27 Cell-like Entities: Scientific Challenges and Future Applications

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27.1 Introduction

In the last few decades, scientists have learned how to manipulate the basic components of life, how to design biomolecular networks, how to evolve biomolecules with unique characteristics, and how to direct and control cellular processes at the molecular level. Using this knowledge as a foundation, it is theoretically possible to conceive of designing biological constructs, which we refer to as cell-like entities (CLEs), that use custom engineered biological machinery to accomplish specified tasks. The practical challenge is: Can we fabricate biological constructs for specific purposes using the same principles and components found in natural biological systems? For example, can CLEs be designed to detect very low levels of specific chemicals and fluoresce to indicate their presence, can they be designed to synthesize functional chemicals on demand, or can they use engineered metabolic pathways to detoxify environmental pollutants? The building of CLEs that could accomplish these goals would result in fundamental breakthroughs in exploiting our understanding of cellular control systems and biochemical information processing.

The concept of the CLE is based on understanding how the biochemical reactions network that we call a living cell functions, how is it controlled, and what the key processes and components are that will provide unique functions when integrated. The goal is not to create a living organism, but to create a biological construct that uses the essence of living systems to provide a wide array of solutions to current technological and medical challenges.

27.1.1 Definition of Nonliving Versus Living

The emergence of cellular life is one of the major transitions in history. The critical factor was the establishment of a closed boundary encapsulating catalytic reactions and genetic information in a well-defined compartment. This co-containment allowed for the parallel evolution of biochemical processes and the information that defines those processes. Although there are significant differences of opinion as to

the definition of what constitutes "living," there are several characteristics of living organisms that all agree are components of life. First, living organisms are open dynamical systems—energy and matter flow across the physical boundary of the system in both directions. The internal components of the organism are not in equilibrium and can be characterized as a quasi-steady state, but are subject to major state transitions. Compared to that of macrochemical systems, the behavior of cellular reactions at the molecular level is unique in several ways. Processes such as diffusion, binding, and catalysis occur in parallel within a confined three-dimensional microvolume. Many of these processes are stochastic in nature, and this stochastic behavior often contributes to functionality (McAdams and Arkin, 1997). Second, living organisms can reproduce. Their physical structure and information content are passed on from generation to generation, and in theory the lineage of any given cell can be traced back to its primordial ancestor. The physical structure of a living organism persists in time, although it may undergo significant transformations. On the other hand, the informational content is relatively stable on a macro scale. Somatic mutations and stochastic phenomena result in individual variability in a population of cells whereas genomic mutations result in population drift over time. This variability permits the exploration of novel solutions at the edges of the available reaction space in response to environmental perturbations. Finally, an important characteristic of living systems is the ability to perform useful (meaningful) work. The definition of "useful" must ultimately relate to ensuring the survival of the species.

As a consequence of the physical chemical nature of the biomolecular networks that constitute the living cell, living cells exhibit several unique properties. Although the basic complement of cellular components is transmitted from parent to daughter cells at the time of cell division, the full structures of the daughter cells require additional assembly and growth. This is accomplished as a consequence of the self-assembling properties of the basic molecular components of the cell combined with a supply of external energy and global control exerted by regulated gene expression. Furthermore, if the cellular structure is physically or chemically damaged, the nature of the damage is recognized and repair mechanisms are activated. The capacity to self-repair allows the cell to exist and maintain normal functions for periods of time that exceed the lifetime of individual biomolecular components, such as proteins and lipids. Another unique feature of cellular systems results from the modular nature of expression of gene sets in response to both internal and external stimuli. The genome contains specific subroutines that are run when biochemical switches are activated. This allows for adaptive responses and reprogramming of cellular functions to ensure survival in a chaotic environment. The responsiveness of cellular systems is mediated by a wide spectrum of receptor molecules that detect environmental signals, transduce the message in the context of the current state of the cellular system, and activate appropriate responses. This capability is facilitated by the exquisite sensitivity

and selectivity of biomolecular reactions. All of these features combine to allow cells to behave in an autonomous yet collective manner and exhibit emergent behaviors that are not manifested by the individual components. The complex regulation of cellular performance results in a robust and stable system that exhibits the properties of a "living" organism.

27.1.2 Useful Components and Functions of Cells

When exploring the properties of biological systems to identify unique capabilities that can be exploited for human purposes, several additional features of biological systems are attractive. Cells are the fundamental unit of living systems and can be thought of as low energy micro-scale building blocks. Their small size is important in constructing intricate microstructures that are highly adapted for efficient utilization of particular niches in the environment. The low energy requirements of cells needed to support internal biochemical and biophysical reactions are provided by a small number of high-energy biochemicals (e.g., ATP, GTP, NADH, NADPH) that are derived from a diverse range of environmental resources. Another important aspect of biological systems is that all information processing is accomplished through biochemical reactions. Unlike silicon-based computer hardware that requires the input of external software programs to perform useful functions, biological systems are run by wetware—the functional program is a fundamental consequence of the properties of the biomolecular components and their interactive biochemical network. Finally, biological systems are capable of interacting with the nonliving world through communication channels that use common message exchange media—photons, electrons, and chemicals. These features can be manipulated to perform useful functions and form the basis of new technologies.

