Advanced Review

# The engineering of artificial cellular nanosystems using synthetic biology approaches

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Artificial cellular systems are minimal systems that mimic certain properties of natural cells, including signaling pathways, membranes, and metabolic pathways. These artificial cells (or protocells) can be constructed following a synthetic biology approach by assembling biomembranes, synthetic gene circuits, and cell-free expression systems. As artificial cells are built from bottom-up using minimal and a defined number of components, they are more amenable to predictive mathematical modeling and engineered controls when compared with natural cells. Indeed, artificial cells have been implemented as drug delivery machineries and *in situ* protein expression systems. Furthermore, artificial cells have been used as biomimetic systems to unveil new insights into functions of natural cells, which are otherwise difficult to investigate owing to their inherent complexity. It is our vision that the development of artificial cells would bring forth parallel advancements in synthetic biology, cell-free systems, and *in vitro* systems biology.


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## INTRODUCTION

In 1953, Stanly L. Miller built an apparatus that circulated methane, ammonia, water, and hydrogen in mimetic primitive-earth conditions. Glycine and alanine were observed among the products that suggested the synthesis of biomolecules from nonliving substances.[1] This work offered a possible explanation regarding how life might be created from nonliving materials on earth. Furthermore, this work established a new scientific area that attempted to use minimal and well-defined chemical systems to mimic cellular evolution in natural environments. Approximately 20 years later, Ronald Kaback isolated membrane vesicles from *Escherichia coli* (*E. coli*) that contained functional lactose transporters LacY.[2] On the basis of this minimal system, significant insights were gained with regards to the simultaneous transport of proton and lactose through bacterial membranes. The two examples were among the first efforts that harnessed minimal cellular systems to mimic and study natural biological systems.

Minimal biomimetic systems have indeed been used extensively for both biological studies and biotechnological applications in the past decades. Minimal cellular circuits have been created outside cells to mimic genetic activities of cells.[3] Cell-free systems have been used to synthesize RNA *ex vivo*.[4] A cell-free system that reconstituted both metabolic and protein synthesis machineries has been used to synthesize desired biological products.[5,6] Biomimetic vesicles have been applied in genes and drugs delivery.[7–9] These studies establish a solid foundation for the engineering of more complex cellular nanosystems. In fact, it is now possible to combine individual biomimetic components to create multifunctional cellular systems that mimic functions of natural cells, including gene expression,[10–14] membrane transport,[14] and subcellular localization.[15] Specifically, scientists have synthesized artificial cells (or protocells) by integrating synthetic gene circuits and bio-membranes.[14,16] Feedback gene circuits have been used to control intracellular functions.[17] In such bottom-up approaches,

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artificial cells are created by encapsulating desired protein synthesis machineries and informational polymers into synthetic membranes.

This review focuses on addressing two questions: How do researchers improve the control and engineering of artificial cellular systems? How do the systems help scientists to learn and harness functional mechanisms of natural cells to create new biotechnological applications? Answers to these questions have tremendous implications on studying the origin of primitive cells,[4,18–20] revealing functioning principles of natural cells,[10,21–23] and producing proteins and drugs using synthetic approaches.[6,24–28] Here, we discuss the development of cellular compartments (the shell), synthetic machineries (the engine), and informational components (the information) for the construction of artificial cells. For each of the components, their contributions to basic biological research and biotechnological applications are described (Figure 1). Furthermore, we discuss important development of the components that could advance the field of artificial cells.

## THE SHELL: THE ENGINEERING OF ACTIVE ARTIFICIAL CELLULAR COMPARTMENTS

The construction of stable membranes is essential to robust functioning of artificial cells. The membranes serve as protective shells and provide confined boundaries for artificial cells to evolve and to conduct biosynthesis without interruption from extracellular environments.[19,30] The membranes are commonly constructed by the self-assembly of amphiphilic building blocks, such as fatty acids[30,31] , phospholipids,[24,32] or polymeric copolymers.[12,33] The amphiphilicity of fatty acids allows the spontaneous formation of either spherical micelles or vesicles in aqueous solution. The conversion between lipid micelles and vesicles can be modulated by amphiphile concentration, ionic content, and pH.[30] Furthermore, the stability of these membrane vesicles is affected by environmental factors, such as presence of ionic contents,[20] osmotic pressures,[20] and pH changes.[31] The stability of the membranes can be improved by altering liposomal composition. Specifically, fatty acids were mixed with their corresponding alcohols[31] or glycerol monoesters[20] to form stable vesicles under broad pH ranges and high ionic concentrations (Figure 2).

Membranes of artificial cells can also be constructed using phosphatidylcholine (PC) molecules (a class of phospholipid), which have one diacylglycerol and one phospholipid group. Their vesicles can be spontaneously assembled with low amphiphile concentrations and they maintain high stability under various ionic contents, pH, and temperature.[30,34] Thus, the relative stability of PC vesicles makes them a good candidate for the construction of artificial cellular membranes. Polymeric copolymers composed of hydrophilic and hydrophobic monomers can also be used as amphiphilic building blocks for artificial cellular compartments (the vesicles formed are called polymersomes).[33]

To allow the intake of desired materials and discharge of waste by artificial cells, membranes will need to be permeable to specific molecules. In general, lipid bilayer membranes have low permeability to polar and large molecules because of their hydrophobic cores. Recent studies have shown that the permeability can be modulated by varying the composition of lipid building blocks.[30] The passage of molecules through the membranes is proposed to be mediated by the fluidity of lipid bilayers.[35] The size of lipid head groups and the lengths and saturation degrees of hydrophobic tails influence the fluidity and thus affect the permeability of the constructed membranes. In general, PC vesicles have lower permeability to large molecules than fatty-acid bilayers.[30] To further improve permeability of membranes, certain membrane transporters and channels can be incorporated into the membranes. Many studies have used pore-forming proteins $\alpha$-hemolysin to improve the permeability of membranes toward molecules that are less than 3 kDa.[36–38] $\alpha$-hemolysin forms a pore with a diameter of 1.4 nm for non-selective transportation without destroying PC vesicles.[37,38] MIP26 proteins form channels on liposomes and facilitate sucrose transport though the membranes. MIP26 channels are also regulated by calmodulin and calcium ion, which could be exploited for the control of molecular transport through liposomes.[39] Other proteins, such as porins[40] and CHIP28,[41] have also been shown to form channels that facilitate the transport of peptides and water respectively through phospholipid membranes.

## THE SHELL: APPLICATIONS OF ARTIFICIAL MEMBRANES IN BIOLOGICAL AND BIOTECHNOLOGICAL STUDIES

The enhanced control on permeability and stability of artificial membranes establishes a foundation toward applications of the membranes in biological studies. An artificial cellular system has been used to study complex membrane dynamics during exocytosis. An important step of exocytosis is the release of cargo molecules through fusion pores. The expansion of these pores during the release process is

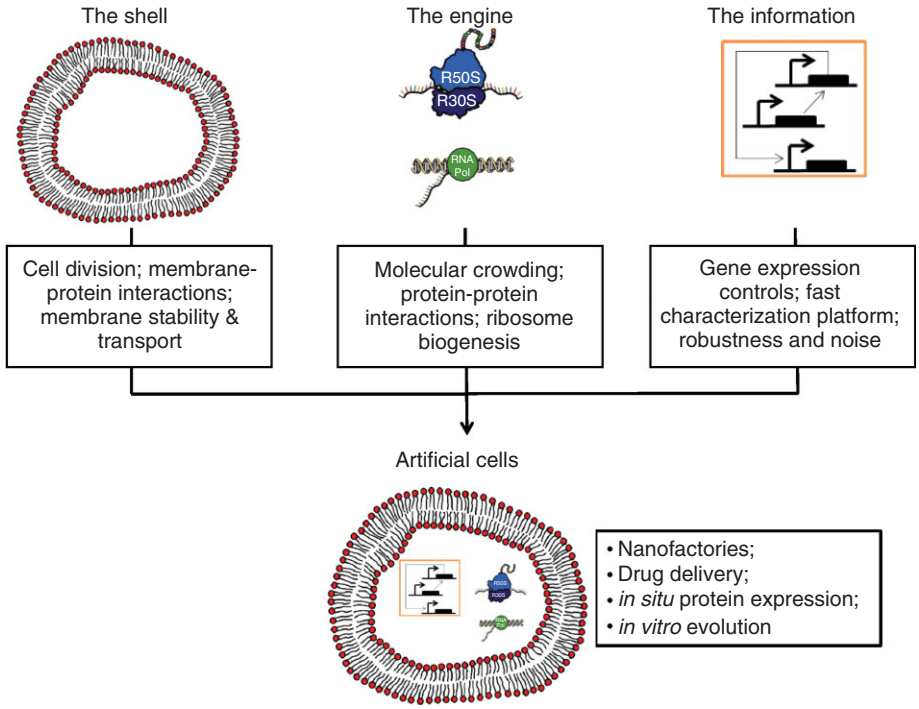


**FIGURE 1** | The mutual impacts of research in artificial cells and synthetic biology. Artificial cells are constructed by encapsulating synthetic gene circuits (the information) and expression systems (the engine) inside membranes (the shell). These artificial cells have been used as biomimetic systems for biological studies and as *in situ* expression systems for biotechnological applications. Furthermore, we envision that the development of artificial cells would bring forth parallel advancements of each of the subcomponents. Specifically, the engineering of membranes for artificial cells could lead to new insights into cellular division and membrane–protein interactions. The optimization of cell-free systems for artificial cells could generate new findings into molecular crowding, protein–protein interactions, and ribosome biogenesis. Finally, the engineering of gene circuits in cell-free systems could unveil new insights into dynamics of gene expression and establish a new platform for fast characterization of synthetic gene circuits. (Reprinted with permission from Ref 10. Copyright 2013 Nature Publishing Group; Reprinted with permission from Ref 29. Copyright 2013 Nature Publishing Group)

poorly understood owing to the complexity of natural cells.[23] Using micro-electrofusion technology,[42] a small vesicle was connected to the inside membranes of a surface-immobilized liposome through a lipid nanotube.[23] Fluid was injected at a constant rate into the small vesicle from the opposite side of nanotube. The small vesicle grew and the nanotube shortened. The nanotube underwent a transformation from a cylindrical tube to a toroidal fusion pore and released molecules to an environment (Figure 3(a)). On the basis of this artificial exocytosis model, it was demonstrated that the later stages of exocytosis could be driven by minimizing membrane surface tension without the presence of proteins.

