Biochemistry Perspective:

Synthetic biology for fundamental biochemical discovery

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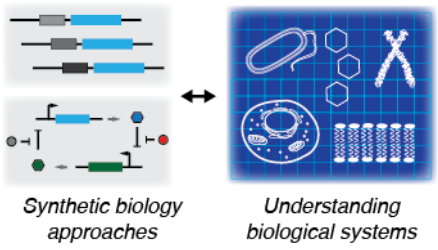
Abstract

Synthetic biologists have developed sophisticated molecular and genetic tools in order to engineer new biochemical functions in cells. Applications for these tools have focused on important problems in energy and medicine, but they can also be applied to address basic science topics that are not easily accessible by classical approaches. We focus on recent work that has utilized synthetic biology approaches – ranging from promoter engineering to the *de novo* synthesis of cellular parts – to investigate a wide-range of biochemical and cellular questions. Insights obtained by these efforts include how fatty acid composition mediates cellular metabolism, how transcriptional circuits act to stabilize multicellular networks, and fitness trade-offs involved in the selection of genetic regulatory elements. We also highlight common themes about how 'discovery by synthesis' approaches can aid fundamental research. For example, re-wiring of native metabolism through metabolic engineering is a powerful tool for investigating biological molecules whose exact composition and abundance is key for function. Meanwhile, endeavors to synthesize cells and their components allow scientists to address evolutionary questions that are otherwise constrained by extant laboratory models.

TOC Figure:



Main text

The rapid development of synthetic biology over the past twenty years has drawn comparisons to the rise of organic chemistry in the 19th century[1]. In both cases, the philosophical underpinnings of the nascent field – discovery through forward engineering instead of reverse analysis – serve as more of a pronounced shift than the specific techniques performed by its scientists. Where synthetic biology has differed is its focus and dependence on the development of tools: research processes and molecular parts that allow scientist to engineer cell and organisms functions faster, more precisely, and with greater reliability (Figure 1). The complexity of even the simplest biological systems necessitates robust tools for their manipulation, but it also aides tool creation. Many components in the synthetic biologists toolset originated from molecular and biochemical studies on their native functions. Synthetic biologists generally refine these tools to engineer cells for new applications, such as producing a commodity chemical or detoxifying contaminated groundwater. Along the way, however, synthetic biology tools become highly developed, and can then be reapplied to probe basic biological and biochemical questions. In this Perspective, we summarize the relationship between tool development and fundamental discovery in biochemistry, and then briefly describe several examples in which synthetic approaches have been fruitful in generating mechanistic insight. These examples span a diverse set of topics, covering membrane lipid composition, cofactors in cell metabolism, fitness trade-offs in gene regulations, mechanisms behind intercellular communication, and processes that could have allowed for the chemical origin of cells.

A classic example of a synthetic biology development cycle is that of bacterial promoters, a commonly used part for manipulating gene expression and protein levels in cells. In *E. coli*, pioneering studies on the regulation of bacterial operons, such as araBAD for arabinose catabolism[2], led to a deep mechanistic understanding of these genetic elements. The promoters from these systems were then cloned into expression vectors that allowed novel capabilities, such as inducible, on-demand expression of toxic genes in the case of the arabinose-induced promoter (P_{BAD})[3]. Because balancing expression

levels in a biochemical pathway is often critical for optimized performance, titratable promoters became an early cornerstone of synthetic biology. However, these applications led to the limitations of native promoters to become apparent. For example, inducible promoters generally feature an 'all or none' behavior, so intermediate induction levels in a population actually represent changing proportions of two populations. This behavior results from a positive feedback loop, in which the expression of genes encoding transporters for the inducer (e.g. arabinose) are also under induction. For P_{BAD}, engineering of strains with constitutive expression of arabinose transporters then allowed for true homogenous, titratable expression[4]. Promoter engineering efforts have driven the identification and optimization of versatility promoters in a variety of systems[5], including ones that take on more complex responses, such as dynamic regulation in response to pathway intermediates[6]. Tools for post-transcriptional regulation, such as targeted and inducible bacterial proteases developed by Cameron and Collins[7], can be used to further tune protein levels in a temporal manner.

