial clay[24] to cell-free expression of a minimal divisome.[25]

Because of the intricacies involved in reconstituting all the proteins involved in the divisome, simpler and more robust systems to bypass this complexity and divide lipid systems artificially are needed. In the case of fatty acid membrane or liposomes, new fatty acids are able to be fed externally in the form of resulting in that vesicle or liposome becoming large enough that the surface area to volume ratio surpasses the stable limit. Ultimately, the consequence is membrane collapse and the formation of several daughter vesicles.[26] Another advancement in fatty acid liposome division is the use of hydroxypyrene and thiols to generate reactive oxygen species. These, in turn, associate with the fatty acids and induce membrane pearling and division.[27]

Alternatively, vesicles naturally change into large filamentous structures with the incorporation of additional phospholipids. After this transition, the liposomes can be physically sheared, similar to the report discussed previously.[28] MreB is an essential component for rod formation in bacteria but lacks this rod forming activity when expressed in synthetic cells.[29] Recently, it has been shown that the spatial interactions of membrane proteins, also known as 2D crowding, can be mimicked with crowding agents.[30] Under these conditions, MreB changes the morphology of liposomes from spheres to rods, a shape more amenable to division by shearing.[31] This morphological change is an essential first step toward building a synthetic true-to-life system that undergoes cellular division.

Further basic research into vesicle growth in response to size, osmotic pressure, and fatty acid concentration resulted in a phase diagram detailing the parameters liposomes underwent stable growth, unstable growth, or bursting. In addition, this study was able to demonstrate that stable membrane growth is achievable with expression of a simple set of bacterial cytoskeletal proteins inside the liposome.[32]

Another popular method for fusing and fissuring liposomes is the freeze-thaw method, in which subsequent freezing and thawing of small liposomes cause them to fuse together into larger ones.[33] One of the more impressive uses of this method is coupling it with inner RNA replication. After encapsulation of RNA and an RNA replicase, subsequent freeze/thawing serves two purposes: 1) It causes the liposomes to fissure and reform into daughter vesicles and 2) During the fission process, the membranes are disrupted enough to allow diffusion of nutrients into the liposomes to sustain future RNA replication. Using this method, it is possible to achieve sustainable passage of liposomes through ten generations, marketing this development as a milestone in synthesizing a culturable liposomal "cell" line.[34]

Other areas of the field focus on investigating a feasible way for liposomes to divide on a primordial Earth. It is known that montmorillonite, a clay, has the ability to catalyze the formation of RNA from ribonucleotides,[35,36] but a group also discovered that this clay accelerates the formation of fatty vesicles from micelles, allowing for an aqueous compartment for this RNA formation to take place. Importantly, it is possible for these vesicles to divide via extrusion through small pores in the clay. This is an important revelation for potential origin of life work, but may also be a useful technology in some applications of synthetic cells since it provides framework for vesicle formation, division, and RNA synthesis.[24]

Perhaps more important for the development of a self-dividing synthetic cell system is the identification of a mutant form of FtsZ that is sufficient to remodel a phospholipid membrane. Immediately prior to bacterial cell division, FtsZ locates to the division point and forms filaments of repeating FtsZ subunits, which is termed the Z-ring. It is FtsZ that recruits the other proteins involved in bacterial cell wall synthesis such as the Fts family of proteins. It is the coordination of these proteins that direct the synthesis of the new cell wall and the cinching motion of the Z-ring that ultimately ends up in bacterial cell division. By coupling an amphipathic alpha helix to its terminus, FtsZ directionally inserts itself into the membrane in vitro, bypassing the need for a separate membrane localization element. Depending on which terminus this alpha helix is fused to, the protein is able to programmatically create a physical concave or convex bend in the membrane.[37] Additionally, introduction of FitA with the mutant FtsZ causes a small percentage of liposomes to successfully form z-rings and undergo division.[38] This method was further explored within giant vesicles when the group expressed and imaged fluorescently tagged cell division proteins—FtsZ, FtsA, and ZipA via the PURE cell-free protein synthesis system. After expression, the group visualized deformation of the liposomal membrane and postulated that further refinement with more proteins could lead to full membrane division.[39]

Yet another method to induce membrane division was developed by creating liposomes composed of both POPC, POPG, and previously characterized amphipathic molecules. With the addition of a bolaamphiphilic molecule (one that is polar at both ends, but has a significant non-polar region in the middle), the liposomes would undergo fission into two roughly equally sized daughter vesicles. Additionally, this technology was coupled to a PCR-controlled DNA-replication reaction inside the liposomes and, interestingly, the presence of DNA as a negatively charged molecule enhanced

the liposome's ability to divide. As the liposomes continue to divide, the membrane composition changes towards a higher percentage of this synthetic molecule causing it to act undesirably.[40]

Similarly, work has been done to fuse liposomes utilizing charge. Depending on the concentration of POPC versus POPG as well as the overall charge of the membrane, liposomes can be made to fuse with a giant unilamellar vesicle (GUV).[41] This work is useful to the general synthetic cell community because it is relatively low-complexity, which makes it much more versatile than other systems that rely on internally expressed components.

Many possible approaches have been explored for the development of a self-dividing system and there potentially still remains technology to be developed to engineer a reliable and efficient self-dividing liposomal system (**Figure 2**). Other approaches not touched upon here are discussed in a review from Caspi and Dekker.[42] In short, the orchestra of membrane division is difficult due to the precise requirements for temporal, spatial, and stoichiometric control over the event. In contrast, lipid synthesis is relatively easier to accomplish with several groups already establishing different phospholipid synthesis pathways in synthetic cellular systems.

2.3. Encapsulation

There is a wide variety of methods to encapsulate cell-like machinery within liposomes to effectively create synthetic cells or liposomal bioreactors. One of the earliest methods and still widely utilized today is the emulsion-based method. The process involves passing water droplets through an oil phase creating water-in-oil droplets. These droplets are then passed back into an aqueous phase to make liposomes.[43] This process has been improved upon in both speed and efficiency with the incorporation of microfluidic advances. In a process termed octanol-assisted liposome assembly, liposomes within the 5–20 μm size range are synthesized by two streams (an aqueous and oil) with the oil stream containing 1-octanol.[44] With the whole setup on a chip, the system is quick, reliable, and the chip can be manufactured within 3 h. It is hard to imagine a future where synthetic cell technologies are not all done in conjunction with microfluidics due to the numerous benefits it offers. Refinement and creative applications of microfluidics have already seen a rise in new protocols such as encapsulating coacervates as proto-organelles within synthetic cells.[45] This field has evolved rapidly and there is much nuance. Further reading can be done from this recent review.[46]