Through evolution, biological systems have solved a wide variety of engineering problems including nano-/micro-scale detectors, mechanical effectors, logic networks, and efficient energy harnessing systems. What are some of the useful functions biological systems perform? They can detect electromagnetic radiation from low-energy infrared to high-energy ionizing radiation. They can detect and respond to physical factors, such as osmotic pressure, mechanical tension, pH, and heat, as well as biochemical factors such as nutrients and signal molecules. Cells have developed selective membranes with associated proteins to control the flux of chemicals ranging from water and ions to macromolecules and, in fact, continuously transport specific molecules in and out of the cell to control the internal environment. At higher levels of organization, cellular membranes consisting of layers of cells with defined gaps filter large volumes of fluids at the molecular level. Semipermeable membranes and biomolecular transport systems can create ionic potentials and serve as batteries. Biological organisms can collect sunlight and produce useful chemical energy with high efficiency. They use flagellar motors to move themselves or to move fluids across

their surface. If they are not free-swimming organisms but are confined to the solid phase, they can still move by systematic rearrangements of their internal scaffolding. Cells can generate photons as luminescence. They can communicate with each other through chemical signals both in the aqueous environment and in the air. They can hibernate or sporulate to protect themselves and survive life-threatening conditions, propagate when conditions are good, and initiate programmed cell death if appropriate. Living organisms have many more amazing capabilities yet to be discovered, all of which may be exploited to solve difficult technical challenges.

27.1.3 Key Features of Cellular Control Systems

As described, living cells are robust autonomous micro-scale agents that possess amazing capabilities to self-organize, self-repair, and evolve new functionalities. From a systems engineering point of view, cells consist of a complex set of nested, nonlinear control systems that, taken together, can ensure survival in the face of large perturbations from "normal" conditions in a dynamic environment. To this end, cells are constantly monitoring their environment with a multitude of sensors and sensor strategies. The inputs from these sensors are integrated into an overall control strategy to survive adverse perturbations and maintain essential functions. Cells are extremely complex, high-performance systems.

The more complex the system is, the more critical the issues concerning control are. The real world is highly chaotic from the perspective of the cell. External perturbations can have catastrophic consequences without adequate control mechanisms. Hence, through evolution, biological systems have explored and exploited novel solution spaces to provide robust biochemical control systems. These biological control networks are dominated by the segregation of information and feedback loops over multiple levels of molecular organization. The stochastic nature of reactions in the intracellular space requires a robust control system that operates in a highly noisy environment. In this situation, the concept of robustness does not mean maintaining the system in a fixed steady state, but rather maintaining a dynamic nonequilibrium system in a condition that is recognizable as a particular phenotype.

Cells must make appropriate decisions to successfully respond to challenges in the environment. There are many examples of specific control systems in biological systems. The metabolic response of *E. coli* to changes in nutrients through the lac operon (Ozbudak et al., 2004) involves changes in the regulation of gene expression that ultimately control the energy metabolism in the cell. The decision to divide and subsequent regulation of the cell cycle to successfully generate daughter cells (Pomerening, Sontag, and Ferrell, 2003) require the synchronized expression of a spectrum of genes in response to internal signals. At a more fundamental level is the interaction between bacteria and viruses (bacteriophages). The interplay between the genome of the bacteria and the bacteriophage determines not only the survival of the cell but the behavior of the virus (see, e.g., the interaction of *E. coli* and the λ-

phage; Arkin, Ross, and McAdams, 1998). In all of these cases, decisions within cells are achieved by biochemical switches.

Research into complex cellular control systems is providing new insights into the molecular mechanisms of biocontrol processes. The study of "minimal cells" provides clues as to which genes and processes are the most fundamental to a viable cell (Hutchison et al., 1999; see also chapters 3 and 16). Another approach to understanding the complexity of biological systems employs perturbation analysis that is accomplished by combining genetic manipulations to perturb the system and techniques to measure global gene expression in response to the perturbation (Ideker et al., 2001; Rao and Arkin, 2001). Using this research approach, researchers discovered that specific changes in the expression of genes in a single pathway have repercussions on gene and protein expression of almost every major biochemical pathway in the cell, emphasizing the interconnectedness of cellular reactions and the importance of distributed control systems to maintenance of the system in its entirety.

27.2 The Cell-Like Entity

27.2.1 Concept of CLEs

The basic concept of the CLE is that of a biological construct engineered on the principles of cellular systems, but not an engineered cell. CLEs will have an artificial genome that contains the blueprints of the construction and functioning of the CLE. It will have synthetic systems to transcribe genes and translate mRNA to produce functional proteins. But it will not in fact be a living organism; that is, once constructed, the CLE cannot reproduce or evolve. By this definition, the CLE falls well into the nonliving world (figure 27.1), no different from many products based on biomolecules. CLEs can be engineered using molecular techniques to integrate a spectrum of biological components and processes that provide signal transduction, signal fusion, and decision-making capabilities based on the nested control strategy

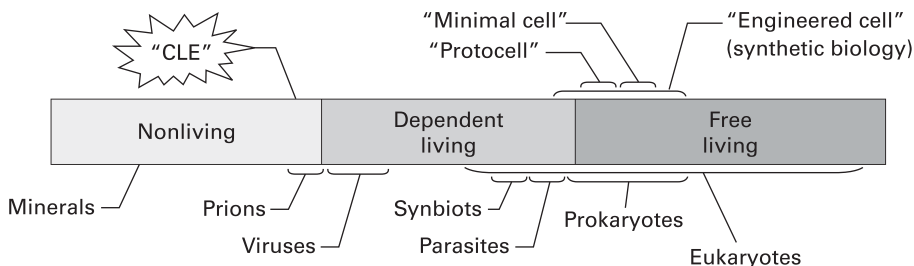

Figure 27.1
The location of bioengineered products on the spectrum of nonliving to living. Synthetic biology and the minimal cell are top-down approaches (reengineering of living organisms). The cell-like entity (CLE) and protocells are bottom-up approaches.

of cells. Custom engineering of extracellular adhesion molecules on the surfaces of CLEs will enable programming of complex network architectures for sensing and control functions. CLEs have the potential to serve as the interface between silicon- and carbon-based technologies. The vision of the fully operational CLE will be a multifunctional, robust, self-organizing bioengineered entity that can be integrated into a wide range of systems and devices and at the same time be affordable, self-repairing, and sustainable.