While titrating gene expression has become a mainstay of metabolic engineering, it can also be reapplied to studying native biochemical pathways in cells. This approach is especially well-suited for investigating complex molecular compositions, such as lipids in cell membranes. Cells contain a multitude of distinct lipids, which vary widely tremendously between different cells, tissues, organelles, and growth conditions, suggesting that these molecules play distinct functional roles[8]. The synthetic pathways for common lipids have been illuminated by a combination of biochemical and genetic approaches. Understanding functional roles for specific lipids remains a challenge, however, because of poor tools for manipulating their composition *in vivo*[9]. Standard genetic approaches – e.g. gene knockouts – provide little functional information for the essential lipid species that make up the bulk of membranes. Instead, it is the stoichiometry between different lipids species – sterols vs. phospholipids, saturated vs. unsaturated acyl chains, glycerol lipids vs. sphingolipids etc. – that determines the physicochemical properties of membranes and varies most dramatically between membrane compartments. Classic chemical approaches for manipulating lipids, such as feeding cells specific fatty acids[10], are hampered by their dependence on complex uptake processes,

which lead to poor stoichiometric control. Recent advances in chemical biology tools do show promise for manipulating lipid composition in a more precise manner. In one recent example[11], Rudd and Devaraj used a chemical ligation strategy to control ceramide synthesis in mammalian cells. However, while chemical approaches can be powerful for characterizing acute effects of lipid composition, such as ceramide-induced apoptosis, homeostatic pathways in cells eventually compensate against such perturbations.

To carry out functional studies of steady-state lipid composition in cells, careful genetic rewiring of lipid metabolism is needed to achieve experimental control of these parameters *in vivo* (Figure 2). Pioneering studies by Dowhan and colleagues first used genetic tools to characterize the effects of cardiolipin levels on supercomplex assembly in yeast[12] and phospholipid head groups on protein translocation in *E. coli*[13]. We have recently taken this approach to study how cellular functions are dictated by the viscosity or fluidity of their membranes. In *E. coli*, titrating expression of a single gene in unsaturated fatty acid (UFA) biosynthesis using the P_{BAD} promoter system allowed us to arbitrarily manipulate UFA levels and control inner membrane viscosity over a ten-fold range[14]. Physiological experiments then uncovered a specific process – cellular respiration – that was mediated by membrane viscosity. This insight led us to develop a mechanistic model for the electron transport chain (ETC) based on the diffusion of electron carriers (quinones). In this model, membrane viscosity determines the rates of diffusion-mediated reactions in the ETC, and thus sets a 'speed limit' for cellular respiration. We also observed that UFAs levels mediate mitochondrial respiration rates in engineered yeast strains in which expression of *OLE1*, encoding for the sole yeast desaturase, was titrated. Notably, the inner mitochondrial membrane lacks rigidifying lipids, sterols and sphingolipids, which are abundant elsewhere in the cell. Lipid composition in eukaryotic cells could thus be optimized for maintaining membrane fluidity in respiratory membranes.

In previous work, we also used rational manipulation of UFA synthesis by *OLE1* modulation to characterize the yeast transcriptional response to changes in membrane fluidity[15]. This study revealed how lipids mediate a major pathway for yeast flocculation

(cell-cell adhesion) triggered by hypoxia, which represses Ole1 activity during fermentation. Membrane fluidity, which is sensed by specific proteins in the ER membrane, likely plays a major role in mediating the global transcriptional response to oxygen restriction, as yeast lack any direct means of oxygen sensing. Using global transcriptional analysis on lipid-engineered strains, we identified a large (>90) set of genes whose expression is regulated by UFA levels during yeast oxygen restriction.