Artificial cells have been used to mimic cell growth and division. Division of primitive membranes is thought to be spontaneous because of the lack of modern biomolecular machineries in primordial soup that catalyzed the cell-division process.[44] Before a primitive cell can produce its own lipids, its membrane has been proposed to grow through self-fusion.[45] Indeed, liposomes can grow in size by incorporating available building blocks in the environments.[46,47] Vesicles can fuse to one another through specific interactions between lipid bilayers.[19,43,48,49] For example, vesicles with either positive or negative charged amphiphiles can fuse selectively to each other and integrate their intracellular contents.[48] Formation of fatty-acid vesicles can be accelerated by mineral particles,[19] which may imply the existence of such growth pathways under primitive-earth conditions.

Membrane dynamics can be modified through molecular crowding. Molecular crowding effect arises owing to highly packed macromolecules in intracellular environments of natural cells. The crowding environment can reduce the volume of accessible solvent for other molecules and therefore enhance effective concentrations of the molecular species.[10,50,51] Electro-fused vesicles that contained macromolecules exhibited a budding shape as a result of the depletion volume effect[43] (Figure 3(b)). The decline of depletion volume due to the budding shape provided additional free volume for macromolecules, which was thermodynamically favorable.[43] Molecular

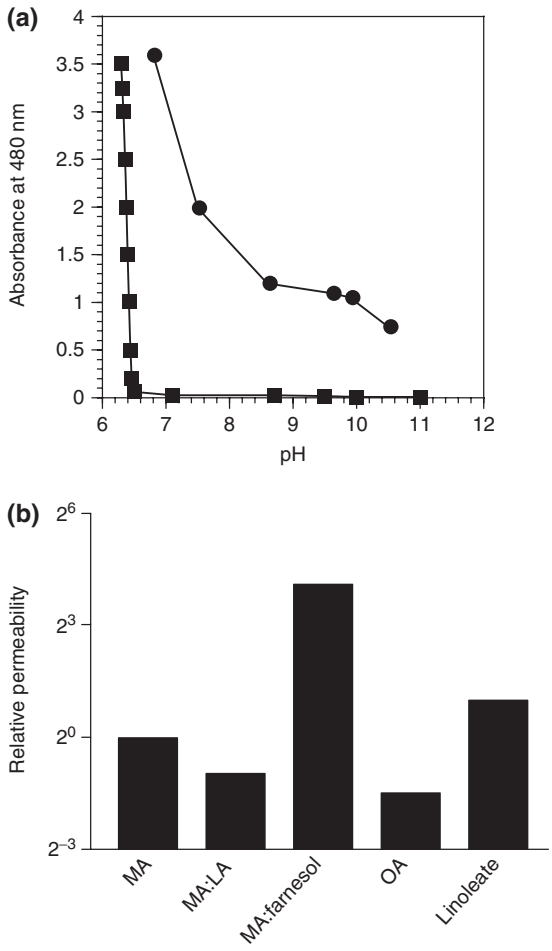


**FIGURE 2 |** The shell: stability and permeability of artificial cellular membranes. (a) The concentrations of nonanoic acid micelles with (filled circles) and without (filled squares) nonanol were estimated by their absorbance under various pH conditions. The slope of the curves corresponds to a transition from micelles to droplets. Mixing of the fatty acid and alcohol remarkably slowed the transition and stabilized the vesicles under pH changes. (Reprinted with permission from Ref 31. Copyright 2002 Elsevier). (b) Relative permeability of liposomes to ribose with various membrane compositions, myristoleic acid (MA, C14:1), lauric acid (LA, C12:0), farnesol, oleate acid (OA, C18:1), and linoleate (C18:2). MA displayed higher permeability than longer fatty acid (OA). Linoleate liposomes were more permeable than OA liposomes that had the same length, but with a higher degree of saturation. Mixing of farnesol with MA increased fluidity and yielded higher permeability relative to pure MA. In contrast, the addition of LA in MA decreased fluidity and lowered the permeability. (Reprinted with permission from Ref 18. Copyright 2008 Nature Publishing Group)

crowding was also demonstrated to drive bending of membranes.[52] These studies showed that physical interactions between intracellular contents and membranes could impact cell growth and division.

Artificial cellular systems allow experimenters to study spatial effects of membranes on biochemical reactions inside cells. Giant multilamellar vesicles (GMLVs) prepared by the freeze-dried empty liposomes (FDELs) method were shown to be stable under mechanical stress.[32] This discovery allowed quick scanning of green fluorescent proteins (GFPs) in the vesicles using fluorescence-activated cell sorters (FACS). Using this technique, the synthesis of GFP was quantified in thousands of GMLVs and found to be active in only parts of the internal volume of artificial cells. In addition, the ratio of this reactable volume was constant and independent from the size of the vesicles.[53] In another study, GFP synthesis system was encapsulated inside small liposomes with radius around 100 nm. Interestingly, the observed production of GFP was sixfold higher in this over-concentrated condition than that in bulk solution.[24] Tan et al. also demonstrated that reaction rates in large reaction volumes were affected more significantly by molecular crowding than in small reaction volumes.[10] Although the underlying biophysical mechanisms in the above studies remain unclear, the use of artificial cellular systems provides potential clues for future studies and insights into how spatial effects may impact cellular activities.

Nanofactories are artificial cellular systems that consist of four components: an active structural shell that allows passage of signals and products, synthetic machinery, a targeting domain, and an initiation or termination mechanism.[54] Nanofactories provide novel approaches for *in situ* drug synthesis and delivery by producing desired materials near target sites.[54] Recently, nanofactories were constructed using polymersomes that allowed the inflow of excess phenylalanine within the nanofactories. The encapsulated phenylalanine ammonia-lyase was able to decrease the level of phenylalanine *in vivo*.[9,55] This design can potentially be used to treat phenylketonuria.[9,54,55] Nanofactories were also constructed using antibodies and fusion proteins. The antibodies served as binding domains by bacteria. The fusion proteins, which included S-adenosylhomocysteine nucleosidase (Pfs) and S-ribosylhomocysteinase (LuxS), were immobilized to the anti-*Salmonella* IgG through $His_6$-tagged protein G. The fusion protein Pfs-LuxS sensed S-(5′-deoxyadenosin-5′)-L-homocysteine (SAH) and synthesized bacterial signaling molecules autoinducer-2 (AI-2).[56] The nanofactories successfully produced AI-2 and triggered quorum sensing response of *Salmonella typhimurium*. A chemical system that synthesized quorum sensing molecules was encapsulated inside liposomes to trigger quorum sensing response of *Vibrio harveyi*.[37] These studies suggest that more complex artificial cells could potentially be employed as low maintenance systems to modulate dynamics of cellular populations *in vivo*.

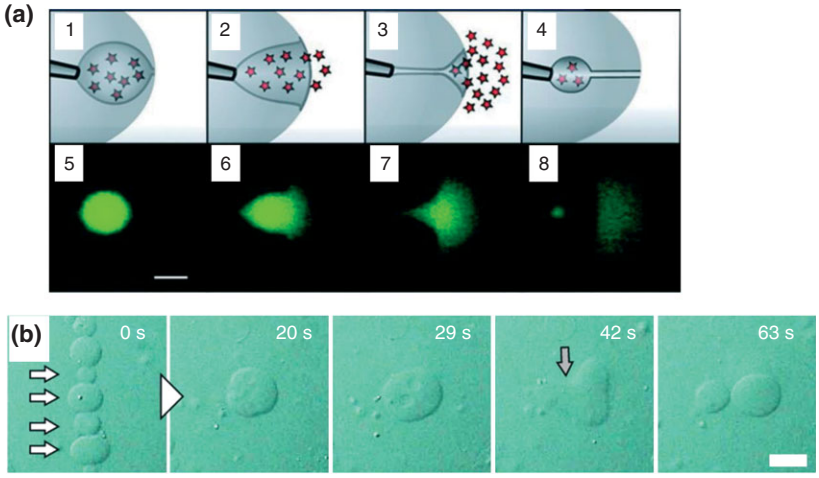


**FIGURE 3** | The shell: The modulation of membrane dynamics. (a) Modeling of a late stage of exocytosis using liposomes connected to nanotubes. (1–4) A small liposome grew with the injection of fluid. The nanotube changed its shape from cylindrical to toroidal and the enclosed materials were released. After one round of stimulation, a new nanotube was formed. (5–8) Visualization of exocytosis using fluorescein-filled vesicles. Fluorescence images 5–8 correspond to the events in 1–4. The scale bar represents 10 μm. (Reprinted with permission from Ref 23. Copyright 2003 The National Academy of Sciences). (B) Electrofusion and budding formation of vesicles containing macromolecules. Voltage supply was turned on or off at the top and bottom sides of a chamber that contained the vesicles. Vesicles started to fuse at time 0 s (indicated by white arrows). The fusion was completed after 20s. The budding transformation started at 42 s (indicated by a gray arrow). Budding shape was observed after 63 s. The scale bar represents 10 μm. (Reprinted with permission from Ref 43. Copyright 2012 The National Academy of Sciences)