The strategy to build CLEs from the bottom up was selected to overcome some of the disadvantages associated with using engineered living cells. First, the behavior of cells is highly dependent on the environment in which they live. For a given organism, growth, survival, and functional properties are maintained in a "normal" range only when environmental physical/chemical conditions are controlled within a limited range. Extremeophiles can thrive in conditions that are adverse to most other cells; however, they perform poorly under more normal conditions. Few cells have large dynamic ranges and therefore have limited robustness with respect to device engineering. Second, it is difficult to express foreign genes that are toxic to biochemical machinery within cells. These products of gene expression often trigger unforeseen reactions that are detrimental to the cell and limit the design space for engineering functionality. Third, many engineered cells exhibit genetic instability with respect to the engineered components. Even when the construct is relatively stable, there is a large range of functionality in supposedly identical constructs. This diversity is beneficial to free-living organisms, allowing subsets of the population to use environmental noise, but the efficiency of the system is less than the theoretical maximum from an engineering point of view. Fourth, the design of synthetic circuits is complicated by the requirement that control elements should not cross-talk with normal cellular control processes. This limits the spectrum of possible control elements that can be used in any particular cell type. And finally, there are ethical issues concerning the use of engineered organisms in the general environment. The concern is that such organisms can impact the stability of natural systems and have unintended consequences that are socially unacceptable. The CLE concept does not necessarily solve all of these problems, but if it can be demonstrated that CLEs have an advantage over living organisms in any of these problem areas, then they will have an operational niche.

A basic tenant of this project is that the necessary components needed to construct a CLE for a given function can be found in nature. The concept that one can artificially integrate the molecular scale interactions of cellular components to achieve a defined function is relatively recent (Pohorille and Deamer, 2002). However, the feasibility of such a concept has been demonstrated already by the work of Libchaber and Noireaux (Noireaux and Libchaber, 2004; Noireaux et al., 2005; Yu et al., 2001) and Ishikawa and cowokers (2004). Noireaux and Libchaber constructed an encapsulated bioreactor, an initial step toward developing a CLE. The system con-

sisted of a plasmid containing the enhanced green fluorescence protein (eGFP) gene encapsulated in a unilamellar lecithin vesicle that contains a commercial in vitro transcription translation system. The researchers measured the formation of eGFP in the vesicles as an indicator of functionality. They addressed the requirement of nutrient and energy availability by incorporating the α-hemolysin gene into the plasmid. The protein product from this gene spontaneously inserts in the encapsulating membrane, forming pores to allow influx of substrates. In another study, Ishikawa and colleagues (2004) constructed a genetic network by expressing a transcriptional activation cascade (SP6 RNA polymerase makes T7 RNA polymerase that transcribes GFPmut1-His6) in a cell-free transcription-translation system. This system was encapsulated in liposomes and its functionality demonstrated by observing the increase in fluorescence emission at 545 nm (excitation at 488 nm) from the reaction mixture. Both of these studies demonstrate the feasibility of the CLE concept—to use biological machinery at the molecular level to perform specific tasks—and provide insights into the creation of self-assembling synthetic pathways.

27.2.2 Components of CLEs

The basic components of a fully functional CLE are illustrated in figure 27.2, and the key features of these components are described in table 27.1. The first five components (vesicle, artificial genome, synthetic system, energy converting system, and input system) are essential to the basic CLE platform. The vesicle is the functional

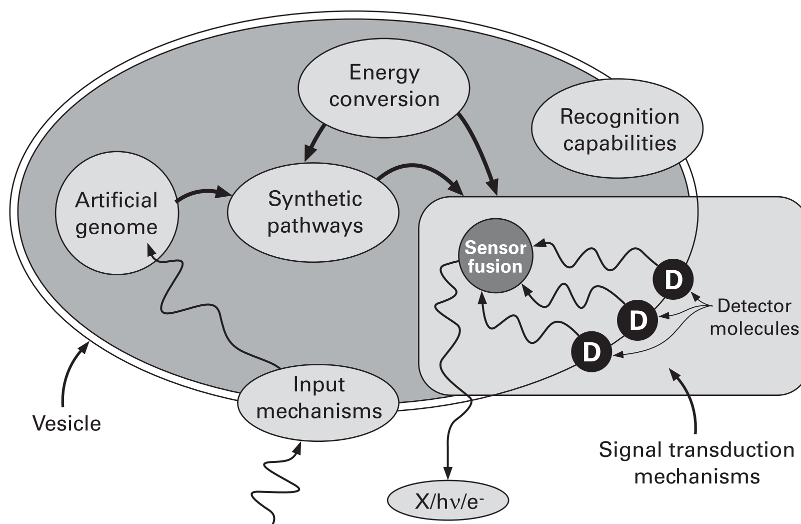

Figure 27.2
Schematic diagram of a CLE illustrating basic components and an integrated functionality (signal detection).