Compared to titratable systems, a more generalizable strategy for manipulating protein abundance is through promoter libraries – either native[16] or synthetic[17]– that sample a range of expression strengths. These tools have allowed researchers to ask broader questions about the relationship between expression levels and biological function. In one recent example, a synthetic promoter library was used to systematically probe the effects of gene expression on growth of yeast[18]. Not surprisingly, the authors observed that the fitness-expression relationship was highly dependent on growth conditions and the specific gene tested in a manner that was consistent with their biochemical function. They also observed that cell to cell variability in expression was lowest in genes that had a tight fitness peak surrounding endogenous expressions levels, suggesting active tradeoffs in the evolution of noise in gene expression. Another elegant study used a library of synthetic enhancers – genetic elements neighboring promoters that additionally regulate expression levels – to examine the trade-offs in gene expression during vertebrate development[19]. Farley and colleagues generated an enormous library of barcoded random enhancers for the *Otx* gene, which controls neural plate patterning in sea squirts (*Ciona intestinalis*). They found that optimized enhancer sequences – which led to strong expression compared to the native sequence – were common in their library, but these led to ectopic expression of *Otx* and nonspecific patterning. Imperfect enhancers generally led to weak expression with specific patterning, and the native sequence balanced these properties. Such a 'goldilocks effect' nicely mirrors biochemical pathway optimization efforts, where efficient chemical production often requires intermediate levels of enzyme to balance the metabolic pathway instead of simple maximum overexpression[20].

Synthetic biology projects, especially those aiming for the production of complex chemicals in non-native hosts, often require both the manipulation of native metabolism and the expression of heterologous genes. For example, robust production of the anti-malarial drug precursor artemisinic acid in yeast[21] required the upregulation and downregulation of native genes involved in isoprenoid synthesis in conjunction with the introduction of a set of genes from *Artemisia annua.* Similar endeavors have led to considerable technology development for the identification, handling, expression, and optimization of genes or whole pathways, allowing their functional transfer from native organisms to a production chassis. One way this technology can be harnessed for basic research is to allow for the study of exotic molecules and pathways in a controlled host. In the context of membrane biology, many unique lipid species are produced in extremophilic or otherwise unculturable organisms, and their production in classic model systems could allow for their functional investigation or identification of unknown genes in their biosynthesis. An example of this approach is the impressive effort is the introduction[22] and further development[23] of a pathway to heterologously synthesize archaeal, isoprenoid ether lipids in *E. coli*. The latest work on this front[24] has used classical metabolic engineering to optimize the pathway, generating *E. coli* strains with up to 30% archaeal phospholipids. These strains allow for the characterization of the effects of these poorly-understood lipids on fitness and chemical tolerance. In a more modest example, we have introduced the pathway for branched chain fatty acid biosynthesis from *Bacillus subtilis* into *E. coli*, which allowed us a second means of controlling membrane viscosity in addition to lipid unsaturation[14].

Heterologous gene expression can also be used to rewire cellular metabolism in order to optimize it for chemical production[25] or study its responses to perturbations. The latter has been demonstrated by work that has used soluble oxidase enzymes from bacteria to investigate metabolic questions in eukaryotes, such as the Crabtree Effect in yeast[26]. More recently, this approach has been applied to interrogate functions for the universal cofactors NAD^+ and NADH in HeLa cells[27]. Titov et. al. characterized a soluble NADH oxidase from *Lactobacillus brevis* (*Lb*NOX) that donates electrons to water, forming NAD^+ and water in a non-productive reaction, in contrast to cellular oxidases which participate

in ATP production by the ETC. Strikingly, expression of *Lb*NOX rescued cell growth when the ETC was non-functional due to specific inhibition. This result indicates that defects in ETC activity arrests cell growth not because of a loss of ATP production, which can occur through glycolysis, but because of an unbalanced NAD^+/NADH ratio, which many synthetic pathways depend on. In a follow up study by the same group[28], a mutated *Lb*NOX variant was generated that is highly specific for NADPH oxidation (TPNOX). Experiments then used both NADH and NADPH oxidases to find that the mitochondrial $NADP^+$/NADPH and NAD^+/NADH reduction potentials are asymmetrically linked.