## THE ENGINE: THE ENGINEERING OF SYNTHETIC METABOLIC AND PROTEIN MACHINERIES

Synthetic machineries for protein synthesis are critical for any living systems as well as artificial cells. The synthesis of proteins consists of two major steps: transcription and translation. During transcription, information carried in DNA is converted into messenger RNAs (mRNAs) by RNA polymerases. Ribosomes then translate the mRNAs into desired proteins using amino acids.[57] Synthesis of proteins outside natural cells in artificial systems was difficult because of the complex and unspecified transcription–translation machineries. This issue was resolved with the development of approaches to produce proteins *in vitro* using cell-free systems, which allowed researchers to study complex biological processes without using intact living cells.[6,21,22,58–62] In one type of cell-free system, cytoplasmic components of cells, typically *E. coli* or wheat germ, are extracted by eliminating cell membranes and native genomic regulation (DNA and mRNA). Synthetic genetic circuits can then be added to the systems to synthesize target proteins in the systems. Bacteriophage T7 RNA polymerases are commonly supplemented to carry out transcription owing to their well-characterized kinetics and functions.[63,64] Another type of cell-free systems is called protein synthesis using recombinant elements (PURE) in which the synthesis machineries are reconstituted from purified components. Specifically, PURE system is generated by 3 initiation factors, 3 elongation factors, 3 release factors, 1 termination factor, 20 aminoacyl-tRNA synthetases, methionyl-tRNA transformylase, T7 RNA polymerase, and ribosomes.[59] The two types of cell-free systems have different properties and thus are different in their advantages and applications. For example, cell extracts produce more proteins per ribosome with less cost than PURE systems.[65] Therefore, cell extracts are good choices for commercial production of desired proteins. Unlike cell extracts, every component in the PURE system is known, which allows tremendous freedom to manipulate and engineer the system. This property makes PURE system an excellent expression system for constituting protein expression systems inside artificial cells.

Nutrient and energy consumption are main factors that limit protein synthesis in cell-free systems.[57] Protein synthesis in a cell-free system lasted for a few hours (Figure 4(a)) in a batch mode reaction, and an optimal ATP concentration was necessary to prolong the protein synthesis.[25] Hydrolyzed ATP was regenerated by coupling a recycling system, such as creatine phosphate and its kinase, to a protein synthesis system. The optimization of ATP recycling systems was shown to prolong and promote protein synthesis[25,66] (Figure 4(a)). Another way to overcome the limitation of nutrient and energy was by creating a continuous exchange system. A continuous transcription

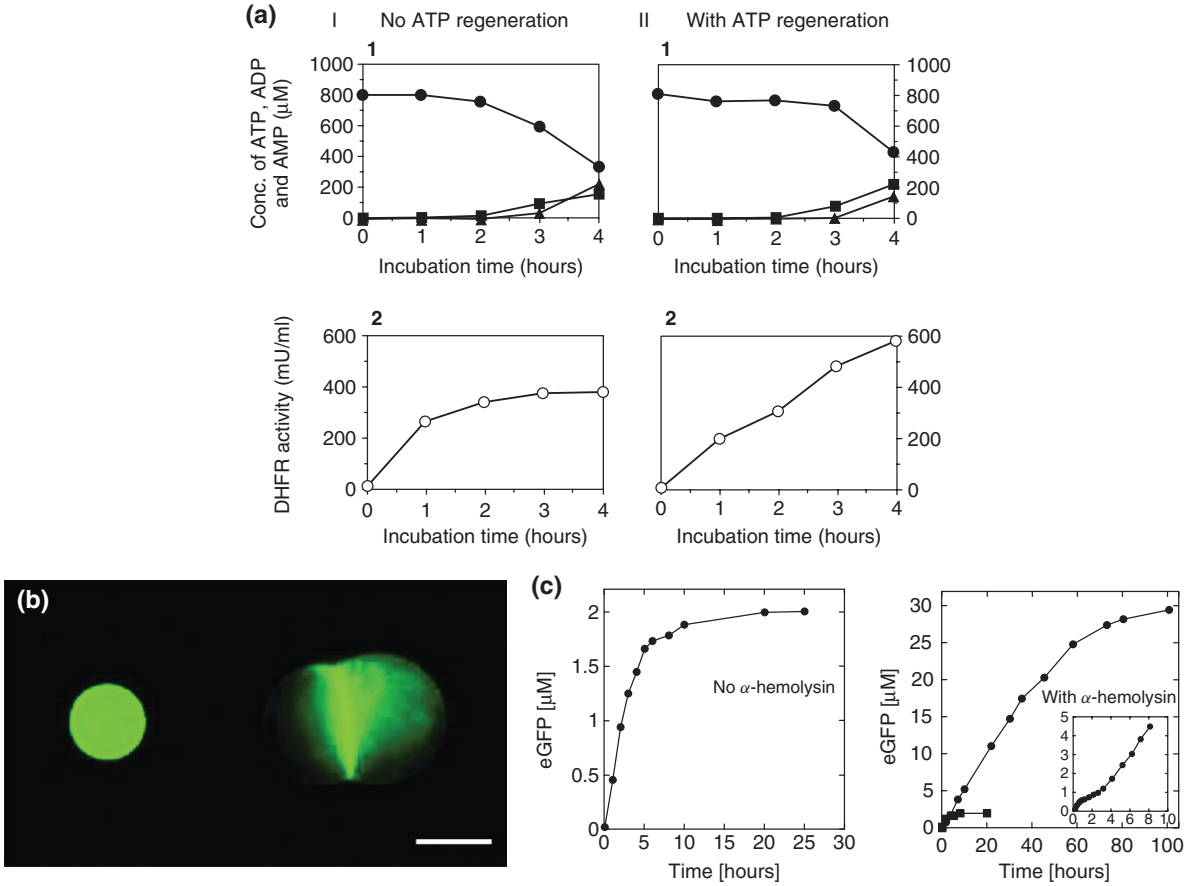


**FIGURE 4 |** The engine: the modulation of protein synthesis rates in cell-free systems. (a) Correlation of ATP (panel 1) and protein (panel 2) concentrations with (II) or without (I) ATP regeneration. Spheres represent ATP; squares represent ADP; triangles represent AMP. Without ATP regeneration, the concentration of ATP declined rapidly in 4 h and dihydrofolate reductase (DHFR) synthesis almost stopped. With regeneration (supplemented with dithiothreitol), the decrease of ATP concentration was slowed and DHFR synthesis was prolonged. Reprinted with permission from Ref 25. Copyright 1994 Japan Society for Bioscience, Biotechnology and Agrochemistry (b) Synthesis of enhance GFP (eGFP) in liposomes using a cell-free system. *E. coli* extract and plasmid encoding eGFP were encapsulated in a vesicle (left) and a doublet (right). eGFP expression was detected using fluorescence imaging. The scale bar represents 15 μm. (Reprinted with permission from Ref 38. Copyright 2004 The National Academy of Sciences). (c) eGFP expression in vesicles with (right) or without (left) $\alpha$-hemolysin. With $\alpha$-hemolysin, protein synthesis was prolonged significantly to over 100 h, which was much longer than without $\alpha$-hemolysin. The inset represents GFP intensities in the first 10 h. Filled spheres (right) represent eGFP expression with $\alpha$-hemolysin; filled squares (right) represent eGFP expression without $\alpha$-hemolysin. (Reprinted with permission from Ref 38. Copyright 2004 The National Academy of Sciences)

and translation system was created by combining a cell-free system with a reservoir of nutrients through a dialysis membrane. The combined system was shown to prolong the protein synthesis from a few hours to a few days.[67]

To mimic a cell, many studies have implemented protein synthesis in artificial compartments. This implementation requires the presence of all necessary components in one liposome simultaneously, which can be achieved by encapsulating either cell extracts[12,16,68] or PURE systems[32,53,69–72] inside lipid bilayers (Figure 4(b)). Liposomes encapsulating the PURE system and plasmids successfully produced GFP. Interestingly, GFP in liposomes had longer lifetime (5 h) than that in bulk solution (2 h).[38] Addition of other cellular components could improve the protein expression. For example, the addition of $\alpha$-hemolysin pore proteins in liposomes allowed media exchange between liposomes and their environment, and could prolong protein expression inside artificial cells for up to 4 days[38] (Figure 4(c)).

## THE ENGINE: THE APPLICATIONS OF SYNTHETIC EXPRESSION SYSTEMS IN BIOLOGY AND BIOTECHNOLOGY

Protein synthesis systems have been valuable tools to study isolated cellular components outside cells,

such as crowding molecules and protein–protein interactions. Using a cell-free system, Tan et al. demonstrated that molecular crowding plays an important role in regulating gene expression dynamics. Cell-free systems were supplemented with inert macromolecules of various sizes to mimic crowding environments.[10] For small inert molecules, a biphasic response was observed where the expression of cyan fluorescent protein (CFP) increased and then declined with increasing crowding densities. In contrast, crowding with big inert molecules gave rise to a monotonic increase in gene expression rates with increasing crowding densities[10] (Figure 5(a)).