Table 27.1
Basic components of a cell-like entity (CLE)

Component	Description
Artificial genome	Permanently stores information required to construct CLEs and enable them to accomplish specific functions.
Vesicle	The envelope that encapsulates the components of the CLE and regulates transport of molecules between the external and internal environments.
Energy conversion system	Provides required energy in a suitable chemical currency to self-assemble and maintain structure of CLE.
Synthetic systems	Synthetic capability to take external substrates and synthesize molecular components of CLE.
Input system	Capability to activate specific functional cassettes of the artificial genome to program CLE functionality.
Output systems	Spectrum of processes to output information captured by CLE to operator.
Recognition systems	Provide capability to organize CLEs into higher-order structures, either in 2 or 3 dimensions.
Signal transduction system	Augments basic CLE with the capability to detect environmental signals and communicate that information through the output system.

container for the individual CLE. The artificial genome contains the blueprints for the structure and function of the CLE. The information content of the artificial genome is converted by the synthetic system into operational components of the CLE that self-assemble into the functional CLE using energy provided by the energy generation system. The input system allows for reprogramming the CLE from one functionality to another in a way analogous to how cytokines reprogram cells for specific functions. Given the basic CLE platform consisting of these five components, additional capabilities can be introduced to detect environmental signals, organize CLEs into hierarchical structures, and output information collected by the CLE to the device operator. These systems must be integrated to generate a micro-scale entity that will conduct useful work.

27.3 Status of CLE Development

27.3.1 Research Plan

The CLE concept is to use the principles and processes inherent in cellular control systems to engineer a unique entity that possesses the functionalities required for autonomous control of physical devices. It is the understanding of the nature of these molecular processes that will enable the development of novel capabilities. The objectives of the program are to demonstrate feasibility, prototype functionality, and control capability of CLEs. Following are the major efforts of the research program:

1. Component engineering: Identify and exploit existing biochemical/physiological processes that will provide essential components of a CLE (lipid vesicle, artificial genome, energy conversion systems, and synthetic pathway to assemble and repair vesicles).
2. Systems integration: Develop a proto-CLE, a simple organic (liposome-like encapsulated) system based on known biochemistry and physiology of single-celled organisms and demonstrate proto-CLE functionality, for example, signal transduction or synthetic function.
3. Advanced development: Optimize basic components and integrate new functionalities to produce fully functional CLE platform and demonstrate CLE control of electromechanical component or subsystem.
4. Manufacturing and fabrication: Develop microfabrication techniques to manufacture large quantities of functional CLEs at low cost for systems applications.

The necessary research and development of CLEs require a staged effort to accomplish. The initial goal is to develop and demonstrate the proto-CLE, consisting of an encapsulated in vitro transcription-translation system with a defined synthetic function. In essence, the proto-CLE will be a vesicle construct that is on intensive care. All substrates and energy (e.g., ATP and GTP) will be provided externally. This construct will serve as the benchmark for future CLE development. The next step is to evolve the CLE from the proto-CLE to a minimal CLE, which consists of a system that functions with minimal life support, that is, it will generate its own chemical energy from an external carbon source and synthesize and self-assemble the majority of its synthetic and functional components. The third stage will produce the fully operational CLE platform. This bioconstruct will self-assemble all of its biomolecular components, harvest energy from external resources, and self-organize into higher-order architectural structures. Along the way, it is anticipated that various spinoff products will be developed from CLEs at different levels of evolution.

To execute this research and development plan, a series of research efforts must be undertaken. Each effort—encapsulation, artificial genome, energy conversion, synthetic systems, and functional systems—addresses the development of one of the major components of the CLE. The current status of these efforts is summarized in the next section. It should be noted that most of these issues are also critical for the assembly of minimal cells (see, e.g., chapter 16).

27.3.2 Encapsulation—Confinement of Components

Development of an encapsulation system for a CLE presents a technological hurdle, since this encapsulation system must allow substrates, chemical energy, and monomer subunits (i.e., amino acids and ribonucleotide triphosphates) to cross the membrane while maintaining the proper barrier to retain CLE components and exclude

undesired foreign components. As a critical step in the development of a CLE, the main focus is on capturing the in vitro cell-free transcription/translation (CFTT) system within liposomes (see, e.g., discussion in chapter 3). Alterations to the encapsulation process or the lipid components of the liposomes provide strategies to increase the efficiency and functionality of encapsulation. After the CFTT is determined to have been captured within the liposome, expression tests will determine whether the synthetic system is still actively transcribing DNA and translating proteins inside liposomes.

A large technical gap exists between incorporating the CFTT system into an artificial membrane system and having a functional CLE. The major challenge is to move necessary substrates or precursor components across the membrane faster than they are consumed in the interior of the liposome. Lipid membranes are permeable to small molecules, including water and ions, to varying degrees (Chakrabarti and Deamer, 1992; Paula et al., 1996). To increase the permeability, multiple lipid species can be used and, because of the slight mismatch in packing between lipid species (i.e., saturated versus mono- or diunsaturated), the permeability of the liposome membrane can be manipulated (Mouritsen and Zuckermann, 1987). As the lipid complexity increases, macroscopic membrane domains will form, the boundaries of which are considerably mismatched (Leidy et al., 2001). Such a system will probably be necessary when large components must cross the membrane or when integral membrane proteins are incorporated into the system. At the outset, two or three lipid component systems may be sufficient to achieve the appropriate flux across the membrane. Another technical hurdle to be addressed is whether the liposomes fuse with each other during the course of CLE operations. Using a low percentage of lipids that have a charged head group could induce the appropriate repulsion between adjacent liposomes. As an alternative, giant unilamellar vesicles (GUVs) can be used. GUVs are on the order of tens of micrometers in diameter, are effectively "flat" because their radius of curvature is relatively large and, thus, have little affinity to fuse (Fisher, Oberholzer, and Luisi, 2000). By using a combination of these techniques, it is possible to optimize the membrane for incorporation and efficient operation of the proto-CLE.