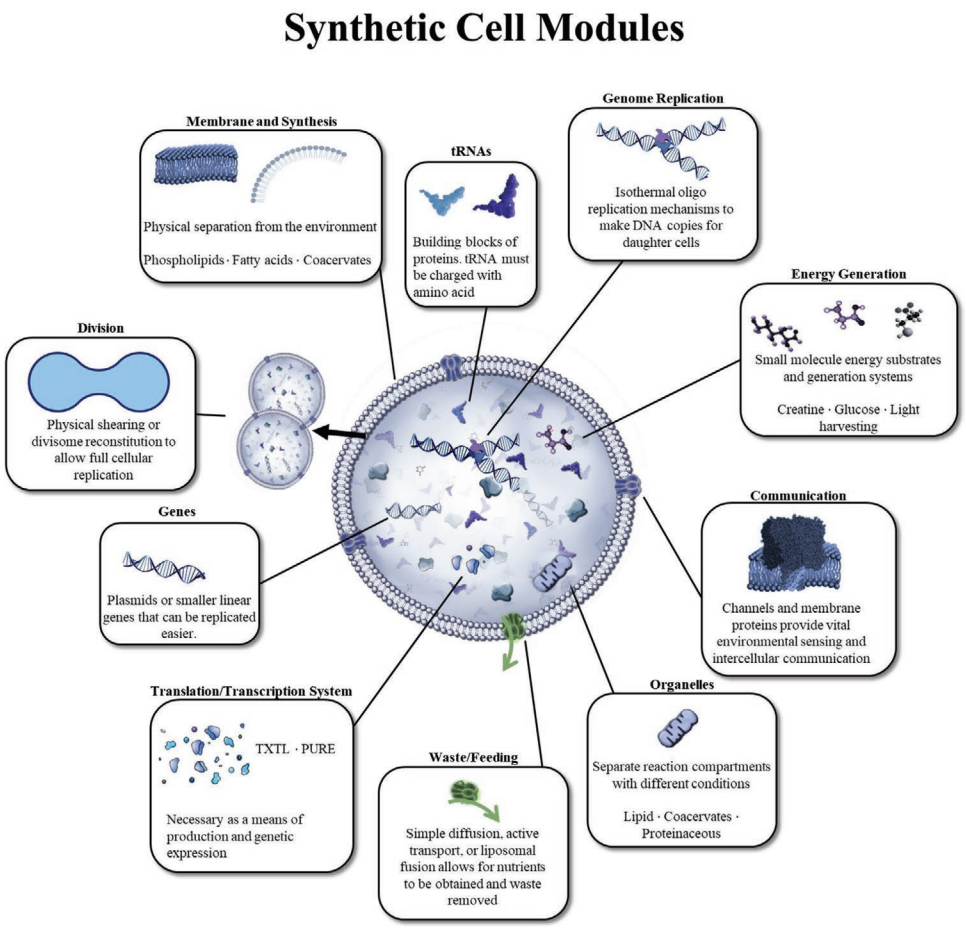

Figure 2. Different "modules" can theoretically be assembled together to create a fully synthetic cell. Created with BioRender.com.

3. Replication of Genetic Material

To replicate a synthetic cell, genetic material needs to be replicated between generations. Synthetic cell genomes can be designed using RNA or DNA, with different isothermal amplification methods used for copying those genomes.

3.1. RNA genome: Self-reproducing RNA Polymerase Ribozyme

A common theory for the emergence of life on Earth is coined "RNA world,"[47] where RNA performed as both the genetic information molecule and as the machinery required to replicate this information. Developing this concept into a synthetic cellular system would not only validate the possibility of an RNA origin of life, but would also allow for the bottom-up construction of a synthetic cell to skip over the technically challenging feats of reconstituting minimal: DNA replication, RNA transcription, protein expression, and tRNA synthesis systems. The search for such a ribozyme began with the utilization of in vitro evolution to evolve an RNA ligase into a ribozyme RNA polymerase.[48] From random libraries, sequences that demonstrated polymerase activity were isolated, and through subsequent selections, were able to improve upon this activity. As it stands, the ribozyme is able to synthesize an RNA sequence as long as itself with the caveat that it is not robust enough to synthesize itself and thus needs a favorable template. They have also succeeded in using this ribozyme to synthesize another active ribozyme, demonstrating the possibility of ribozyme-synthesized ribozymes acting in place of proteins as the main catalytic agents in a primordial Earth. Further advances in this ribozyme's efficiency were made by utilizing water ice;[49,50] although, this is not a feasible technology for use in lipid membranes due to their fragility during freeze-thaw cycles. Currently, a true self-synthesizing ribozyme remains elusive.

Another group took a similar approach, by creating a system that had pieces of itself under self-replicative control. In this system, the "genome" consists of two complementary pieces of RNA, that when one is replicated via an RNA-dependent RNA polymerase, it synthesizes the other. One of the RNA strands expresses the beta-subunit of the Qbeta replicase enzyme. This gene can be expressed in a synthetic cell system as described previously so as to continually replicate the genome. Obviously, the limitations in this system are that all the essential genes needed for RNA replication and protein expression are unable to be expressed in such a system; however, this is an important proof of principle technology that demonstrates the first steps for a fully self-replicating system.[51]

3.2. DNA Genome

Perhaps the most robust method for replicating genetic material in liposomes is PCR. As far back as 1995, PCR was reconstituted in liposomes that were stable even under the high temperatures involved.[52] Later, another group expanded upon this idea of DNA replication in liposomes by using purified thermophilic bacterial replication machinery inside liposomal compartments.[53]

This technology was advanced yet again with liposomes containing DNA and a heat-stable DNA polymerase undergoing temperature fluctuations in accordance with PCR protocols. The result was similar to the above groups; however, the group also periodically added a bolaamphiphilic molecule (one that is polar at both ends but has a significant non-polar region in the middle), which induced liposome division into two generally equal sized vesicles. This is groundbreaking in that genetic material was replicated and passed down into daughter vesicles, a feat that until that point was specific to cells. It is important to note that the replication and division were still researcher controlled and this is far from a true self-replicating system. But at the same time, this group has built a strong foundation for other technologies to be coupled together. One other important note is that this division cannot be maintained indefinitely. They comment that a natural next step would be to couple a mechanism that would allow for either synthesis or recruitment of phospholipids to the daughter vesicles, allowing this replication to continue indefinitely.[40]

A synthetic means to replicate plasmid sized DNA was designed through the dual use of rolling circle amplification, an isothermal DNA amplification system, and a recombinase. Rolling-circle amplification entails using the Φ29 DNA polymerase to continue in full circles, replicating a single strand in a repeating product, while a second Φ29 synthesizes the complement. After this step, the Cre recombinase recognizes a single recombinase site in each plasmid repeat and stitches them together into full circular daughter plasmids.[54] This work is particularly impressive because the Φ29 polymerase was encoded within the plasmid, allowing for the genome to synthesize crucial components of its own replication machinery. This dual reaction system was developed and termed transcription-translation-coupled DNA replication (TTcDR) in a previous study by the same group.[55] This system was used in successive studies and appears to be one of the more robust DNA replication methods due to its simplicity.

Due to the presence of exonucleases in TX-TL, it is more difficult to use linear DNA fragments as a genome than circular plasmids. However, due to it being much easier to synthesize linear DNA than circular, work has been done to circumvent the degradation inherent in TX-TL. This was done by the addition of GamS, a truncated form of lambda gam, a nuclease inhibitor, which effectively decreased degradation of linear template and increasing overall yield of the reporter protein GFP.[56] This was an important advancement in the field by providing a means to use linear DNA in TX-TL, making it theoretically much easier to synthesize genomic DNA in a synthetic cell.

However, the most recent advancement is the use of cell-free protein expression to synthesize Φ29 viral DNA replication machinery inside liposomes. This machinery is sufficient to replicate linear DNA comprising the liposomes' genome.[57] This is a major step forward in the synthetic cell community in that they successfully created a synthetic cell that can synthesize all the required parts for effective genome replication. One of the more recent advancements built upon this technology was using the Φ29 replicase to replicate the PURE transcription factor genes in tandem with the PURE system expressing the proteins themselves. Half of the proteins expressed were expressed to concentrations equal to or greater than the input

concentrations.[58] EF-TU was not expressed because it is by far the most abundant of the PURE proteins (5× higher than the average PURE protein concentration) and has classically been purified or expressed separately to achieve the higher needed concentrations Through further refining, this system should be able to replicate EF-TU as well, the main obstacle will be achieving a high enough yield of EF-TU in the same pot as the other protein products.

4. Energy

Just as cells utilize the energy stored in molecules to carry out many biological processes, the processes in synthetic systems also require energy. Traditionally, researchers have used molecules with high energy phosphate bonds to regenerate ATP levels in the system. There are multiple challenges with this system that have been addressed to varying degrees. One of these is the inherent instability of phosphate bonds due to phosphatases. Advancements were made by changing the energy source to 3PGA (3-phosphoglyceric acid) instead of the more traditional PEP (phosphoenol pyruvate)[59,60] Another sample technology for alternative energy sources is the Swartz lab using a mutant yeast extract instead of *E. coli* that is able to metabolize glucose, a much cheaper energy substrate than the previously described molecules.[61]

Currently, most TX-TL models utilize 3-PGA as the energy substrate due to a study that was done showing remarkable increase in ATP-regeneration when coupled with a newly designed metabolism based on maltose. Using this system, inorganic phosphate was able to be recycled, glucose more efficiently used, and protein production lasting longer.[62]

Traditionally and in most current cell-free protein expression platforms, ATP is supplied through the form of ATP-regeneration systems containing high-energy phosphate compounds that phosphorylate the ADP yielding ATP. However, because natural cells synthesize their own ATP through either metabolism or from light energy, there is interest in developing a pseudo photosynthesis system for use in synthetic cells. Research has shown it is possible to encapsulate rhodopsin, a light-gated proton pump, and ATP synthase in vesicles to convert light energy into a proton gradient and subsequently the proton gradient into ATP via a phosphorylation reaction. In this manuscript, these ATP producing vesicles are encapsulated in a larger vesicle that contains actin, which gets polymerized with the light induction of the ATP-synthesis reaction. This demonstrates the ability of the technology to power synthetic reactions within synthetic cells through harnessing light energy.[63]

5. Transcription and mRNA Maturation

Currently, because synthetic cellular systems remain relatively simple, complex eukaryotic regulatory pathways have not been necessary to reconstitute. However, as these technologies increase in complexity, further gene regulation and epigenetic systems may need to be designed to regulate and control these larger systems. Examples of more complex regulatory pathways that will need to be addressed in synthetic systems are mRNA maturation and epigenetic regulation.