Protein–protein interaction (PPI) is another important factor that affects protein synthesis in cells. The minimal PURE system was used to reconstruct a PPI network using *E. coli* open reading frames (ORFs).[22] The study revealed that 12% of over 4000 ORF products influenced GFP synthesis in a cell-free expression system; 34% of the identified ORF products might physically interact with the minimal components involved in gene expression[22] (Figure 5(b)). These studies that were enabled by protein synthesis system provide insights into how nongenetic and genetic factors could modulate gene expression. Furthermore, the discovered factors might be useful parameters to improve protein production *in vitro* for biotechnological applications.

Cell-free synthetic biology has also been used to study ribosome biogenesis. Ribosomes are primary proteins that translate information encoded in mRNA into proteins. Ribosomes were synthesized *in vitro* for the study of ribosome biogenesis.[29,74–76] A conventional method was established to reconstitute 50S ribosomes by incubating 23S RNA, 5S RNA, and total proteins from 50S subunits in two sequential steps with optimized $Mg^{2+}$, $NH_4Cl$, and incubation durations.[76] However, this method is limited by low efficiency and non-physiological conditions.[29,76] A one-step integrated rRNA synthesis, ribosome assembly, and translation (iSAT) method was developed, which synthesized rRNA *in vitro* from plasmids using RNA polymerases and assembled the rRNAs into ribosomal proteins. The functionality of the assembled ribosomes was verified by the expression of luciferase proteins in ribosome-free cell extracts.[29] rRNA synthesis, ribosome assembly, and ribosome translation were achieved in the same reaction environment under a physiological condition, which could make this method a valuable tool for self-assembly of ribosomes inside artificial cells. In addition, this study provided a convenient method and useful tool to synthesize ribosomes *in vitro* and to study ribosome biogenesis.

Owing to the minimality of cell-free systems, they are particularly amenable for high-throughput testing of proteins and screening of antibiotics. Antimicrobial peptides (AMPs) are short peptides synthesized by innate immune systems to combat invading bacteria.[77,78] Numerous peptide sequences have been discovered from a rich pool of natural occurring AMPs.[77] The AMPs could be optimized by exploiting the inherent modularity within the peptide sequences.[79] The modular sequences were formalized as linguistic grammars and ~700 grammars were derived by examining ~500 well-studied AMPs from database. Forty designed AMPs were synthesized and about half of the synthetic peptides showed antimicrobial activities toward *E. coli* or *Bacillus cereus* under a minimum inhibitory concentration (MIC) of 256 μg/mL.[79] This study is valuable for the design of new functional AMPs. More importantly, it offers a novel high-throughput strategy to develop antibiotics using cell-free systems. High-throughput proteomic studies are also enabled by cell-free systems. Self-assembling protein microarrays were engineered by expressing target proteins in each local spot using DNAs and mammalian reticulocyte lysates[73] (Figure 5(c)). The target proteins were tagged with epitopes, which were then fused to the spot *in situ*. This method allowed on-demand generation of protein arrays and avoided the issues of protein degradation in long-term storage. These studies of cellular components and the development of self-assembly systems using cell-free systems would benefit the development of complex artificial cells that exploits functioning components of natural cells.

Cell-free systems are being developed for the production of biocommodities, including vaccines and biofuel. Vaccination is thought to be a potent way to combat malaria caused by *Plasmodium falciparum* (*P. falciparum)*. However, production of vaccines using cell-based methodologies is limited owing to a high A/T content in genetic sequences of vaccine candidates and glycosylation machinery in natural cells that can produce inappropriately glycosylated proteins.[28] Using a wheat germ cell-free system, three *P. falciparum* proteins Pfs25, PfCSP, and PfAMA1 were synthesized.[28] The proteins were shown to induce highly specific production of antibodies in mouse.[28] Cell-free systems can also be used in the production of butanol, which is limited by the negative impact of butanol on cellular metabolism and growth.[80,81] To overcome the limitation, cell-free systems have been proposed as alternative methods for the production of butanol.[81] Unlike cell-based methods, native

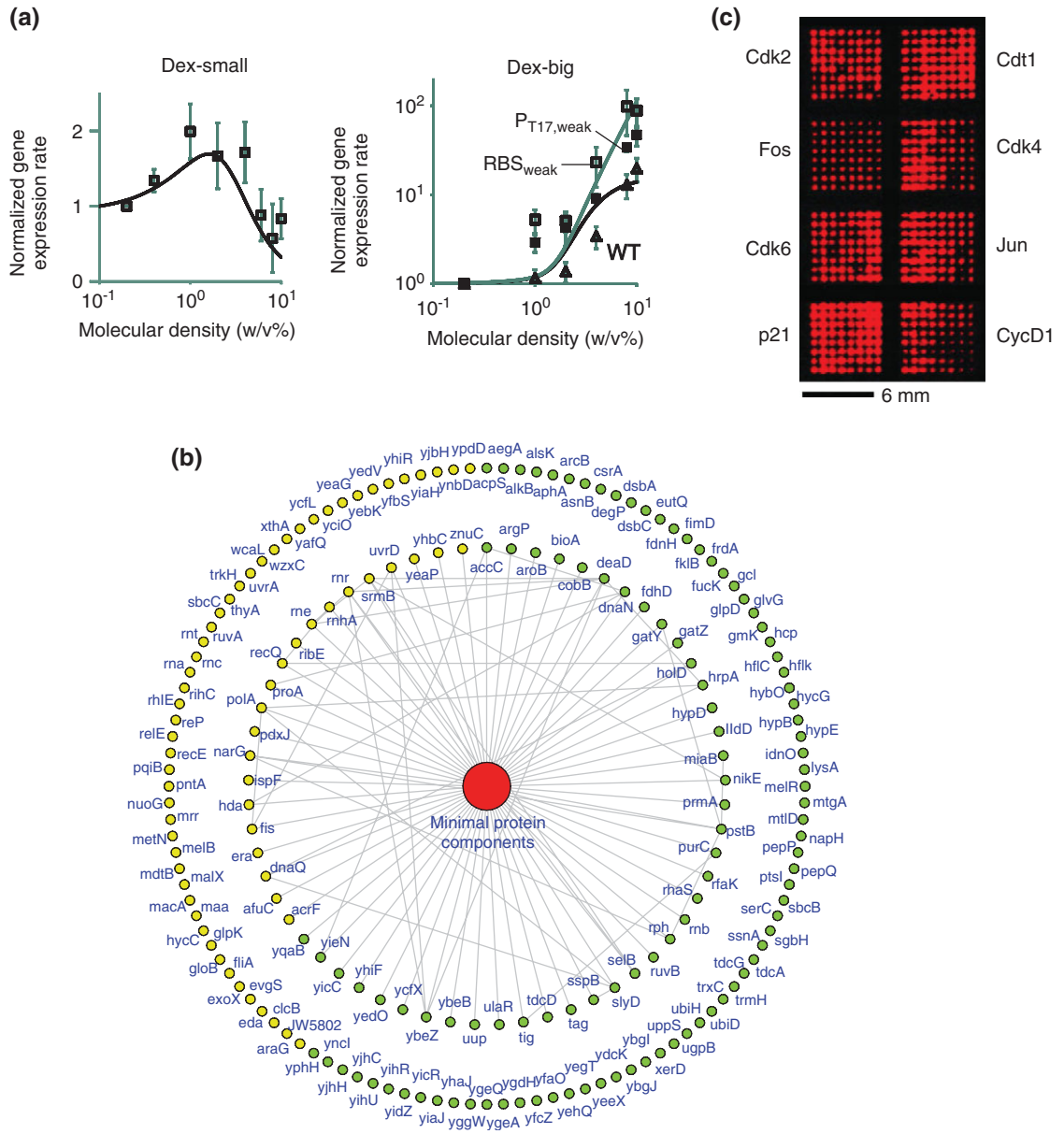


**FIGURE 5 |** The engine: the impact of genetic and nongenetic factors on cell-free expression systems. (a) The impact of molecular crowding on gene expression. Systems with a small crowder (left) showed a biphasic shape in gene expression with increasing crowding densities. Systems with a big crowder (right) exhibited monotonic increase of gene expression with increasing crowding densities. Weak genetic components resulted in faster increase in normalized gene expression rates when compared to a wild type component. $P_{T7,weak}$ represents a weak T7 promoter. $RBS_{weak}$ represents a weak ribosomal binding site. (Reprinted with permission from Ref 10. Copyright 2013 Nature Publishing Group). (b) Mapping of interactions between a protein synthesis system and ORF products. ORFs in the inner circle directly affected the minimal system, while those in the outer circle did not affect protein synthesis. Green circles represent proteins that caused beneficial effects. Yellow circles represent proteins that caused deleterious effects. (Reprinted with permission from Ref 22. Copyright 2008 The American Society for Biochemistry and Molecular Biology). (c) Expression of target proteins in microarrays. Eight different DNAs encoding target proteins fused to glutathione S-transferase (GST) tag were immobilized in microarray format on a glass slide. Cell-free systems were added to carry out the protein synthesis. The protein expression was confirmed using GST antibodies. (Reprinted with permission from Ref 73. Copyright 2004 The American Association of the Advancement of Science)

cellular activities could be significantly reduced in cell-free systems. Purified enzymes and coenzymes in butanol fermentation pathway could be assembled *in vitro* to minimize metabolic by-products.[81] These examples of vaccines and biofuel production suggest that cell-free systems could benefit biocommodity production that is limited by traditional cell-based methodologies.