In addition to the uptake of essential "nutrients," the CLE vesicle will have to include physical pathways to dispose of biochemical waste and reaction byproducts, the accumulation of which may limit the efficiency of synthetic reactions and render the CLE nonfunctional. Porins and other active and nonactive transport proteins incorporated as part of the encapsulation could potentially assist in eliminating unneeded chemicals. In addition, similar mechanisms can be used to construct CLEs capable of responding to stimuli by exporting chemical signals into the surrounding milieu and stimulate synergistic responses in other CLEs by quorum sensing circuitry. Pohorille, Schweighofer, and Wilson (2005) have recently discussed the adapt-

ability of membrane channels to diverse functional demands, which can be applicable to the design of CLEs.

CLE encapsulation systems must be tested under a variety of environmental conditions to provide experimental data as to which systems perform best. Under real-world conditions, temperature and humidity are not regulated. Lipid membrane fluidity is affected by temperature, causing it to undergo transitions from liquid crystalline to gel at temperatures around the phase transition temperature (T_m). In the gel state, the membrane permeability is considerably decreased. However, during the phase transition, the membranes are considerably more permeable than either above or below the phase transition temperature (Cruzeiro-Hansson and Mouritsen, 1988; Hays et al., 2001). Furthermore, the temperature range in which the membrane will be stable is dictated by the lipid composition and characteristics of any membrane proteins present (Oberholzer, Albrizio, and Luisi, 1995; Oberholzer et al., 1995). There are extremophiles that live in hot, cold, and dry environments, and understanding how their membrane components are adapted for such environments and mimicking those components in a CLE membrane system would increase the range in which CLEs could be used (Kiran et al., 2004; Macalady et al., 2004). Another environmental stress that CLEs may encounter is dehydration. Under functional conditions, lipid membranes must be hydrated and the associated water molecules help to maintain the fluidity of the membrane. There are techniques that can "trick" the membrane into thinking that it is still hydrated, and the most common of these is to add the disaccharide trehalose to the membrane suspension before it is dehydrated. The hydrogen bonding between the trehalose and the lipid headgroups mimics that between the lipid headgroups and water, so that the trehalose effectively traps the lipids in the fluid phase when dehydrated and maintains its integrity (Crowe, Carpenter, and Crowe, 1998; Crowe, Reid, and Crowe, 1996).

One drawback of lipid-based membranes for encapsulating CLEs is that they are biologically based, and therefore are labile to environmental stresses. Alternative membrane systems, termed polymerosomes, are based on diblock copolymers. Diblock copolymers have hydrophilic moieties on both ends and a hydrophobic region in the center of the molecule. These diblock copolymers spontaneously form vesicles, similar to liposomes, consisting of a monolayer of copolymers with a hydrophilic compartment in the center sandwiched between the polar ends. Diblock copolymer vesicles can be made and tested for their permeability to various ions and CLE components (Discher and Eisenberg, 2002; Taubert, Napoli, and Meier, 2004). Depending on their permeability properties, polymerosomes can be employed as the encapsulation system for CLEs and tested for efficient performance compared to the liposome-based encapsulation system. Polymerosomes have the advantages that they are mechanically stronger than liposomes, are not as biologically labile,

and may not be affected as much as lipid-based membranes under extreme temperature conditions.

Optimal performance of the CLEs will depend directly on the establishment of adaptive metabolite transport and transmembrane signal transduction coupled to the desired responses. Although useful applications with semipermeable membranes based on mixtures of lipid components will be achieved with the CLEs, in many cases, these will prove insufficient and more complex asymmetric membranes will be necessary. As the CLE system develops and becomes increasingly more complex, technical advances in membrane systems will continue to be evaluated with the goal of optimizing membrane components. A combinatorial approach can be taken to determine which lipids, polymers, or hybrid combinations are the most efficient for optimal CLE functionality. In addition, it will be necessary to incorporate a spectrum of proteins into the encapsulation membrane to transport a variety of cofactors and substrates across the membrane. It is yet to be determined which membrane transport proteins are appropriate for the efficient loading of the CLEs, and they will have to be tested to determine whether they can be incorporated into various vesicle systems. Recent studies have been concerned with the activity of integral membrane proteins in polymer-based vesicles, and these will be used as a basis to test the use of polymerosomes in our CLE system (Haefele, Kita-Tokarczyk, and Meier, 2006; Nallani et al., 2006; Ranquin et al., 2005).

27.3.3 Artificial Genomes—Genes and Control of Gene Expression

The artificial chromosome serves as the blueprint for self-assembly of the operational CLE. It consists of the set of genes required to provide the basic functions of the CLE as well as the control mechanisms at multiple levels of molecular organization—genome, transcriptome, proteome, and metabolome—to efficiently regulate expression of genes in biomolecular constructs. Specific technical challenges to designing and engineering the artificial chromosome are

- identifying the minimal gene set to provide the CLE with sufficient basal activity to self-assembling given a starter set of substrates and components,
- synthesizing an artificial chromosome that contains the required gene set, and
- developing biomolecular switches to regulate gene expression at multiple levels of biological organization.