5.1. Transcriptional Regulation

An issue particularly disruptive to groups attempting to synthesize eukaryotic proteins is that in vitro mammalian transcriptional processes are normally incompatible with in vitro mammalian translation. This becomes a hurdle for groups needing to utilize the chaperone, post-translational modifying enzymes, or folding partners of eukaryotic systems to properly synthesize their protein of interest. Several workarounds exist for groups that must use a eukaryotic in vitro protein expression system. In one, the researchers spatially separated the mammalian transcription and translation processes so that the incompatibilities were not a problem. Once the mRNA properly expresses in the first populations of liposomes, that population was able to be controllably fused with another containing the translation machinery, allowing translation to proceed as normal.[64]

As of now, the T7 polymerase is the most common RNA polymerase used for cell-free transcription by far. This is due to the fact of its already widespread use, relative low-price, and ease to work with. However, in using a single polymerase, the synthetic biology community is potentially missing out on having orthogonal transcriptional activities happening in a synthetic cell. To combat this issue, a group cataloged a pool of different *E. coli* RNA polymerases (RNAP) and their respective orthogonality to each other, meaning how promiscuous each one is with other RNAP promoters.[65] With this information, much more complex genetic circuits can be constructed utilizing RNAP expression as an on-switch and regulating gene expression via promoter strength instead of plasmid concentration.[31] The benefit of this is having separate genetic circuits that have genes that do not compete with a single RNAP. This benefit is also specific to the TX-TL system. Whereas TX-TL retains many of the endogenous sigma factors, polymerases, and required cofactors for multi-promoter expression, the PURE protein expression system lacks these proteins. The standard PURE reaction only uses the T7 polymerase; however, it is true that other polymerases can be added to yield more versatile expression.

5.2. mRNA Maturation

One major problem with eukaryotic cell-free protein expression is the lack of mRNA processing prior to translation. In simple one-pot reactions, it is not feasible to expect accurate and efficient processing of RNA prior to exposure to ribosomes. Several groups have been working on potential workarounds for this problem. One such example is work that uses internal ribosome entry sites (IRES), which are naturally found in 5′untranslated regions of mRNA, to enable 5′-cap-independent translation of transcripts. In their work, the group was able to design an expression vector that could express a variety of complex and diverse eukaryotic proteins in a cell-free eukaryotic system representing a milestone in designing useful and productive eukaryotic cell-free protein expression systems.[66]

Another solution that approaches the problem from another angle utilizes a mixture of ribosomes taken from different cell lines. The differing ribosomes and their differing IRES specificity work to allow translation of mRNA products that may not be optimally translated in a cell-free protein expression system that is from a single cell-line. This "hybrid" system marks another advancement in bringing eukaryotic cell-free technologies up to speed with their prokaryotic counterparts.[67]

As transcriptional pathways become more and more complex in synthetic systems, an obvious need arises to be able to control and regulate these transcriptional systems. Work on this has been done as discussed previously with promoter design. However, other technologies include aptamer regulatory elements upstream of the RNA transcript. These work to control ribosome initiation via binding a small molecule like an on-off switch. Work has been done to develop the theophylline aptamer as a controller in a genetic circuit within a liposomal synthetic cell.[68] Further development of aptamers as regulators can be done to make a larger network of orthogonal transcriptional regulators.

6. Translation

An argument can be made for the "alive-ness" of viruses, biological machines that do not require lipid synthesis, division, or even DNA. However, even these biological entities, along with all other life forms, require protein synthesis to perform enzymatic reactions for self-replication. Protein synthesis is also a cornerstone of evolution as introduction of new amino acid chemical properties exponentially expands the functional abilities of proteins. Following is a brief description of recent developments in synthetic translation. A more detailed study on the current state and near future for bioengineered translation systems was recently published.[69]

6.1. TX-TL and Protein Folding

One of the most fundamental processes of life is DNA storing cellular information that is then transcribed to RNA which can then be translated to protein products. Since the 1950's researchers have been using cell extracts to express proteins in vitro from mRNA. It was not until recently that researchers have been able to fine-tune this system well enough to fully reconstitute the DNA to RNA to protein workflow and successfully express proteins in an encapsulated liposomal system. Depending on the desired purity and cost barriers, two systems have arisen for use as cell-free protein expression systems (**Figure 3**).

The first method makes use of crudely purified cell extract[76,77] where *E. coli* is lysed and purified through several centrifugation steps and re-supplemented with small molecules lost in the purification (nucleotides, tRNA, energy substrates, etc.). This extract can then be encapsulated in phospholipids along with a gene to be expressed (in the form of plasmid or linearized DNA). A major advantage for this system is its versatility in terms of which organismal extracts can be made from. *E. coli* offers the most cost-efficient and best protein production of any extract, while extracts from higher organisms allow for the synthesis of larger or more complexly folded proteins, albeit at lower yields and higher preparation costs.[78] A testament to this method's versatility is that a cell-free protein expression system has been made from: E. coli, archea, yeast, insect, CHO, and human cell lines.[79] Currently, glycoproteins have successfully been made in yeast and even more heavily modified proteins have been synthesized using CHO or human cell lines.[79]

Similar to classical protein expression, different cell extracts can be utilized for different desired protein products in TX-TL (**Table 2**).

6.2. The PURE System

The other method for cell-free protein expression is the PURE system.[80] PURE was developed through the identification of the minimum number of proteins and their required conditions necessary to reconstitute transcription and subsequent translation of a gene. These proteins are then individually expressed and purified allowing for the cell-free expression of proteins from gene products in a similar manner to the lysate method described above. An obvious advantage to this method is that the system is very well defined, as opposed to the unknown composition of cell lysates. The trade-off is lower protein yield, a lesser potential for synthesis of complex or large proteins, as well as requiring more time, equipment, and money to create the components of the PURE system. Although, work is currently being done to optimize all the plasmids of the PURE system in several *E. coli* strains, such that all the protein components can be purified in one batch purification, vastly speeding up the process of purification and preparation of functional PURE system.[81] A similar idea came to fruition by co-culturing strains with different plasmids in them in specific ratios. This allows for all the proteins to be expressed in bulk and purified together, greatly simplifying the time and work that goes into making a functional PURE system.[82]

This PURE system is constantly undergoing improvements and redesign to yield the cheapest, most efficient, and easiest to work with synthetic protein expression system. The most recent of these improvements is a development of PURE 3.0. All the necessary genes for protein expression, ribosome formation, and tRNA synthesis were cloned into three plasmids. Each protein is his-tagged so, after expression, the proteins can be quickly batch-purified to yield the complete protein system for cell-free protein expression. This advancement allows individual labs to create a highly-defined protein expression system without the unknown contaminants present in TX-TL cell extract, while also maintaining the low-cost production method associated with TX-TL production.[83]

Regardless of the protein expression system used, either can be encapsulated inside a phospholipid bilayer with a gene of interest to form a protein synthesis bioreactor, also known as, synthetic minimal cell.