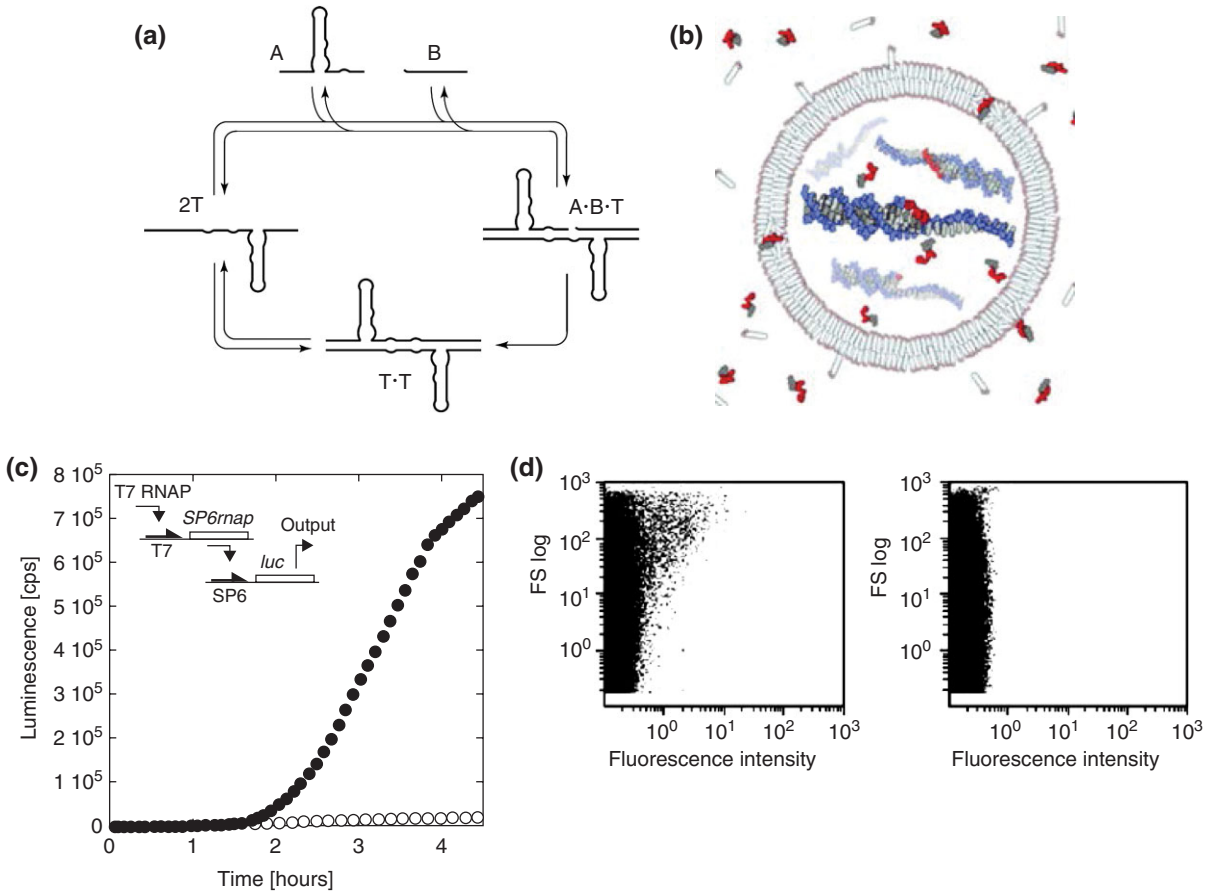


**FIGURE 6 |** The information: the incorporation of genetic modules inside artificial cells. (a) A schematic of R3C ribozyme self-replication. A duplex of R3C ribozyme (T.T) dissociated into two RNAs (T). RNA T-induced ligation of two substrate RNAs A and B through a complex of RNA (A.B.T). A new duplex of R3C ribozyme (T.T) formed after the ligation. Reprinted with permission from Ref 83. Copyright 2002 The National Academy of Sciences (b) A template-directed synthesis of RNA was reconstituted inside protocells that consisted of fatty-acids membranes. Nucleotides from the extracellular environment diffused into the protocells to support the RNA synthesis. (Reprinted with permission from Ref 18. Copyright 2008 Nature Publishing Group). (c) A two-level genetic cascade was reconstituted inside artificial cells. Output luminescence signals (filled circles) increased with time and exhibited a time delay due to the gene cascade. (Reprinted with permission from Ref 84. Copyright 2003 The National Academy of Sciences). (d) A two-level genetic cascade was encapsulated inside artificial cells. A flow cytometer was used to measure gene expression rates inside artificial cells. The results show that artificial cells expressed green fluorescent proteins as an output (left panel). The negative control without DNA did not generate GFP (right panel). (Reprinted with permission from Ref 16. Copyright 2004 Elsevier).

## THE INFORMATION: APPLICATIONS OF GENETIC MODULES IN ARTIFICIAL CELLS

Protein expression systems can be encapsulated inside membranes to create an intracellular environment for the incorporation of genetic modules. Along this line, artificial cells can encode information using DNA and RNA molecules. The use of nucleic acids will allow artificial cells to replicate, evolve, and inherit the information. To start, RNAs could be implemented inside artificial cells as they have been proposed to be the inherited molecules during the origin of cells.[82] For instance, an RNA molecule was demonstrated to self-catalyze its own replication through an R3C ligase ribozyme[83] (Figure 6(a)). The R3C ribozyme catalyzed the ligation of two RNA substrates, which formed the same R3C ribozymes. A recent work created a two-ribozyme system, which catalyzed the synthesis of each ribozyme using four oligonucleotide substrates.[85] As these RNA replications occur in the absence of proteins, they are less complex than DNA replications and would be more amenable to implementation inside artificial cells.

Indeed, a preliminary example of RNA replication has been created inside artificial cells that were constructed using fatty acids[86] (Figure 6(b)). In this work, RNA was replicated using nonenzymatic template-directed synthesis of RNA. An RNA primer was annealed to an oligonucleotide with a

template region. Activated G monomer guanosine 5′-phosphor(2-methyl)imidazolide was then added to induce RNA synthesis. The authors added citrus that chelated magnesium ions and enhanced stability of fatty-acids vesicles.[86] In addition, a complete RNA replication system was reconstituted inside artificial cells.[87] A fusion RNA encoded the beta subunit of a Q$\beta$ replicase, which in turn recognized an RNA structure within the same RNA for RNA replication. The work could lead to the development of artificial cells that evolve and self-replicate their own genetic materials.

Artificial cells could also encode information using alternative forms of DNA. Xeno-nucleic acids (XNA) were created by replacing the canonical ribofuranose ring of DNA.[88] It was demonstrated that these XNA could be replicated using evolved polymerases. The XNAs were also demonstrated to exhibit defined structures that bound to their target specifically. These XNAs could be used to expand ways to encode information inside artificial cells.

Furthermore, artificial cells could exploit either RNA or DNA to enhance their sensing and response capabilities. Through modulating gene expression of DNA promoters, artificial cells could integrate complex environmental signals and respond by modulating either mRNA or protein expression. Linear or circular DNA can be added to the synthetic expression systems, thus allowing *in vitro* expression of synthetic gene circuits. Simple gene circuits with a promoter regulating the expression of a gene were implemented in artificial cells.[10,12,38,69,89,90] Two-cascade gene circuits were created inside artificial cells using orthogonal polymerases[16,84] (Figure 6(c)). Specifically, one of the circuits consisted of a $P_{T7}$ promoter that regulated the expression of SP6 RNA polymerases, which activated expression from a $P_{SP6}$ promoter.[84] A positive feedback loop was created inside artificial cells by using T3 RNA polymerases (RNAP) that auto-regulated its own expression[91] (Figure 6(d)).

## THE INFORMATION: THE STUDY OF GENETIC MODULES USING ARTIFICIAL CELLULAR SYSTEMS

To streamline the construction of artificial cells, gene circuits can be designed and optimized using cell-free systems outside artificial cells. Using a cell-free system, Tan et al. demonstrated that molecular crowding can increase robustness of gene expression[10] (Figure 7(a)). Specifically, crowding densities were modulated using inert dextran polymers that did not react with the system of interest.[94] Next, gene expression in cell-free systems was perturbed using chemicals and found to be more robust to the perturbations under crowded conditions. In addition, Tan et al. showed that a negative feedback loop, DNA promoters, and ribosomal binding sites (RBSs) interact with molecular crowding to fine-tune gene expression rates.

Single-molecule imaging could also be exploited for the design of genetic modules for artificial cells. The imaging method would reveal heterogeneity of molecular interactions, which could be useful for both the modeling and design of genetic components. In the same study,[10] Tan et al. used single-molecule imaging to study transcriptional processes under crowded conditions (Figure 7(b)). Single-molecule experiments were set up by fusing DNA promoters to poly(ethylene glycol) (PEG) surfaces. Next, fusion RFP-T7RNAP molecules were added to track its binding to the DNA promoters. Tan et al. modulated crowding densities using dextran and found that crowded conditions increased the binding of T7 RNAP to the $P_{T7}$ promoters. This work represented the first example of using single-molecule imaging for the design of synthetic biological systems. This work was built upon a rich literature of single-molecule imaging using cell-free systems, which had revealed tremendous insights into interactions between transcription factors and DNA promoters.[95–97] In these experiments, reaction environments and genetic components could each be controlled independently in contrast to their natural counterparts in cells. The modulation would allow experimenters to pinpoint critical design factors of gene expression and to potentially translate the results directly to implementation inside artificial cells, which have the same reaction environments as the cell-free systems.