The expression of individual or sets of genes can be further engineered into arrangements that allow for responsive control in the form of biological circuits. While circuits are inherently present in biological system, e.g. in bacterial operon regulation, much of the initial work on synthetic circuits aimed to mimic motifs found in electrical circuits, such as toggle switches[29] or oscillatory circuits[30]. These studies provided insight on native biological circuitry and the topological organization of signaling cascades, which has been previously reviewed[31]. More recently, there has been an effort to design circuits that govern cell-cell interactions, such as engineering a T-cells to selectively kill cancer cells[32]. Circuits are also likely to underlie much of the unique characteristics inherent to multicellular systems, where neighboring cells must carry out intra-cellular communications and processing in order to organize themselves, coordinate growth, and share nutrients. In one recent study[33], Zhou et al. showed that macrophages and fibroblasts natively form stable two-cell circuits that cause mixed populations to converge to a specific stoichiometry of the two cell types. The macrophage-fibroblast system is based on the exchange of two growth factors across cellular contact sites, and the authors used computation network motif analysis, characteristic of synthetic circuit engineering, to identify negative feedback as the key parameter in maintaining the stability of the circuit.

Because circuits underlie cellular interactions in complex organisms, engineering them into model systems is an avenue for researching the evolution and properties of multicellularity. This has been recently demonstrated by an elegant set of experiments

that use synthetic notch receptors coupled to cell adhesion genes (cadherins) to drive the organization of fibroblasts into multilayer structures[34]. By introducing new components to the circuit, such as additional notch ligands or transcriptional repressors, the authors were able to increase the complexity of the structure through additional layers or asymmetry. The assembly of these multi-cell consortiums was reproducible, reversible, and in specific cases could even regenerate after cleavage. These results suggest that similar motifs could underlie the robust patterning seen in metazoan tissue development.

Beyond genetic pathways and circuits, there has also been increased focused on synthesizing and redesigning whole parts of cells *de novo*. The synthesis and transduction of a small bacterial genomes early in the decade[35] foreshadowed efforts to synthesize whole chromosomes, which is the focus of the ongoing Synthetic Yeast 2.0 project. In this ongoing project, six of the sixteen linear chromosomes of *S. cerevisiae* have so far been replaced by modified versions through heterologous recombination of synthesized fragments in a piece by piece fashion. The ultimate aim of this endeavor is to introduce features, such as recombination sites and easily-modified tRNA genes, that allow for easier genome-wide engineering of yeast functions. These features will also researchers to test how large changes to genome structure mediate function. One insight has already arisen is the surprising level to which large changes in chromosome structure, induced for example by the removal of the rDNA repeat regions of chromosome XII[36], do not necessarily lead to large changes transcriptional profiles and cell fitness. A pair of recent studies has come to the similar conclusion from engineering yeast strains with only two[37] or one[38] chromosomes. Not surprisingly, the reduction in chromosome number dramatically altered their topological organization in the nucleus, which was measured by chromosome conformation capture using high-throughput sequencing. However, yeast with only a single, large chromosome are still viable, and show only minimal defects in fitness or transcriptional profiles. This striking result indicates that functional importance of chromosome topology should not be assumed, at least in yeast. Future work with these strains should uncover what, if any, are the benefits to a multi chromosome arrangement, which is typically found in eukaryotic cells.

Research on synthetic chromosomes is an example where synthetic biology approaches are probing functional and evolutionary questions that are not addressable by previously existing models. The same logic applies to many of the structures and features that define complex organisms, ranging from key macromolecules to whole organelles (Figure 3). It is difficult to track the evolution of cellular structures in the laboratory, but synthesizing intermediate steps of a pathway and testing their functions is an alternative way of addressing the same underlying questions. The evolution of cellular complexity during emergence of eukaryotic cells, for example, has been partially informed by the characterization of eukaryotic-like archaea[39] and mitochondrial ancestors[40] through metagenomics. However, we have very little understanding about key steps in this process – for example, how endosymbiosis progresses to generate highly interconnected metabolic organelles. The mechanisms behind these processes could be explored with creative synthetic biology models for key intermediate steps that are generated with either 'top down' or 'bottom up' approaches. Motivation for the latter approach can be found in the *in vitro* exploration of simpler, protein-based, bacterial organelles – such as carboxysomes – by synthetic biologists in recent years. This field has been motivated by heterologous expression of these structures and their potential to act as scaffolds and platforms for metabolic pathways. However, these same studies have also illuminated many of the structural and biophysical aspects of their assembly and function[41,42].