6.3. Self-Synthesizing Protein Expression

An obvious direction to take cell-free protein expression is to design a system that can self-synthesize the cornerstone of

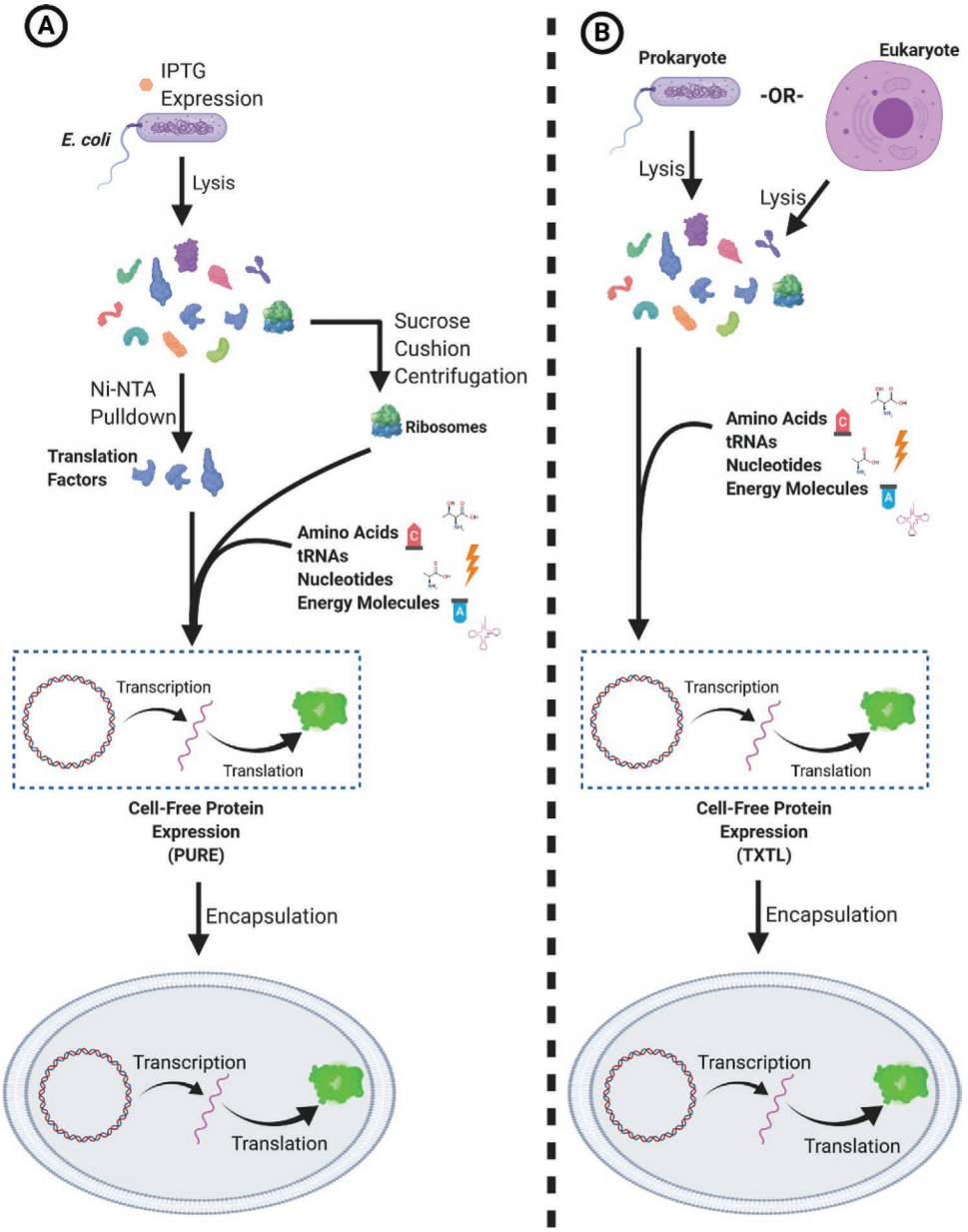

Figure 3. The PURE and whole-cell translation systems. A) *E. coli* containing the expression vectors for the PURE proteins is lysed after expression, and the PURE proteins purified using traditional affinity tag techniques. Ribosomes are purified separately using sucrose cushion centrifugation. This mixture is supplemented with small molecule substrates and energy molecules such as: nucleotides, tRNA, amino acids, etc. At this point, the system is sufficient to express proteins from a DNA template and can easily be encapsulated within liposomes to construct an encapsulated cell-free protein expression bioreactor, or a synthetic minimal cell. B) Depending on the sophistication needed for the minimal cell, a cell type is chosen and lysed. The resulting lysate is supplemented with small molecule substrates and energy molecules such as: nucleotides, tRNA, amino acids, etc. At this point, the system is sufficient to express proteins from a DNA template and can easily be encapsulated within liposomes to construct an encapsulated cell-free protein expression bioreactor, or a synthetic minimal cell. Created with BioRender.com.

biological replication, the ribosome; although, this is not currently possible with current synthetic systems. The major obstacle is successfully synthesizing all the ribosomal proteins; however, by optimizing DNA concentrations, the PURE system can synthesize all 54 r-proteins in batch format.[84] Additionally, the development of iSAT (integrated synthesis, assembly, and translation of ribosomes) technology allows for the synthesis of both rRNA and ribosomal proteins as well as the assembly of the two together in the same compartment, a requirement for future utilization in artificial synthetic cells.[85] With the aid of a crude S150 *E. coli* extract, iSAT allows for the synthesis of ribosomes de novo through mimicking the cytoplasmic environment. This technology has been recently advanced to allow for directed ribosomal evolution, opening the door for the development of ribosomes with new desired characteristics.[86] A major challenge in synthesizing ribosome components in vitro is the inability to perform

Table 2. Comparison of major types of in vitro protein expression.

System	Main advantages	Main disadvantages
E. coli[1]	• Very high protein yield • Relatively tolerant of additives • Cheap and easy to work with	• Many eukaryotic proteins insoluble • No eukaryotic co- and post-translational modifications and folding chaperones
Wheat germ[70]	• Translation of large proteins • No endogenous mammalian proteins • High protein yield	• No complex co- and post-translational modifications and folding chaperones • Premature termination of products
S. cerevisiae[71]	• Eukaryotic translational and folding machinery • Inexpensive large-scale fermentation	• Lack appropriate chaperones for some proteins from higher eukaryotes (e.g., plants and animals) • Not commercially available
Insect[72,73]	• Translation of large proteins • No endogenous mammalian proteins • Certain forms of protein glycosylation	• No mammalian co- and post-translational modifications and folding chaperones
Rabbit reticulocyte[74]	• Mammalian system • Cap independent translation	• No protein glycosylation • Lower yield
Human[75]	• Complex co- and post-translational modifications	• Low yields

the post-transcriptional modifications present on the 16S rRNA. This problem was overcome through utilizing iSAT within a liposomal selection scheme. A single point mutation was found that conveyed 57% activity of native modified 16S rRNA.[87] This single point mutation may sound minor but is significant in opening the door for further study of ribosomal components in the in vitro space. A classical problem with studying ribosomes in vivo is that lethal mutations can not be studied because they result in a dead cell.

The above technologies combined together culminate into R-iSat. R-iSat utilizes the PURE protein expression system to synthesize the 16s rRNA and assemble together the 30s ribosomal subunit. This approach allows for the synthesis and functional test of mutant 30s ribosomal subunits from DNA templates.[88] This technology is groundbreaking as a step towards fully achieving directed evolution of a ribosome. Once this goal is realized, selective pressure could theoretically be used to evolve a native ribosome into one that can self-replicate.

In addition to the ribosomal proteins, there are 31 other required translation factors involved in the minimal protein expression platform of the PURE system. In order to simplify the purification system needed to synthesize the PURE system, a 30-cistron oligo containing 30 of the 31 translation factors needed in the PURE system was constructed. With this oligo split between a few plasmids, it is possible to express all the 30 components of the PURE system from a minimal set of plasmids. Although this system is not currently functional in a cell-free protein expression platform, the availability as BioBricks of plasmids containing all the PURE components will no doubt be useful once cell-free protein expression is able to properly express that magnitude of genes adequately.[83]

6.4. Protein Folding

A problem that has classically plagued protein expression is the inability of prokaryotic systems to express larger and more complexly folded protein products. Just as with classical protein expression, different cell extracts are used for TX-TL systems depending on the protein of interest. *Saccharomyces cerevisiae* is a candidate for more complex protein product expression. Of course, even higher-level organismal extracts can be used for even more complex protein expression, but are more work-intensive and costly. Cell extracts from higher-level organisms are more adept at folding larger proteins due to a wider array of chaperone proteins.[71]

6.5. Post-Translational Modifications

A similar problem to complex protein folding is the lack of post-translational modifications available in bacterial cell extracts. One solution is identical to the above: use higher-level organisms for the cell extract to maintain the more complex protein expression machinery present in eukaryotes. However, work has been done to allow bacterial extracts to produce proteins with disulfide bonds through the addition of iodoacetamide to the cell extracts.[89] Addition of iodoacetamide acts to inactivate several enzymes in *E. coli* that reduce disulfide bonds during translation. However, this additive also nonspecifically inhibits many enzymes in the cell extract, reducing the overall yield of active protein. Recently, a method has been developed for removing these reducing proteins from the cell extract via affinity tags.[90] This is a much more desirable method for production of proteins that require disulfide bond formation due to its specificity of removing only the target reducing proteins without inactivating other cellular enzymes sensitive to iodoacetamide.