Successfully meeting these challenges requires a coordinated research effort.

A circular bacterialike chromosome is the genome of choice for the CLE because of its higher stability. Linear DNA molecules are sensitive to exonuclease degradation and therefore would require additional features for the protection of the DNA ends (Bendich and Drlica, 2000; Ishikawa and Naito, 1999). The CLE genome must

include all necessary genes to maintain the CLE and also integrate genes for specific functions depending on the application of the CLE. The "application" genes will encode proteins involved in performing the CLE function. Several attempts have been made to determine the minimal gene set for bacteria by experimental approaches (Akerly et al., 2002; Forsyth et al., 2002; Kolisnychenko et al., 2002; Sassetti, Boyd, and Rubin, 2001) as well as by comparative analysis of prokaryotic genomes (Gil et al., 2004; Islas et al., 2004; Koonin, 2000). By comparing the genomes of two simple prokaryotic organisms, *Haemophilus influenza* (about 1,700 genes) and *Mycoplasma genitalium* (about 470 genes), Mushegian and Koonin (1996) identified a set of 255 genes (Koonin, 2000) that were orthologous between the two species. Because *M. genitalium* and *H. influenzae* belong to two different bacterial lineages, the genes that are conserved in these two bacteria were thought to be essential for the functioning of a modern-type cell even under the most favorable environmental conditions (i.e., abundance of nutrients and lack of competition or stress). This set was hypothesized to constitute a minimal gene set of a living cell. In a more recent study, Gil and coworkers (2004) suggested a minimal gene set consisting of about 206 genes that encode proteins involved in transcription, translation, DNA replication and repair, cell division, protein folding and secretion, protein and RNA processing and degradation, metabolite transport, energy metabolism, and maintenance of the cell membrane. Since CLEs are not intended to divide, the genes for DNA replication and cell division would not need to be included in the CLE genome. The number of genes can be further reduced by using only one codon per amino acid, so that fewer genes encoding aminoacyl-tRNA-synthetases and tRNA would be necessary (Luisi, 2002). Depending on the nutrients provided to the CLE, the basic maintenance genes might vary and individual genes will have to be added or deleted (see discussion of minimal genome size in chapter 3).

Once it has been decided which genes should be part of the CLE genome, the artificial chromosome must be synthesized. In recent years, multiple attempts have been made to not only improve but also reduce time and cost of de novo DNA synthesis. Different error-correcting techniques such as mismatch-binding protein MutS and enzymatic cleavage of mismatches have been developed to diminish the number of errors in synthesized DNA fragments (Carr et al., 2004; Fuhrmann et al., 2005). Smith, Hutchison, Pfannkoch, and Venter (2003) demonstrated the assembly of a whole genome (5,836 bp) from ∮X174 bacteriophage starting from synthetic oligonucleotides. Further synthesis methods for large DNA fragments have been described in the literature (Kodumal et al., 2004; Xiong et al., 2004).

The third challenge is to establish an in vitro gene expression system that can be controlled at the transcriptional, translational, and protein degradation level. A key operational parameter is how rapidly functional gene expression circuits can be shut

down after challenging the system with a control signal, and how fast they can restart again when the signal is removed. Our hypothesis is that each individual control mechanism will inhibit the gene expression to a certain degree. All three inhibitory mechanisms combined should be able to exert stringent control of the expression of the proteins of interest and shorten the time delay of the response of the system after exposure to the external signal. Our long-term interest in controllable genetic elements is to incorporate them into the artificial genome to regulate the reading of the blueprints for CLEs.

27.3.4 Energy Conversion Systems—Providing Energy

A critical issue to be resolved is that of how to design these constructs to extract energy from their environment and how to program them for energy conservation. Energy coupling will be required for the functional catalysis of biochemical reactions inside the CLE and to establish communication between its interior and the environment. The processes to be carried out by these microdevices must obey the thermodynamic principles of energy conversion. Two types of mechanisms have been identified to drive the energetics of biological constructs: an internal substrate-level phosphorylation (i.e., ATP synthesis through other high-energy intermediates) and generation of an electrochemical proton potential coupled with ATP synthesis (e.g., respiration and photosynthesis). Extensive literature provides insight into how to tackle this issue (Anthony, 1980; Harold, 1986; Nichols and Ferguson, 1992). In bacteria, for example, the oxidation of an aldehyde by NAD drives the formation of a high-energy acyl phosphate. Acyl phosphates serve as phosphoryl donors for ATP formation. Electron-transport–coupled phosphorylation, on the other hand, functions as a free energy converter. Under aerobic conditions in bacteria, the electron-transport chains consist of a dehydrogenase and a reductase, and this redox process is coupled to ATP synthesis by proton translocation across the membrane. In photophosphorylation, electron-transport and proton translocation are driven by light (Lengeler, Drews, and Schlegel, 1999). Both approaches will need to be explored and adapted to the CLE platform for specific applications.

27.3.5 Synthetic Systems—Generating Components

To convert the information stored in the artificial chromosome into functional elements, proteins and metabolites, a set of synthetic pathways is required for the CLE. The first step is the development of these synthetic systems. This involves the establishment of an in vitro transcription and translation system in which genes contained in the artificial genome can be expressed. Protein biosynthesis outside an intact cell has been studied for several decades (Betton, 2003; Spirin et al., 1988; Swartz, 2001; Voloshin and Swartz, 2005). Development of procedures to separate endogenous mRNA from the ribosome was a key discovery that allowed for diverse

applications of in vitro transcription and translation systems (Spirin, 2002). Extracts from *E. coli*, rabbit reticulocyte, and wheat germ are available from commercial vendors that allow protein synthesis of virtually any exogenous mRNA or DNA template (Spirin, 2002). Although these synthetic systems are versatile, many are not energy efficient and, because of the crude nature of the separation from other cellular material, contain many side reactions unrelated to transcription and translation.