The most challenging question on cellular evolution is how biological building blocks can assemble into cell-like structures *de novo* – a process that must have occurred for life to arise on the early earth. Biochemical work on the mechanisms behind the origin and early evolution of life have been almost entirely synthetic in nature. The past few years have seen dramatic advances on synthetic models for replicating primitive membrane compartments[43,44], partially self-copying ribozyme[45], self-sustaining activation chemistries[46], and mechanisms by which early cells could evolve [47]. The most significant hurdle for building synthetic cells in the lab – and potentially during the origin of life – remains efficient chemical copying of RNA or other genetic polymers without the use of highly complex enzymes. Here too, however, synthetic approaches, including the application of non-natural nucleobases[48] and leaving groups[49], have yielded important

insights to the mechanisms behind non-enzymatic polymerization. The development of a fully synthetic cell in the lab is a long standing goal of the field and will provide an invaluable model for investigations on how purely chemical systems can transition into ones capable of Darwinian evolution.

Outlook

Synthetic biology is a discipline that will continue to transform the world of biotechnology and medicine. We anticipate that the tools and approaches it develops will also lead to considerable advances in basic science. We highlight two specific types of biological questions that in our view benefit from synthetic biology approaches. The first involves study of molecules whose exact composition is key for understanding their function. Examples include lipid biochemistry in cell membranes and enzymes and their cofactors in central metabolism. The second involves processes that underlie the evolution of cellular components, such as the emergence of metabolic organelles or optimization of developmental enhancers. In the process of addressing basic questions, new applications will undoubtedly also arise. Lipid components could be explored that make industrial microbes more solvent resistant products, for example, or synthetic cells developed that can then explore new chemistries. It is this continual interplay between fundamental, mechanistic science, and demanding technological applications that has underpinned the history of the field and will continue to do so in the future.

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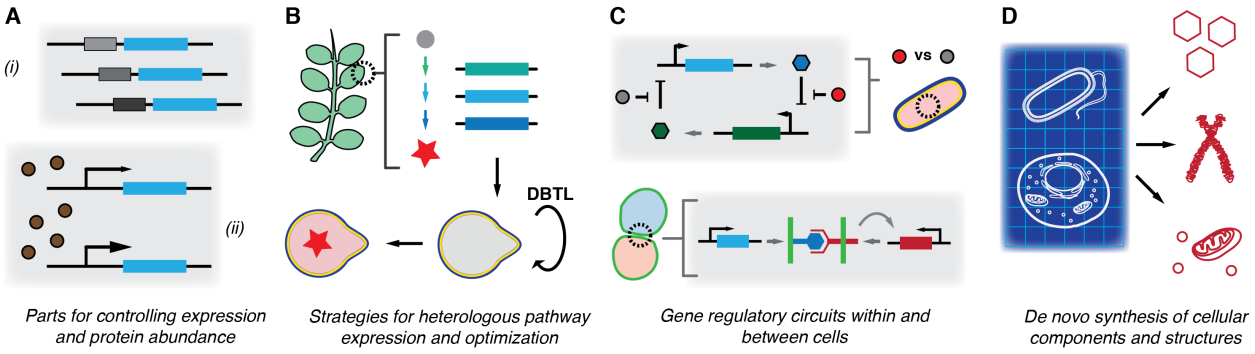

Figure 1: A synthetic biologist's toolkit. The past twenty years of synthetic biology research has led to the development of sophisticated tools – molecular parts, computational models, laboratory techniques, and experimental strategies – that allow for wholesale engineering of biological systems. (**A**) The most fundamental of these tools are parts for manipulating gene expression using promoter libraries (*i*) or titratable promoters (*ii*). Protein abundances can be further controlled by sets of parts targeting transcription, translation, or protein stability. (**B**) Metabolic engineering provides processes for the systematic identification and reconstitution of metabolic pathways from native hosts, such as plants, into industrial ones, such as yeast. Optimization of these pathways through Design Build Test Learn (DBTL) cycles allow for efficient production of new chemical products in engineered cells. (**C**) Rationally designed gene regulatory circuits have been developed to control cellular behavior. Shown is schematic for a synthetic bacterial toggle switch (top), as developed by Gardner et. al.[29], and one controlling simple multi-cell adhesion and organization (bottom), as developed by Toda et. al. (**D**) Approaches are being developed to synthesize or modify whole parts of cells, which can then be rationally re-engineered. These include protein-based capsules from bacteria, whole chromosomes from bacteria and yeast, and membrane-bound organelles.