Similarly, bacterial cell systems lack the machinery to make glycosylation modifications to proteins in vivo or in vitro. Recent work has begun to address this through the synthesis of oligosaccharyltransferase enzymes alongside a protein target. These expressed oligosaccharyltransferases can successfully add glycosylation modifications to specific amino acid residues on the target protein, allowing bacterial extracts to perform feats classically reserved for mammalian cell extracts.[91] In more recent work, TX-TL protein expression machinery from *E. coli* strains was optimized for protein glycosylation. This

system synthesizes proteins from genes followed by efficient and site-specific glycosylation. Not only is this a major step forward in developing a tool for studying protein glycosylation in a highly controlled environment, but it also provides a means for synthetic cells to manufacture their own glycoproteins.[92]

7. tRNA

Although tRNAs are a relatively simple component of synthetic protein expression systems, they are worth mentioning tRNA production will be a non-trivial necessary function that a fully synthetic living cell will need. All protein expression systems discussed here contain aminoacyltransferases. In the case of the PURE system, they are individually purified along with all the other proteins and in the cell extract system; however, to avoid any codon bias, equal amounts of each tRNA is normally added to the reaction mixture.[93]

7.1. Incorporation of Unnatural Amino Acids

A distinct advantage to using a bottom-up approach in the construction of a synthetic cell is the ability to modify fundamental aspects of biology. For example, it has been shown that tRNAs can be charged with unnatural amino acids, that can then be utilized by the PURE system for synthesis of novel unnatural proteins.[94] The potential for a system that utilizes a wider amino acid library is great and largely unexplored. But it stands to reason that a larger and more versatile array of building blocks could aid in the quicker development of enzymes necessary for future synthetic cell technologies that may not exist in nature. It was additionally shown that PURE could function normally using solely in vitro transcribed tRNA, termed iVT-tRNA.[95] By demonstrating the versatility that arises from in vitro synthesized tRNAs, this work is a good step towards being able to incorporate unnatural amino acids into the codon table by working around the problems of tRNA modifications present in natural tRNAs. An interesting feature of this work is that because the new codon table lacks most of the redundancy present in the natural codon table, there are many open codons for which to assign unnatural amino acids.

Several other groups have realized the potential for new technologies that may arise with synthetic amino acids and have worked to develop the technology further. Notable among these is the incorporation of unnatural amino acid residues in GPCR proteins.[96] Other important and groundbreaking studies into the utilization of unnatural amino acid incorporation can be found in other excellent reviews on the subject.[97]

Further synthetic tRNA incorporation methods prove difficult as codons need to be reassigned, endogenous tRNAs need to be depleted, and tRNA modifications need to be taken into account. These restrictions and obstacles are currently being explored.[98] A method to combat some of these challenges is utilizing anti-sense oligonucleotides targeting certain endogenous tRNAs. This allowed the endogenous tRNAs to be sequestered and unavailable to compete with the added unnatural tRNAs during translation.[99] Other methods for endogenous tRNA removal include incubation of cell-extract with RNase coated magnetic beads to degrade the tRNA prior to addition of synthetically synthesized tRNAs.[100]

An unrealized area of study within this field is testing what advancements in unnatural amino acid incorporation have the versatility to be used within the synthetic cell community. For example, utilizing quartet codons via an evolved ribosome not only allows for unnatural amino acids to be incorporated much easier within the codon table but also allows for a codon table with a far greater capacity.[101]

8. Waste Removal and Nutrient Uptake

Metabolism requires uptake of nutrients from the environment and removal of waste products. In natural cells, these ends are achieved by a complex and highly controlled system of membrane transporters. Most of those complex natural systems are extremely challenging to reproduce in synthetic cells (**Figure 4**).

The general way molecules are transported between a cell and the environment is through protein channels. A major obstacle that has been impeding progress in synthetic cells is the inability to reliably make membrane proteins by cell-free means due to the troubles involved with synthesizing a trans-membrane protein outside of a membrane structure and inserting it into the liposomal membrane with directionality. Although advancements are currently being made, functional membrane protein synthesis still remains a challenge. More specific challenges and other recent solutions can be found in a recent review.[102]

8.1. Channels

Due to their complex, membrane-bound, and post-translational modification-heavy nature, membrane channels are difficult proteins to express in artificial systems. A notable channel that has been successfully expressed in cell-free transcription is the α-hemolysin protein, a small monomer protein from *Staphylococcus aureus* that assembles into a heptamer membrane pore. With this protein expressed in liposomes, the pore can be used to diffuse energy molecules and small reagent molecules such as nucleotides and tRNA into the lumen, allowing the protein expression pipeline to continue for an order of magnitude longer than when the pore is not expressed.[103] Because in most cases, there is no simple way to transport nutrients into synthetic cells, this system has major implications in "feeding" these synthetic cells. However, it is important to note that these pores work through passive diffusion and act differently than nutrient uptake does in natural cells.

Another area of membrane channel proteins research involves the ATP-synthase. Rather surprisingly, because of its relative complexity, size, and trans-membrane domains, the ATP-synthase was successfully synthesized utilizing the PURE protein expression system.[104] This finding was innovative in opening the gates for a newer more complex proteins to be added to the PURE expression toolbox. In a similar vein, it was demonstrated that functional proteorhodopsin (a light-activated proton pump) could form a proton gradient within liposomes that could then be utilized by ATP- synthase to

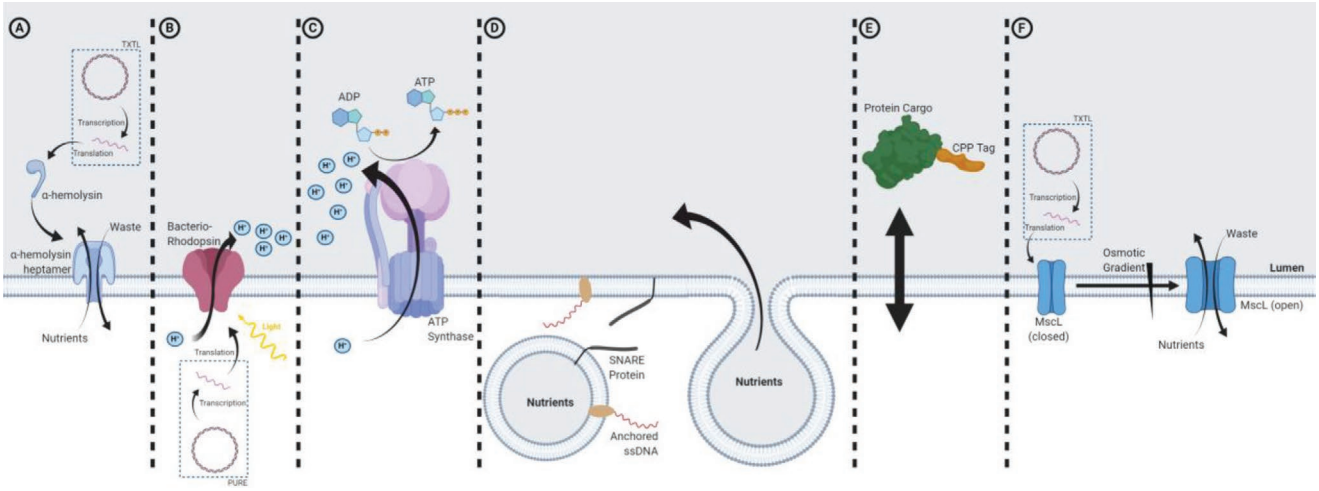

Figure 4. Various methods developed to transport biological molecules across a synthetic cell membrane. A) α-hemolysin is expressed via the TX-TL protein expression system. It spontaneously forms a heptamer and inserts itself within the liposomal membrane forming a pore that allows passive diffusion of waste and nutrient molecules. B) Bacterio-rhodopsin is expressed via the PURE protein expression system and inserts itself within the Giant Unilamelar Vesicle membrane. Utilizing light energy, this trans-membrane protein can pump protons against their concentration gradient and across the liposomal membrane. C) ATP synthase can be either incorporated or expressed within liposomes and is often paired with bacteriorhodopsin to utilize the created proton gradient to synthesize ATP. D) Vesicles presenting a SNARE protein or single-stranded DNA anchored in their outer membranes can be programmed to fuse with a liposome that is presenting the corresponding SNARE protein or complementary single-stranded DNA molecule. Upon fusion, any nutrients or cargo within the vesicle's lumen are delivered to the lumen of the liposome. E) Any protein expressed within liposomes or expressed prior to being encapsulated within liposomes can be tagged with a CPP that has the potential to allow passive diffusion of that protein across a liposomal membrane. F) MscL can be expressed via TXTL and insert itself within liposomal membranes. From there, the mechanosensor can sense a change in osmotic pressure and open up, becoming an open membrane pore allowing anything including nutrients, waste, or signaling molecules to diffuse across the membrane. Created with BioRender.com.