Incorporation of well-defined transcription and translation components into the CLE will improve energy efficiency and eliminate extraneous reactions that may interfere with CLE function. One such defined system has been developed by Shimizu and coworkers (2001). The "protein synthesis using recombinant elements," termed the PURE system, includes 32 individually purified components and has been shown to produce functional proteins. The PURE system uses as a template either mRNA or DNA, allowing the genetic flexibility needed for the CLE. The components have been designed with hexa-histidine tags and GST fusion peptides in order to achieve one-step purification of a desired protein, which would allow for simplified quantification and quality control testing of CLE protein products (Shimizu, Kanamori, and Ueda, 2005). Ueda and coworkers have constructed a system without inhibitory factors such as nuclease, proteases, and other enzymes that hydrolyze nucleotides as well as compensatory reactions for energy regeneration and sulfur bond formation. Well-defined control of the essential processes (transcription, translation, and energy regeneration) of the CLE are crucial for its functionality.

The CLE will include other synthetic processes, and a spectrum of several genes that can be expressed by T7 RNA polymerase under the control of the T7 promoter is being investigated. One case study is focused on demonstrating the self-assembly of the glutathione (GSH) synthetic pathway in an in vitro CFTT system. The key goals are to

- construct a functional self-assembling in vitro metabolic pathway;
- obtain quantitative measurements of system behavior to define operational parameters; and
- investigate and optimize the behavior of the in vitro synthetic system.

The glutathione synthetic pathway was selected for this demonstration because GSH plays an important role in modulating the oxidation-reduction status of the cell. GSH is a ubiquitous tripeptide that participates in diverse biological functions, including gene expression, apoptosis, signal transduction, metabolism, and membrane transport (Sies, 1999). GSH is synthesized in bacteria through a two-step enzymatic process involving γ-glutamylcysteine ligase (GCL) and glutathione synthetase (GS) (Kelly, Antholine, and Griffith, 2002). In the nomenclature of *E. coli* genes, the two enzymes correspond to the two genes *gshA* and *gshB*, respectively. Both GCL and

GS depend on ATP and Mg^{2+} for catalytic activity. In bacteria, GS is functionally active as a homotetramer (Yamaguchi et al., 1993). The rate-limiting step of GSH synthesis is controlled by GCL, which is controlled through feedback inhibition by GSH. Plasmids containing *gshA* and *gshB* have been constructed and the functional gene products have been expressed in both *E. coli* and several commercial in vitro transcription/translation systems. The GCL and GS protein products have been constructed with a hexa-histidine tag and S-tag, respectively, in order to simplify detection, quantitation, and purification for subsequent kinetic parameterization. Formation of reaction products, γ-glutamylcysteine and glutathione, and consumption of amino acid substrates over time are measured by a rapid and sensitive high-performance liquid chromatography (HPLC) method for amino acid detection (a modification of the method of Henderson et al., 2001; and Nardi, Cipollaro, and Loguercio, 1990). ATP utilization and ADP formation are also measured as indicators of GCL and GS enzymatic activity. Such quantitative data will support the mathematical modeling of this biomolecular network and help build the biomolecular engineering tools to enhance CLE design and engineering.

Measuring the rates of transcription, translation, and glutathione synthesis in this system allows for the investigation of this synthetic system under defined conditions. Protein levels, transcripts, all amino acids, γ-glutamylcysteine, glutathione, and all nucleotides are quantitatively measured. These time course data provide the needed reaction parameters to quantitatively predict the behavior of this self-assembling synthetic network model. The next phase of the program will include encapsulating the self-assembling GSH synthetic network into liposomes (see section 3.2). Real-time monitoring methods are under investigation to determine protein production (using a GCL-fluorescent protein fusion product) in the enclosed system. Additional synthetic pathways will be developed for the CLE to provide desired functionality.

27.3.6 Functionality

Building a proto-CLE by successfully integrating the major components (first four components in table 27.1) to provide the basic operational capabilities is an enormous challenge. However, such a basic CLE is not very useful on its own since it lacks any input-output systems and cannot perform useful work apart from its own repair. The next objective is to use the proto-CLE as a basis for design and manufacture of a more complex functional CLE capable of unique actions and functions. If such capabilities can be engineered as separate interchangeable genetic modules, they can be easily added to a basic proto-CLE to produce a variety of functions.

To build a CLE module with a specific function, three separate components are usually required. The first component is a sensor/receptor. The ability to sense envi-

ronmental stimuli is an integral part of almost any biological organism, and living cells use a variety of sensors. Receptors for a variety of stimuli, including metal ions, environmental stress (temperature, osmolarity, UV damage, gamma irradiation, oxidative damage), and organic and inorganic compounds are known (Nivens et al., 2004). These sensors can be engineered into a CLE functional module so that the module gains the ability to detect a corresponding stimulus and respond accordingly. Alternatively, several classes of molecules such as RNA ribozymes and aptamers can be designed to bind to a variety of chemicals and thus serve as sensors (Bayer and Smolke 2005, Breaker 2002). Yet another approach is to use computational tools to redesign an active site of an existing receptor to bind a different molecule (Looger et al., 2003).