Synthetic expression systems and genetic modules can also be incorporated inside micro-emulsion and microchips as artificial cellular systems. Cell-free systems and genetic modules were encapsulated inside water-in-oil emulsion droplets and were demonstrated to exhibit unique reaction kinetics that was dependent on details of the reaction systems.[13] Specifically, the synthesis of a tetramer $\beta$-glucoronidase (GUS) was accelerated in small reaction volumes when compared with a tetramer $\beta$-galactosidase (GAL). It was hypothesized that the synthesis of GAL was limited by gene expression, which was not accelerated in small reaction volumes. In contrast, the formation of GUS was limited by tetramer association, which was accelerated in small reaction volumes. In another work, dynamics of gene expression were tracked using polydimethylsiloxane microchips that contained 20 femtoliter of cell-free reaction mix in each reaction chamber[92] (Figure 7(c)). In these nano-environments, it was demonstrated that

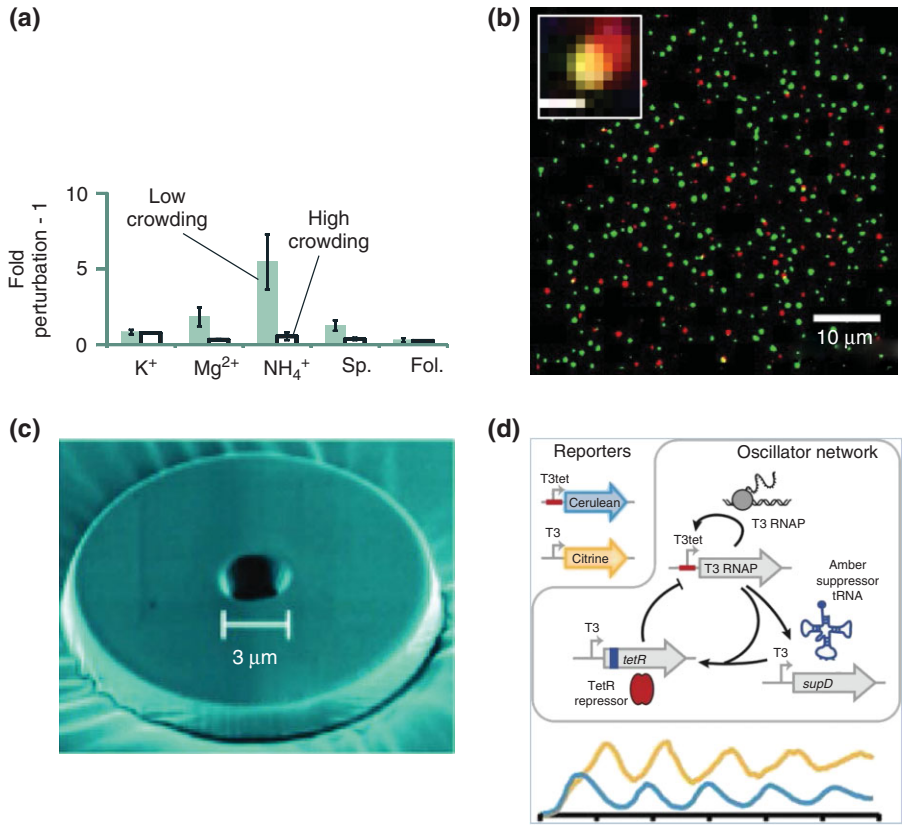


**FIGURE 7 |** The information: applications of genetic modules in artificial cellular systems. (a) A high crowding condition (open bars) reduced fold perturbation of gene expression when compared to a low crowding condition (gray bars). Chemicals were added to perturb gene expression. (Reprinted with permission from Ref 10. Copyright 2013 Nature Publishing Group). (b) Single-molecule imaging was used to study the impact of molecular crowding on T7 RNAP binding to DNA promoter. (Reprinted with permission from Ref 10. Copyright 2013 Nature Publishing Group). (c) Fabrication of a 20 femtoliter nano-chamber for the study of gene expression using cell-free expression systems. (Reprinted with permission from Ref 92. Copyright 2013 American Chemical Society) (d) A delayed gene circuit was used to create oscillations in cell-free systems. T3 RNAP activated its own expression (top panel). T3 RNAP also activated the expression of SupD that activated the expression of TetR. TetR then repressed the expression of T3 RNAP. The circuit generated sustained oscillations in a cell-free system (bottom panel). (Reprinted with permission from Ref 93. Copyright 2013 The National Academy of Sciences)

translational bursting occurred during gene expression. These systems offer bottom-up approaches to investigate gene expression under minimal conditions without complication of cell division, host networks, and cellular organelles.[98–100] In addition, they establish a foundation toward using artificial cells for *in vitro* evolution of cellular components.[101,102]

On the one hand, the development of artificial cells could directly benefit from the characterization of gene circuits in cell-free systems. On the other hand, the same pipeline of measuring gene circuit dynamics using cell-free systems could lead to fast- and high-throughput platforms for the design of synthetic circuits and components.[98,99] Indeed, synthetic expression systems are being established as *ex vivo* systems (outside natural and artificial cells) for fast characterization of synthetic genetic parts, including fluorescent proteins,[103] RBSs,[104] and hybrid DNA promoters. Using PURE system, 17 different fluorescent proteins were synthesized, screened, and quantified for their fluorescence intensities. The fluorescent proteins were then used to evaluate the impact of spacing between RBS and start codons on gene expression.[103] Hybrid DNA promoters typically consist of an RNAP and a transcription factor binding site. Transcription factors either activate or repress gene expression by RNAP. Hybrid promoters that consisted of either a acyl-homoserine-lactone synthase (LasR) or a Tet repressor protein (TetR) operator were tested in cell-free systems.[104,105] LasR was an activator that was induced by quorum sensing signals acyl-homoserine-lactones (AHL). TetR was an inhibitor that was inhibited by anhydrous tetracycline (aTc). Both hybrid promoters responded to their respective small molecule inducers and expressed reporter proteins in cell-free systems. In addition to

simple circuits, the genomic DNA of phage T7 was added to cell-free systems, which generated complete phage T7 particles, suggesting that cell-free systems could indeed support the execution of complex reactions.[106] A delayed negative feedback loop was used to create oscillations of gene expression in a cell-free system that was supplied continuously with nutrients[93] (Figure 7(d)). We foresee that the development of gene circuits using cell-free systems would expand the parts library for the construction of artificial cells.

## CONCLUSION

The advancements in synthetic biology, cell-free expression systems, and liposomes have created a solid foundation for the development of artificial cells. Indeed, tremendous research has been performed in the last decade to improve the engineering of artificial cells and to apply artificial cells in biotechnological applications. Through the engineering of artificial cells, significant insights are also gained into functioning mechanisms of natural biological systems. Furthermore, the development of new technologies in synthetic polymers and cellular components could help advancing the field of artificial cells. We envision that the development of artificial cells would both create and enhance new technologies for synthetic biology, *in vitro* systems biology, and biomimetic nanosystems.

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## REFERENCES

1. Miller SL. A production of amino acids under possible primitive earth conditions. *Science* 1953, 117:528–529.
2. Rudnick G, Schildiner S, Kaback HR. Equilibrium between two forms of the lac carrier protein in energized and nonenergized membrane vesicles from Escherichia coli. *Biochemistry* 1976, 15:5126–5131.
3. Kim J, White KS, Winfree E. Construction of an in vitro bistable circuit from synthetic transcriptional switches. *Mol Syst Biol* 2006, 2:68.
4. Mills DR, Peterson RL, Spiegelman S. An extracellular Darwinian experiment with a self-duplicating nucleic acid molecule. *Proc Natl Acad Sci U S A* 1967, 58:217–224.
5. Chen HZ, Zubay G. Prokaryotic coupled transcription-translation. *Methods Enzymol* 1983, 101:674–690.
6. Jewett MC, Calhoun KA, Voloshin A, Wuu JJ, Swartz JR. An integrated cell-free metabolic platform for protein production and synthetic biology. *Mol Syst Biol* 2008, 4:220.
7. Kikuchi H, Suzuki N, Ebihara K, Morita H, Ishii Y, Kikuchi A, Sugaya S, Serikawa T, Tanaka K. Gene delivery using liposome technology. *J Control Release* 1999, 62:269–277.
8. LaVan DA, McGuire T, Langer R. Small-scale systems for in vivo drug delivery. *Nat Biotechnol* 2003, 21:1184–1191.
9. Chang TM. Therapeutic applications of polymeric artificial cells. *Nat Rev Drug Discov* 2005, 4:221–235.
10. Tan C, Saurabh S, Bruchez MP, Schwartz R, Leduc P. Molecular crowding shapes gene expression in synthetic cellular nanosystems. *Nat Nanotechnol* 2013, 8:602–608.
11. Martino C, Horsfall L, Chen Y, Chanasakulniyom M, Paterson D, Brunet A, Rosser S, Yuan YJ, Cooper JM. Cytoskeletal protein expression and its association within the hydrophobic membrane of artificial cell models. *Chembiochem* 2012, 13: 792–795.
12. Martino C, Kim SH, Horsfall L, Abbaspourrad A, Rosser SJ, Cooper J, Weitz DA. Protein expression, aggregation, and triggered release from polymersomes as artificial cell-like structures. *Angew Chem Int Ed Engl* 2012, 51:6416–6420.
13. Matsuura T, Hosoda K, Kazuta Y, Ichihashi N, Suzuki H, Yomo T. Effects of compartment size on the kinetics of intracompartmental multimeric protein synthesis. *ACS Synth Biol* 2012, 1:431–437.
14. Noireaux V, Bar-Ziv R, Godefroy J, Salman H, Libchaber A. Toward an artificial cell based on gene expression in vesicles. *Phys Biol* 2005, 2:P1–8.
15. Long MS, Jones CD, Helfrich MR, Mangeney-Slavin LK, Keating CD. Dynamic microcompartmentation in synthetic cells. *Proc Natl Acad Sci U S A* 2005, 102:5920–5925.
16. Ishikawa K, Sato K, Shima Y, Urabe I, Yomo T. Expression of a cascading genetic network within liposomes. *FEBS Lett* 2004, 576: 387–390.