synthesize ATP on the outside.[63] If encapsulated within a larger vesicle, this smaller liposome could be used as a pseudo-organelle to synthesize ATP from light energy to drive other synthetic cellular reactions. The culmination of these two technologies was recently realized by utilizing the PURE system to express components of this bacteriorhodopsin/ATP-synthase energy generation system, that was then used to synthesize ATP energy to power more transcription and translation.[105] Because the focus of energy generation within synthetic cells has always centered around an ATP-regeneration system, the ability to power a liposomal bioreactor with something as versatile, cheap, and waste product-free as light is a major step forward for the creation of synthetic cells. Additionally, because these proteins are self-made, this system has the theoretical potential to be used for a future completely self-sustaining system.

Utilizing the protocol as above, a variety of trans-membrane proteins including a potassium ion pump were functionally expressed. Moreover, this technology was used to incorporate p-Azido-l-phenylalanine, an unnatural amino acid, into proteins via stop codon suppression demonstrating this system's utility towards the synthesis of novel synthetic biological constructs.[106]

Recent work has been invested into making use of MscL as a mechanosensitive channel expressed via TX-TL within synthetic cells.[107] The MscL forms a 3 nm pore, big enough to let even smaller peptides through (with MWCO of 6–10 kDa). This technology was expanded to allow for an adaptive response of the synthetic cell, a feature crucial to emulate in artificially made cells. Not only were the synthetic cells able to sense a change in osmolarity and regulate the opening of the MscL channel, but the MscL also allowed for an influx of chemical inducers that upregulated expression of cytoskeletal protein (MreB) that assembled within the cell.[108] This type of work is unprecedented in that it coordinates and couples the function of a tightly regulated gene expression system with a mechanosensitive channel that allows for the synthetic cell to take cues and adapt based on an environmental response. It is safe to say that work like this will remain crucial as we work to build cell models that can interact with each other, the environment, and other cell populations. Short term, the gated property of this channel is significant in comparison to α-hemolysin in that it allows for programmed control of the channel, apart from simple expression regulation.

Similarly, work has been done on utilizing pH-sensitive peptides to form pores in synthetic membranes in response to a pH change.[109] While this work was marketed towards drug delivery or cellular cargo delivery, the potential for allowing synthetic cells to respond to environmental pH changes has the potential to be useful.

8.2. Other Ways of Transport Across the Membrane

Trans-membrane channels are oftentimes difficult to express as a functional protein, so it is beneficial to the biotechnology community to have alternatives to utilize for delivering molecules to liposomal systems.

An alternative method for getting nutrients into liposomes is through fusion with other nutrient carrying vesicles. A preliminary investigation with this technology was done in which SNARE proteins were anchored in liposomal membranes and the SNARE proteins fuse multiple populations of liposomes. In their experiments, each population had a different gene product that would come together to complete the genetic circuit and yield a final product. Instead of a genetic circuit, this technology could be vital in supplying nutrients to synthetic cells by encapsulating "feeder liposomes" that could fuse with the synthetic cell via SNAREs to deliver these nutrient molecules. Additionally, because this fusion confers the lipid membrane as well, it has the potential to be an alternative to lipid synthesis in cases where lipid synthesis is problematic.[110]

8.3. Passive Transport

Another clever solution for membrane transport involves linking the cargo molecule with a cell-penetrating peptide (CPP) tag. CPPs are small peptides that are able to penetrate across the cell membrane and into a cell, which has allowed them to become extremely useful tools in cargo delivery in vivo and in vitro.[111] Covalent CPP tags have been used to bring proteins, nucleic acids, and synthetic molecules across phospholipid membranes for a wide array of biological purposes including: RNA silencing, drug delivery, or probe delivery. Some of the technical challenges are cell toxicity and immunogenicity, which of course are non-problems for use in liposomal systems that are not alive. The advantage for CPPs is in their simplicity; in much the same way as their in vivo uses, CPPs can allow for probe delivery, protein import/export, genetic material import/export, or diffusion of small molecules into liposomes, allowing difficult synthesis products to be supplemented instead of created.[112] By coupling a CPP tag onto a protein, it is possible to have this tag drag the protein across the liposomal membrane.[113,114] This has several implications, the first being that this would allow synthetic cells to excrete proteins that would allow them to interface with their environment or communicate with other populations of cells, synthetic or natural. Additionally, CPPs would allow for a mode of nutrient uptake. For example, because ribosomes are currently unable to be synthesized in a synthetic medium, ribosomal components could be tagged with CPPs and allowed to diffuse into the synthetic cells, replenishing their translation machinery as it begins to decay in activity. Of course, it is important to note that this is not true import/export as it is diffusion based; however, its usefulness is in its simplicity as there is currently no way to selectively import or export desired proteins without reconstituting entire membrane pores and channels, a feat not currently possible in liposomes.

9. Organelles

While simple synthetic cells are modeled after prokaryotic cells without defined organelles, significant effort has been directed towards reconstitution of natural cell organelles and engineering models of eukaryotic cells.[4,115]

9.1. Lipid-Based Organelles

Synthesis of a liposomal system that has distinct compartments for different enzymatic reactions is still in the early stages of development. An example of recent developments in this area is the ability to compartmentalize components of a multi-step pathway in different liposomes that shared a face. The intermediate products were able to be diffuse across this shared membrane to synthesize the final product. This is a major development in spatially compartmentalized reactions within synthetic cells in a manner mimicking that of organelle compartmentation in live cells.[116]

The future of synthetic cells that model eukaryotic cells is described in a recent paper.[117] The current stage of the synthetic biology community involves simply cutting and pasting biological mechanisms together to form simple step-wise pathways in synthetic cells. This group provides a vision and validation of components that would be required to make modular synthetic organelles. The advantage being that they can be combined together in certain combinations to do much more complex biochemistry than is currently possible in single pot synthetic cells.

Because of the difficulty associated with creating distinct compartments with differing inner conditions, other researchers have instead encapsulated live cells within liposomal cells. The two populations were able to work in concert together. In this case, the liposome could act as a physical barrier between the live cells and toxins, while the encapsulated bacterial cells were able to process feedstock and pass on a "metabolite" to the synthetic cell's machinery for further processing. This specific process is less important than the general ability of symbiosis demonstrated between natural and synthetic cells, reminiscent of symbiogenesis.[118]

As described above, a pseudo-chloroplast was made in the form of small vesicles containing functional proteorhodopsin and ATP-synthase. These small vesicles were encapsulated within a much larger one allowing them to synthesize ATP via a phosphorylation reaction to power actin polymerization within the larger vesicle.[63]

Similar work is being done via the incorporation of larger cellular components coupled with synthetically made pieces. An excellent example of such technology is isolating chromatophores from *Rhodobacter sphaeroides* and encapsulating them within giant liposomes. During illumination, the chromatophores were able to phosphorylate ATP from ADP and sustain the transcription of encapsulated gene products. Further work was done via cryo-EM to better visualize the orientation of the proteins and different compartments.[119] Until fully artificial systems can be properly controlled and assembled, work like this is promising for allowing us to harness the organization and structure already present in biological living systems.

In spite of the difficulty in making multi-lamellar liposomes, microfluidics is showing great promise in the ability to synthesize multi-compartment liposomes with great speed, accuracy, and efficiency. Utilizing different reagents during the dewetting process of microfluidic double emulsion liposome construction is the key to allow multiple compartments to form within single larger liposomes.[120] Because microfluidics is a much more precise way of synthesizing liposomes, this ability to

consistently form liposomes has the potential to open the gates for a much wider range of eukaryotic mimics in the synthetic cell community. For example, reactions that have incompatible reaction conditions can now be coupled together within the same synthetic cell by having each one take place within a spatially separate compartment.