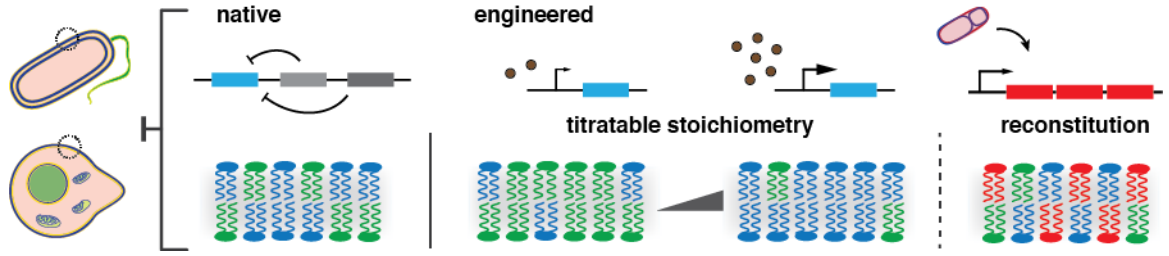

Figure 2: Metabolic engineering strategies for manipulating membrane lipid composition *in vivo*. Membrane composition in cellular lipid bilayers features defined stoichiometries of different lipid species, which is maintained by homeostatic regulation of native lipid biosynthesis pathways. Investigating functions for individual lipid species requires engineering systems where their presence and stoichiometry can be experimentally modulated. One way this can be achieved is to place key genes in the biosynthetic pathway under synthetic regulation with titratable promoters. An additional strategy involves reconstituting lipid species from other organisms into model systems to study their function, and provide additional ways of manipulating membrane properties. These strategies have been successful employing in classic synthetic biology hosts (bacteria and yeast) but similar approaches should allow for functional studies of lipid composition in more complex systems.

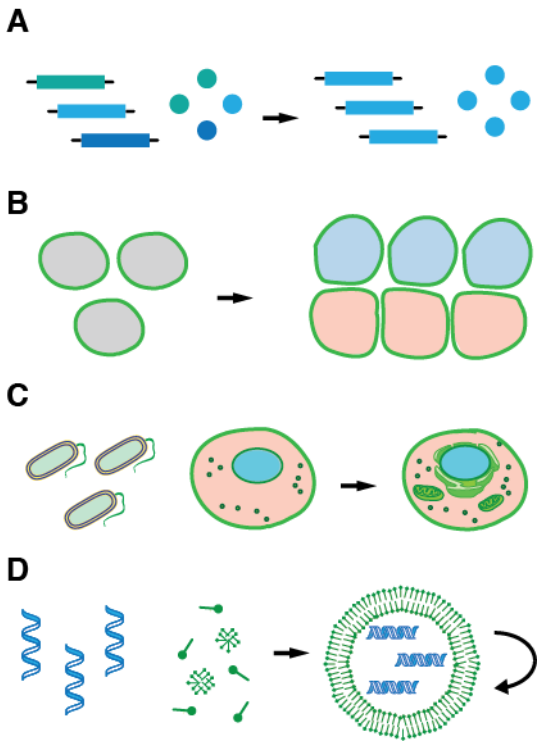

Figure 3: Investigating molecular and cellular evolution using synthetic biology. Major transitions in biology are difficult to study in the lab, but synthesizing their key intermediates or models of them is a potential avenue for understanding their underlying processes and selective advantages. Examples include (**A**) the selection of genes, proteins, or regulatory elements from populations of variants based on their *in vivo* activities, (**B**) the emergence of cooperativity in multicellularity and tissue formation, (**C**) the development of organelles and cellular structures during the evolution of eukaryotes, and (**D**) the assembly and self-replication of primitive cells during the origin of life.

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