17. LeDuc PR, Messner WC, Wikswo JP. How do control-based approaches enter into biology? *Annu Rev Biomed Eng* 2011, 13:369–396.
18. Mansy SS, Schrum JP, Krishnamurthy M, Tobe S, Treco DA, Szostak JW. Template-directed synthesis of a genetic polymer in a model protocell. *Nature* 2008, 454:122–125.
19. Hanczyc MM, Fujikawa SM, Szostak JW. Experimental models of primitive cellular compartments: encapsulation, growth, and division. *Science* 2003, 302:618–622.
20. Monnard PA, Apel CL, Kanavarioti A, Deamer DW. Influence of ionic inorganic solutes on self-assembly and polymerization processes related to early forms of life: implications for a prebiotic aqueous medium. *Astrobiology* 2002, 2:139–152.
21. Bui HT, Umakoshi H, Ngo KX, Nishida M, Shimanouchi T, Kuboi R. Liposome membrane itself can affect gene expression in the Escherichia coli cell-free translation system. *Langmuir* 2008, 24:10537–10542.
22. Kazuta Y, Adachi J, Matsuura T, Ono N, Mori H, Yomo T. Comprehensive analysis of the effects of Escherichia coli ORFs on protein translation reaction. *Mol Cell Proteomics* 2008, 7:1530–1540.
23. Cans AS, Wittenberg N, Karlsson R, Sombers L, Karlsson M, Orwar O, Ewing A. Artificial cells: unique insights into exocytosis using liposomes and lipid nanotubes. *Proc Natl Acad Sci U S A* 2003, 100:400–404.
24. Pereira de Souza T, Stano P, Luisi PL. The minimal size of liposome-based model cells brings about a remarkably enhanced entrapment and protein synthesis. *Chembiochem* 2009, 10:1056–1063.
25. Kawarasaki Y, Nakano H, Yamane T. Prolonged cell-free protein synthesis in a batch system using wheat germ extract. *Biosci Biotechnol Biochem*, 1994, 58:1911–1913
26. Nakano H, Tanaka T, Kawarasaki Y, Yamane T. An increased rate of cell-free protein synthesis by condensing wheat-germ extract with ultrafiltration membranes. *Biosci Biotechnol Biochem* 1994, 58:631–634.
27. Zawada JF, Yin G, Steiner AR, Yang J, Naresh A, Roy SM, Gold DS, Heinsohn HG, Murray CJ. Microscale to manufacturing scale-up of cell-free cytokine production--a new approach for shortening protein production development timelines. *Biotechnol Bioeng* 2011, 108:1570–1578.
28. Tsuboi T, Takeo S, Iriko H, Jin L, Tsuchimochi M, Matsuda S, Han E-T, Otsuki H, Kaneko O, Sattabongkot J, et al. Wheat germ cell-free system-based production of malaria proteins for discovery of novel vaccine candidates. *Infect Immun* 2008, 76:1702–1708.
29. Jewett MC, Fritz BR, Timmerman LE, Church GM. In vitro integration of ribosomal RNA synthesis, ribosome assembly, and translation. *Mol Syst Biol* 2013, 9:678.
30. Monnard PA, Deamer DW. Membrane self-assembly processes: steps toward the first cellular life. *Anat Rec* 2002, 268:196–207.
31. Apel CL, Deamer DW, Mautner MN. Self-assembled vesicles of monocarboxylic acids and alcohols: conditions for stability and for the encapsulation of biopolymers. *Biochim Biophys Acta* 2002, 1559:1–9.
32. Sunami T, Matsuura T, Suzuki H, Yomo T. Synthesis of functional proteins within liposomes. *Methods Mol Biol* 2010, 607:243–256.
33. Harada T, Discher DE. Materials science: bubble wrap of cell-like aggregates. *Nature* 2011, 471:172–173.
34. Oberholzer T, Albrizio M, Luisi PL. Polymerase chain reaction in liposomes. *Chem Biol* 1995, 2:677–682.
35. Lande MB, Donovan JM, Zeidel ML. The relationship between membrane fluidity and permeabilities to water, solutes, ammonia, and protons. *J Gen Physiol* 1995, 106:67–84.
36. Song L, Hobaugh MR, Shustak C, Cheley S, Bayley H, Gouaux JE. Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore. *Science* 1996, 274:1859–1866.
37. Gardner PM, Winzer K, Davis BG. Sugar synthesis in a protocellular model leads to a cell signalling response in bacteria. *Nat Chem* 2009, 1:377–383.
38. Noireaux V, Libchaber A. A vesicle bioreactor as a step toward an artificial cell assembly. *Proc Natl Acad Sci U S A* 2004, 101:17669–17674.
39. Girsch SJ, Peracchia C. Lens cell-to-cell channel protein: I. Self-assembly into liposomes and permeability regulation by calmodulin. *J Membr Biol* 1985, 83:217–225.
40. Nikaido H, Rosenberg EY. Porin channels in Escherichia coli: studies with liposomes reconstituted from purified proteins. *J Bacteriol* 1983, 153:241–252.
41. Zeidel ML, Ambudkar SV, Smith BL, Agre P. Reconstitution of functional water channels in liposomes containing purified red cell CHIP28 protein. *Biochemistry* 1992, 31:7436–7440.
42. Karlsson M, Sott K, Davidson M, Cans AS, Linderholm P, Chiu D, Orwar O. Formation of geometrically complex lipid nanotube-vesicle networks of higher-order topologies. *Proc Natl Acad Sci U S A* 2002, 99:11573–11578.
43. Terasawa H, Nishimura K, Suzuki H, Matsuura T, Yomo T. Coupling of the fusion and budding of giant phospholipid vesicles containing macromolecules. *Proc Natl Acad Sci U S A* 2012, 109:5942–5947.
44. Szostak JW, Bartel DP, Luisi PL. Synthesizing life. *Nature* 2001, 409:387–390.