Of course, in addition to making functional organelles that produce energy, metabolites, or substrates important for cellular function, communication between organelles remains another hallmark of life that will need to be reconstituted within synthetic cells. This was successfully done in a synthetic cell harboring two different vesicles encapsulated within itself. One of these protorganelles contained glucose oxidase and the other contained horseradish peroxidase and Amplex red. When glucose was allowed to diffuse through the larger vesicle membrane through a pore, it was oxidized to make hydrogen peroxide, which continued on to the horseradish peroxidase containing vesicles to oxide Amplex red into resorufin as a reporter.[121] This technology demonstrates a sought after result of having two synthetic cell organelles engage in signal communication. This proof of concept paves the way for further study in redox reactions being used to modulate intra-synthetic cell communication, or for other methods of protorganelle cooperation. A system that shares similar themes was developed that makes use of light as the means for regulation instead of redox chemicals. By incorporating diacetylene functional groups on some lipids, these are able to crosslink in response to UV irradiation that forms pores within the membrane. By incorporating this technology on a liposomal membrane inside a larger vesicle, light can be used to induce the release of compounds initially segregated from the cytosol of the larger vesicle.[122] In the study β-galactosidase was released allowing for the catalyzation of a substrate (fluorescein di-β-D-galactopyranoside) to free fluorescein, acting as a reporter; however, like the previous study, this technology of light-controlled protorganelles is flexible and can be coupled with limitless other reactions.

Proof of concept work was done in which synthetic amphipathic molecules were used to encapsulate enzymes and reagents composing a three enzymatic cascade reaction. The resulting "artificial organelles" were then encapsulated inside a much larger compartment also composed of synthetic amphipathic molecules to create a functional cell mimic. The organelles work collectively to synthesize the final product.[123] Although the encapsulating molecules were not biological, this is an important proof of concept that shows enzymatic reactions can work between organelles within synthetic cells, acting as a more accurate model of eukaryotic cellular life than single compartment liposomes.

A similar idea of a programmable response through the use of engineered microcompartments is explored through the use of amphiphilic block polymers. These reduction-sensitive molecules are self-assembled to encapsulate either enzyme substrates or ion channels and ultimately encapsulated within a GUV. When a reducing agent is added to diffuse across the GUV membrane, it disrupts the block polymers and allows for the release of the cargo, triggering either an enzymatic reaction or ion flux from pore incorporation within the GUV membrane.[127] The strength of this system is its versatility in mimicking cellular signaling. Any type of cargo can be incorporated within these block polymer subcompartments and thus a virtually limitless number of potential reactions can be programmed within synthetic cells in response to addition of a reducing agent.

9.2. Membraneless Organelles

Multi-lamellar liposomes that act as compartmentalized sectors of a synthetic cell are hard to develop because of the difficulty in consistently synthesizing these multi-lamellar liposomes. Recent work has developed an alternative approach for the synthesis of artificial organelles from protein cages. They were able to express self-assembling proteins in *S. cerevisiae* that can selectively target heterologous proteins for compartmentalization. They comment on the ability of these encapsulins to act as separate compartments for housing enzymatic reactions.[124]

An alternative approach to developing enclosed spaces in liposomes makes use of the interaction of RNAs and cationic peptides to form complex coacervate acting as compartments. This self-assembling activity can be mediated via enzymatic phosphorylation of the peptides. Being able to control compartment formation is an extremely useful tool to have when attempting to construct a cell. Applying this technology to synthetic cells would allow organelles containing release molecules to the rest of the cell in response to stimuli through the form of genetic circuits.[125] This idea is explored through work in making a pH-controlled coacervate compartment within liposomes. This reversible process relies on the ability of a poly-cation to form the compartment when subjected to a pH under its pK_a and ultimately trigger liquid–liquid phase separation. This work is even coupled with cellular function showing how coacervate formation can trigger a stronger enzyme response due to the crowding that occurs within the coacervate droplet.[126] This technology is already impressive and has the potential to grow into a unique way to regulate both reaction conditions within a liposome as well as trigger a cellular response to a pH change.

Similar work has been done by studying bacterial microcompartments, small proteinacious compartments found in bacterial species.[128] By studying how these microcompartments are formed and how protein cargo can be selectively targeted to the inside, the goal is to eventually utilize the same technology to form small protein-bounded organelles in synthetic cells. These may even have advantages over traditional membrane-bound organelles for specific applications due to how they are formed in a fundamentally different manner. Much more work outside the scope of this review has been done on the role microcompartments will play in synthetic biology. Further reading can be found from an excellent review on the subject.[129]

10. Inter-Cellular Communication and Environment Interaction

Just as live cells sense changes in the environment, interact with other species/cell types, and exchange chemical messages with each other, synthetic cells must be able to do so as well. Affording synthetic cells the ability to communicate with

each other would open up the doors to the study of population dynamics as well as genetic circuits between multiple populations to build larger and more complex responses. More general environmental interaction is even more fundamental as that allows for everything from nutrient collection to maintaining homeostasis in the face of adverse conditions.

Work is already being done on designing nano-scale bioreactors that can communicate between populations. For example, synthetic cells were programmed to express α-hemolysin in response to an induction signal. α-hemolysin self-assembled into pores within the membrane, allowing for the free diffusion of encapsulated glucose. A second population of proteinosomes was designed such that their proteinaceous shells oxidized the glucose to produce hydrogen peroxide as a by-product, which triggered the HRP-mediated peroxidation of the reporter, Amplex red within the proteinosomes.[130] The importance of demonstrating that both communication between populations of synthetic cells is possible and that fundamentally different types of synthetic cells can be made to communicate cannot be overstated.

Additionally, allowing synthetic cells to interact with live cell populations has the potential to provide an unintrusive and highly-programmable interface with cell populations, be it within the human body or hard to assay bacterial communities. A good example of this is through the design of a genetic circuit created between *E. coli* and synthetic cells. The expression of α-hemolysin is controlled via a theophylline inducer, triggering α-hemolysin expression in the presence of theophylline and allowing the encapsulated IPTG to diffuse out of the lumen. The IPTG is then allowed to diffuse into the *E. Coli* and trigger a cellular response via a classical IPTG-induction cassette.[131] This work works as a proof of concept for both network construction between different synthetic cell communities as well as demonstrates an unintrusive way to modulate the behavior of natural or unmodified cell populations. This work was expanded upon with a two-way design and better characterization of the relationship that emerges when synthetic cells are allowed to be "seen" by bacteria via quorum sensing. In this study, the responses of both synthetic cell populations expressing quorum sensing molecules and several bacterial species are assayed to determine if synthetic cells can pass a "Turing Test" of sorts- meaning is it possible to have the bacterial cells sense the synthetic cells as other bacteria.[132] The results were exciting and intriguing in that only one of the three populations assayed was able to reliably reconstitute the cycle of sensing and relaying the signal that is the cornerstone to quorum sensing. This was most likely due to both a lack of complete understanding of quorum sensing mechanisms as well as a lack of sensing enzymes in the synthetic cell membrane; however, this was an important first step in both beginning to understand the relationships that can emerge from live-synthetic cell interactions as well as the beginnings of constructing these relationships.

In addition to bacteria-like modeling, eukaryotic tissue-like modeling is an important facet of synthetic biology that has only recently found the technology to make it feasible. The first of these is 3D-printing, which allows for synthetic cells to be printed into a range of 3D structures.[133] Although this is done with droplets, it is only a matter of time that synthetic cells are offered the same level of flexibility. A second area of study necessary to allow for synthetic tissues is that of the communication. Just as how gap junctions allow adjacent cells to pass along a signal and communicate, two α-hemolysin pores were covalently linked to span across the membranes of two liposomes linking them and thus allowing chemical signals to travel between the two lumens.[134] Similar technology includes the use of the Delta-Notch signaling system to send and receive a signal between adjacent cells creating a positive feedback loop. The way it works is a Notch membrane protein mechanically senses the Delta membrane protein on an adjacent membrane. This induces a conformational change that results in the Notch intracellular domain on the lumen side of the protein to be cleaved, travel to the nucleus, and induce expression of the Delta protein that localizes to the membrane. This Delta protein signals an identical response in other adjacent cells, causing the signal to propagate.[135] Both of these technologies are impressive and allow for communication on a population-scale of synthetic cells that was not previously possible; however, there is still much work to be done in relaying the number and complexity of signals both within and among multiple synthetic cell populations.

Lastly, demonstration of synthetic cells sensing environmental conditions is demonstrated nicely in recent work that combines a multi stimuli adhesion unit with an energy conversion unit. The technology combines the light and redox-sensitive protein interaction of iLID and Nano with pH-sensitive and metal ion mediated binding property of His-tags as well as a light to ATP conversion module. With these modules, the synthetic cell is able to use the presence of light, non-oxidative conditions, neutral pH, and the presence of metal ions in order to determine if it will adhere to a particular surface.[136] It is this complex multi-stimulation sensing ability that will allow synthetic cells to approach the complexity of environmental interaction that is apparent in live cells.

11. Summary and Further Reading

Most live cell processes have been reconstituted, at least in one form, in synthetic cells. The notable exceptions are those of self-replication of the translation system and the spontaneous reproduction of the membrane.

It is difficult to speculate when will a fully alive, self-replicating synthetic cell is capable of undergoing Darwinian evolution. The recent progress in the field suggests such live artificial cell is only a few years away, and the synthetic cell technologies are rapidly evolving to take advantage of the newest capabilities.

This is a fast-growing field with many applications emerging from different implementations of above-described advancements.

Applications of synthetic cells span many areas of basic research and practical fields. Synthetic cells are used as chassis for investigating biological processes, including reconstitution of signaling pathways[137,138] and construction of complex genetic cascades.[110] Synthetic cells can be chassis for constructing biosensors,[139–141] for metabolic engineering,[142–144] for production of viruses;[145] it was even demonstrated that synthetic cells can shrink solid tumor in live mice.[146]

Table 3. A list of excellent and relevant reviews on the subjects discussed.

Title	Keywords/grouping	Refs.
Bottom-Up Synthetic Biology: Reconstitution in Space and Time	Bottom-up synthetic cell; gene regulation; transcription; translation; cytoskeleton	[3]
Artificial Cells: Synthetic Compartments with Life-Like Functionality and Adaptivity	Bottom-up synthetic cell; compartmentalization; genetic circuits; growth; division; environmental interaction; sub-system compatibility	[4]
Shaping Up Synthetic Cells	Bottom-up synthetic cell; cytoskeleton; shape; morphology	[147]
Chapter Nine: TX-TL-Based Approach to Synthetic Cells	Methods; TX-TL; encapsulation; membranes	[1]
The Hallmarks of Living Systems: towards Creating Artificial Cells	Requirements of life; modules	[8]
MaxSynBio - Avenues towards Creating Cells from the Bottom Up	Bottom-up synthetic cells; collaboration	[9]
Synthetic Biology: Integrated Gene Circuits	Genetic circuits	[148]
Cell-Free Protein Synthesis in Micro Compartments: Building a Minimal Cell from Biobricks	Bottom-up synthetic cell; sub-system compatibility; self-organization	[149]
Mastering Complexity: Towards Bottom-up Construction of Multifunctional Eukaryotic Synthetic Cells	Bottom-up synthetic cell; sub-system compatibility; eukaryotic mimics; microfluidics; DNA nano-technology	[115]
Tailoring the Appearance: What Will Synthetic Cells Look Like?	Bottom-up synthetic cell; membranes	[11]
Cell-Free Systems in the New Age of Synthetic Biology	Cell-free protein expression; synthetic biology	[150]
Overview of Cell-Free Protein Synthesis: Historic Landmarks, Commercial Systems, and Expanding Applications	Cell-free protein expression	[151]
Open Problems in Artificial Life	Requirements of life; modules	[6]
Communicating artificial cells	Intercellular communication	[152]
Progress Toward Synthetic Cells	Compartmentalization; genome replication; sub-system compatibility	[22]
The emerging age of cell-free synthetic biology	Genetic circuits; cell-free protein expression	[153]
How to Make a Minimal Genome for Synthetic Minimal Cell	Minimal genome; genome replication	[154]
Minimal Cell Mimicry	Minimal genome;genome replication; growth	[155]
Cell-Free Biology: Exploiting the Interface between Synthetic Biology and Synthetic Chemistry	Bottom-up synthetic cell; synthetic biochemistry	[156]
Semi-Synthetic Minimal Cells as a Tool for Biochemical ICT	Environmental interaction; signaling	[157]
Semi-Synthetic Minimal Cells: Origin and Recent Developments	Bottom-up synthetic cells; encapsulation; Cell-free protein expression	[158]
Approaches to Chemical Synthetic Biology	Synthetic biochemistry; synthetic biology	[159]
Cell-Free Synthetic Biology: Thinking Outside the Cell	Cell-free protein expression; genetic circuits	[160]
Piecing Together Cell-like Systems	Bottom-up synthetic cells; sub-system compatibility	[161]
Build Life to Understand It	Synthetic biology	[162]
Designing Synthetic Biology	Synthetic biology	[163]
Synthetic Biology Moving Into the Clinic	Synthetic biology; clinical applications	[164]
Towards Synthesis of a Minimal Cell	Bottom-up synthetic cells, minimal genome, Genome replication, cell free protein expression	[165]
Engineering Protocells: Prospects for Self-Assembly and Nanoscale Production-Lines	Bottom-up synthetic cells; sub-system compatibility	[166]
Integration of Biological Parts toward the Synthesis of a Minimal Cell	Bottom-up synthetic cells; sub-system compatibility	[167]
Artificial Cells: Building Bioinspired Systems Using Small-Scale Biology	Bottom-up synthetic cells	[168]
A Chemical Engineering Perspective on the Origins of Life	Synthetic biochemistry	[169]
The Ten Grand Challenges of Synthetic Life	Obstacles to synthetic cells	[170]
Cell-Free Protein Synthesis: Applications Come of Age	Cell-free protein expression	[171]
Synthetic Organelles	Organelles	[172]

In a field as diverse as synthetic biology, there is much more work and technologies that could not be fit within this discussion. For further reading, please see **Table 3** for a list of excellent reviews and opinions on the subject.

12. Speculations and Future Directions

In this review, we highlighted the development of key elements necessary to perform most crucial functions of live cells in

synthetic, engineered systems. We understand that this is not a complete list of all elements of natural cells, nor is it an exhaustive overview of all possible activity in the field of synthetic cell engineering. We focused on elements of natural cells that are, in our opinion, key to building universal and lineage agnostic chassis for building synthetic cells.

We speculate that the next main areas of rapid progress in this field will be the replication of synthetic cell genome and compartment. We are aware of many groups actively working on solving membrane synthesis, genome replication, and compartment replication problems we described in this review.

One area where there is need for more focused effort is combining existing modules into functional, coordinated chassis. We have a lot of modules that provide some key functionality of live cells, but not a lot of work has been done on integrating various functionalities, and on testing robustness of the system comprised of more than one of those modules. Problems like managing resource allocations, gene expression control, or creating genetic and metabolic networks across various elements of synthetic cells will need to be addressed. As the synthetic cell grows in complexity, those problems will become more pressing and crucial for the success of the whole field. We hypothesize this might become the greatest challenge in the next 5 years of synthetic cell engineering. Given how complex, and still poorly understood, are the mechanisms natural cells use to control cell cycle, we speculate that synthetic cell community will build new, artificial ways of controlling and integrating all subsystems. Instead of replicating the natural control systems, we will engineer new checkpoints, genetic circuits, and feedback mechanisms for synthetic cell chassis.

Many elements still remain to be built. However, it is very hard to create a specific list of missing parts, as this would require generating exhaustive and complete list of elements necessary for life, thus creating an objective definition of life. Such a definition does not exist. The most commonly agreed in the field is the NASA definition of life: a chemical system capable of Darwinian evolution. This definition does not provide a list of necessary components, and no other definition of life is nowhere nearly universally accepted. It is, therefore, hard to define what elements of natural cells are still missing from the reconstitution efforts.

The definition of life, and thus the specific finish line in the effort to build the first living artificial cell from nonliving components, is very elusive. We speculate that once we engineer a minimal cell that propagates itself using substrates from the environment, most people will agree that this synthetic cell is „alive". Therefore, the minimal list of elements necessary to perform life will be defined by the very existence of the first artificial living cell built from nonliving components.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

artificial life, bioengineering, cell-free protein expression, synthetic cells

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