45. Hanczyc MM, Szostak JW. Replicating vesicles as models of primitive cell growth and division. *Curr Opin Chem Biol* 2004, 8:660–664.
46. Walde P, Wick R, Fresta M, Mangone A, Luisi PL. Autopoietic self-reproduction of fatty acid vesicles. *J Am Chem Soc* 1994, 116:11649–11654.
47. Kurihara K, Tamura M, Shohda K, Toyota T, Suzuki K, Sugawara T. Self-reproduction of supramolecular giant vesicles combined with the amplification of encapsulated DNA. *Nat Chem* 2011, 3:775–781.
48. Caschera F, Sunami T, Matsuura T, Suzuki H, Hanczyc MM, Yomo T. Programmed vesicle fusion triggers gene expression. *Langmuir* 2011, 27:13082–13090.
49. Zan GH, Tan CM, Deserno M, Lanni F, Losche M. Hemifusion of giant unilamellar vesicles with planar hydrophobic surfaces: a fluorescence microscopy study. *Soft Matter* 2012, 8:10877–10886.
50. Zimmerman SB, Trach SO. Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of Escherichia coli. *J Mol Biol* 1991, 222:599–620.
51. Minton AP. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J Biol Chem* 2001, 276:10577–10580.
52. Stachowiak JC, Schmid EM, Ryan CJ, Ann HS, Sasaki DY, Sherman MB, Geissler PL, Fletcher DA, Hayden CC. Membrane bending by protein-protein crowding. *Nat Cell Biol* 2012, 14:944–949.
53. Hosoda K, Sunami T, Kazuta Y, Matsuura T, Suzuki H, Yomo T. Quantitative study of the structure of multilamellar giant liposomes as a container of protein synthesis reaction. *Langmuir* 2008, 24:13540–13548.
54. Leduc PR, Wong MS, Ferreira PM, Groff RE, Haslinger K, Koonce MP, Lee WY, Love JC, McCammon JA, Monteiro-Riviere NA, et al. Towards an in vivo biologically inspired nanofactory. *Nat Nanotechnol* 2007, 2:3–7.
55. Bourget L, Chang TM. Phenylalanine ammonia-lyase immobilized in microcapsules for the depletion of phenylalanine in plasma in phenylketonuric rat model. *Biochim Biophys Acta* 1986, 883:432–438.
56. Fernandes R, Roy V, Wu HC, Bentley WE. Engineered biological nanofactories trigger quorum sensing response in targeted bacteria. *Nat Nanotechnol* 2010, 5:213–217.
57. Noireaux V, Maeda YT, Libchaber A. Development of an artificial cell, from self-organization to computation and self-reproduction. *Proc Natl Acad Sci U S A* 2011, 108:3473–3480.
58. Karzbrun E, Shin J, Bar-Ziv RH, Noireaux V. Coarse-grained dynamics of protein synthesis in a cell-free system. *Phys Rev Lett* 2011, 106:048104.
59. Shimizu Y, Inoue A, Tomari Y, Suzuki T, Yokogawa T, Nishikawa K, Ueda T. Cell-free translation reconstituted with purified components. *Nat Biotechnol* 2001, 19:751–755.
60. Matsuura T, Kazuta Y, Aita T, Adachi J, Yomo T. Quantifying epistatic interactions among the components constituting the protein translation system. *Mol Syst Biol* 2009, 5:297.
61. Shin J, Noireaux V. Efficient cell-free expression with the endogenous E. coli RNA polymerase and sigma factor 70. *J Biol Eng* 2010, 4:8.
62. Liu DV, Zawada JF, Swartz JR. Streamlining Escherichia coli S30 extract preparation for economical cell-free protein synthesis. *Biotechnol Prog* 2005, 21:460–465.
63. Martin CT, Coleman JE. Kinetic analysis of T7 RNA polymerase-promoter interactions with small synthetic promoters. *Biochemistry* 1987, 26:2690–2696.
64. Studier FW, Moffatt BA. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J Mol Biol* 1986, 189:113–130.
65. Jewett MC, Forster AC. Update on designing and building minimal cells. *Curr Opin Biotechnol* 2010, 21:697–703.
66. Kim DM, Swartz JR. Prolonging cell-free protein synthesis with a novel ATP regeneration system. *Biotechnol Bioeng* 1999, 66:180–188.
67. Spirin AS, Baranov VI, Ryabova LA, Ovodov SY, Alakhov YB. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* 1988, 242:1162–1164.
68. Yu W, Sato K, Wakabayashi M, Nakaishi T, Ko-Mitamura EP, Shima Y, Urabe I, Yomo T. Synthesis of functional protein in liposome. *J Biosci Bioeng* 2001, 92:590–593.
69. Murtas G, Kuruma Y, Bianchini P, Diaspro A, Luisi PL. Protein synthesis in liposomes with a minimal set of enzymes. *Biochem Biophys Res Commun* 2007, 363:12–17.
70. Kuruma Y, Stano P, Ueda T, Luisi PL. A synthetic biology approach to the construction of membrane proteins in semi-synthetic minimal cells. *Biochim Biophys Acta* 2009, 1788:567–574.
71. Sunami T, Kita H, Hosoda K, Matsuura T, Suzuki H, Yomo T. Chapter 2 - Detection and analysis of protein synthesis and RNA replication in giant liposomes. *Methods Enzymol* 2009, 464:19–30.
72. Sunami T, Hosoda K, Suzuki H, Matsuura T, Yomo T. Cellular compartment model for exploring the effect of the lipidic membrane on the kinetics of encapsulated biochemical reactions. *Langmuir* 2010, 26:8544–8551.
73. Ramachandran N, Hainsworth E, Bhullar B, Eisenstein S, Rosen B, Lau AY, Walter JC, LaBaer J. Self-assembling protein microarrays. *Science* 2004, 305:86–90.
74. Traub P, Nomura M. Structure and function of E. coli ribosomes. V. Reconstitution of functionally active 30S

ribosomal particles from RNA and proteins. *Proc Natl Acad Sci U S A* 1968, 59:777–784.

75. Maki JA, Culver GM. Recent developments in factor-facilitated ribosome assembly. *Methods* 2005, 36:313–320.
76. Nierhaus KH, Dohme F. Total reconstitution of functionally active 50S ribosomal subunits from Escherichia coli. *Proc Natl Acad Sci U S A* 1974, 71:4713–4717.
77. Zasloff M. Antimicrobial peptides of multicellular organisms. *Nature* 2002, 415:389–395.
78. Hancock RE, Chapple DS. Peptide antibiotics. *Antimicrob Agents Chemother* 1999, 43:1317–1323.
79. Loose C, Jensen K, Rigoutsos I, Stephanopoulos G. A linguistic model for the rational design of antimicrobial peptides. *Nature* 2006, 443:867–869.
80. Jones DT, Woods DR. Acetone-butanol fermentation revisited. *Microbiol Rev* 1986, 50:484–524.
81. Zhang YH. Production of biocommodities and bioelectricity by cell-free synthetic enzymatic pathway biotransformations: challenges and opportunities. *Biotechnol Bioeng* 2010, 105:663–677.
82. Atkins JF, Gesteland RF, Cech T. *RNA Worlds: From Life's Origins to Diversity in Gene Regulation*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 2011.
83. Paul N, Joyce GF. A self-replicating ligase ribozyme. *Proc Natl Acad Sci U S A* 2002, 99:12733–12740.
84. Noireaux V, Bar-Ziv R, Libchaber A. Principles of cell-free genetic circuit assembly. *Proc Natl Acad Sci U S A* 2003, 100:12672–12677.
85. Lincoln TA, Joyce GF. Self-sustained replication of an RNA enzyme. *Science* 2009, 323:1229–1232.
86. Adamala K, Szostak JW. Nonenzymatic template-directed RNA synthesis inside model protocells. *Science* 2013, 342:1098–1100.
87. Ichihashi N, Matsuura T, Kita H, Sunami T, Suzuki H, Yomo T. Constructing partial models of cells. *Cold Spring Harb Perspect Biol* 2010, 2:a004945.
88. Pinheiro VB, Taylor AI, Cozens C, Abramov M, Renders M, Zhang S, Chaput JC, Wengel J, Peak-Chew SY, McLaughlin SH, et al. Synthetic genetic polymers capable of heredity and evolution. *Science* 2012, 336:341–344.
89. Nourian Z, Roelofsen W, Danelon C. Triggered gene expression in fed-vesicle microreactors with a multifunctional membrane. *Angew Chem Int Ed Engl* 2012, 51:3114–3118.
90. Schroeder A, Goldberg MS, Kastrup C, Wang YX, Jiang S, Joseph BJ, Levins CG, Kannan ST, Langer R, Anderson DG. Remotely activated protein-producing nanoparticles. *Nano Lett* 2012, 12:2685–2689.
91. Kobori S, Ichihashi N, Kazuta Y, Yomo T. A controllable gene expression system in liposomes that includes a positive feedback loop. *Mol Biosyst* 2013, 9:1282–1285.
92. Karig DK, Jung SY, Srijanto B, Collier CP, Simpson ML. Probing cell-free gene expression noise in femtoliter volumes. *ACS Synth Biol* 2013, 2:497–505.
93. Niederholtmeyer H, Stepanova V, Maerkl SJ. Implementation of cell-free biological networks at steady state. *Proc Natl Acad Sci U S A* 2013, 110:15985–15990.
94. Laurent TC. The interaction between polysaccharides and other macromolecules. 5. The solubility of proteins in the presence of dextran. *Biochem J* 1963, 89:253–257.
95. Wang Y, Guo L, Golding I, Cox EC, Ong NP. Quantitative transcription factor binding kinetics at the single-molecule level. *Biophys J* 2009, 96:609–620.
96. Friedman LJ, Gelles J. Mechanism of transcription initiation at an activator-dependent promoter defined by single-molecule observation. *Cell* 2012, 148:679–689.
97. Hammar P, Leroy P, Mahmutovic A, Marklund EG, Berg OG, Elf J. The lac repressor displays facilitated diffusion in living cells. *Science* 2012, 336:1595–1598.
98. Billerbeck S, Harle J, Panke S. The good of two worlds: increasing complexity in cell-free systems. *Curr Opin Biotechnol* 2013, 24:1037–1043.
99. Harris DC, Jewett MC. Cell-free biology: exploiting the interface between synthetic biology and synthetic chemistry. *Curr Opin Biotechnol* 2012, 23:672–678.
100. Forster AC, Church GM. Towards synthesis of a minimal cell. *Mol Syst Biol* 2006, 2:45.
101. Fujii S, Matsuura T, Sunami T, Kazuta Y, Yomo T. In vitro evolution of alpha-hemolysin using a liposome display. *Proc Natl Acad Sci U S A* 2013, 110:16796–16801.
102. Agresti JJ, Antipov E, Abate AR, Ahn K, Rowat AC, Baret JC, Marquez M, Klibanov AM, Griffiths AD, Weitz DA. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. *Proc Natl Acad Sci U S A* 2010, 107:4004–4009.
103. Lentini R, Forlin M, Martini L, Del Bianco C, Spencer AC, Torino D, Mansy SS. Fluorescent proteins and in vitro genetic organization for cell-free synthetic biology. *ACS Synth Biol* 2013, 2:482–489.
104. Chappell J, Jensen K, Freemont PS. Validation of an entirely in vitro approach for rapid prototyping of DNA regulatory elements for synthetic biology. *Nucleic Acids Res* 2013, 41:3471–3481.
105. Karig DK, Iyer S, Simpson ML, Doktycz MJ. Expression optimization and synthetic gene networks in cell-free systems. *Nucleic Acids Res* 2012, 40:3763–3774.
106. Shin J, Jardine P, Noireaux V. Genome replication, synthesis, and assembly of the bacteriophage T7 in a single cell-free reaction. *ACS Synth Biol* 2012, 1:408–413.