The second component of a functional module involves signal transduction. This component serves as a conduit of information flow from the sensor to the output mechanism. In many cases, signal transduction is an integral part of receptor regulation (Bayer and Smolke, 2005), as is the case for the two-component signal transduction systems (Stock, Robinson, and Goudreau, 2000) and bacterial transcriptional regulation. In many cases, a link between sensor and output can be engineered to create different receptors with the same output function. Alternatively, different output functions can be coupled to the same receptor (Bayer and Smolke, 2005).

The third component of the functional module is the output function. As in previous cases, many choices are available for the output function. Examples include reporters that produce a measurable quantitative signal (fluorescent proteins, luciferases, enzymes participating in a colorimetric reaction) and proteins that can participate in biodegradation of a specific compound (bioremediation of organic pollutants, heavy metals, etc.). Not all three components of a functional module must to be present at the same time; one can, for example, design an ‘‘always-on’’ module that will not require any sensing or signal transduction but will have only an output function. For example, a genetic oscillator can periodically produce a chemical and thus serve as a molecular time clock (Elowitz and Leibler, 2000).

Current research is focused on an effort to combine several recent advances in signal transduction and gene regulatory circuits to synthesize a sophisticated biosensor module in a bacterial system with the ultimate goal of incorporating the module into the CLE. The proposed module is designed to sense the presence of bivalent mercury ions (a toxic water contaminant) and translate the signal into an output in the form of a fluorescent protein. Bivalent mercury binds to a Mer repressor protein, which then becomes an activator of the Mer promoter. Transcription from the Mer promoter produces several proteins: yellow fluorescent protein, which serves as a measurable real-time output; mercury reductase, which converts bivalent mercury into the neutral species that can diffuse from the environment; and a T7 RNA

polymerase, which activates the expression of the second operon. Once the signal (bivalent mercury ions) is dispersed and no new contamination is introduced, the operon returns to its original repressed state and thus ceases to produce yellow fluorescent protein output. However, T7 RNA polymerase transcribed from the Mer promoter will jump-start a second autoinducible operon consisting of a cyan fluorescent protein and another gene for T7 RNA-polymerase. This operon will continuously produce a persistent, "mnemonic" output to document the past presence of the contaminant. A version of such a module can potentially be used in CLEs as a mercury biosensor and bioremediator.

At the current stage of the program, we have successfully built the first operon that was shown to respond to the addition of mercury chloride, with the magnitude and length of the response (as measured by the total fluorescence of bacterial cultures) dependent on the initial concentration of mercury. Experiments are under way to implement the second operon and to combine both operons into one functional module.

27.3.7 Outstanding Issues

At this stage of development of the CLE technology, several technical challenges remain. Many potential applications of the CLEs will require that they be functional situations other than water-based applications. In these cases, the application will require that gases and fluids be able to interact with CLEs in order to generate the desired responses. These responses will be more efficient and controllable if CLEs are constructed using microfluidic channels on solid or semisolid supports. In addition, microfabrication would allow arraying CLEs of multiple functionalities capable of transducing signals across the microchannels and increase their versatility. Several groups have established protocols for microfabrication of gels and other polymers within microfluidic channels; progress in this field has recently been reviewed (Peterson, 2005). Other examples of sophisticated methods to control microfluidic applications both temporarily and spatially have been developed, as well as a renewed understanding of the ability of microfluidics to use the particular properties of fluids at the micro-scale, where viscosity and surface tension are most important (Atencia and Beebe, 2005). These technologies can be merged with that of the CLEs to harness the full potential of these hybrid systems. It should be noted that the same trend using programmable microfluidics is seen in the development and possible programming of protocells (see chapter 12).

One final issue is the mass production of CLEs. Fabrication techniques will undoubtedly be affected by microfluidic technologies. Recent developments have been made using microfabricated flow cytometers that can detect, analyze, and sort cells or particles, through an integration of microfluidics, optics, and electronics (Huh et al., 2005). These high-speed devices will allow for the analysis and separation of CLEs following encapsulation of the molecular components.

27.4 Applications

The CLE program seeks to create novel technologies for civilian and military applications, including smart biosensors that are able to respond to changes in the environment, tracking devices to locate personnel, and monitoring devices to detect physiological change with built-in response systems to modulate any adverse conditions that may be detected. CLEs, being the biological equivalent of an integrated circuit, can provide multifunctional capabilities that support combined detection and response functionalities or "smart sensors." Proposed applications for this technology include sensing of physical, chemical, and biological events in the environment; monitoring of human health and fatigue status; decision making on the basis of incongruent, incomplete, or corrupted information (fuzzy logic); and control of micro-/nanoelectromechanical devices (MEMS, NEMS).

Even without exploiting the potential of a gene system for sensing or responding, the CLE may serve as a platform for nanoscale motors or similar devices. The potential for producing customized hybrid cell-like platforms with specific molecule adhesion and recognition properties offers new immobilization options that may survive extreme environments better than engineered cells.

27.4.1 Remote Operations Sensing

CLE-based multidimensional sensing (CBMS) exploits cellular signal transduction components, processes, and systems to perform as real-time surveillance and reconnaissance, to decrease information processing time, and to improve integration and decision-making capabilities based on multidimensional information (heat, light, chemical, biological, ionizing radiation, and vibration signatures). Remediation can be integrated with the detection functions, resulting in numerous applications. The utilization of cells in detection systems is not new (DeBusschere and Kovacs, 2001; Gilchrist et al., 2001; Gray et al., 2001; Pancrazio et al., 1998; Park and Shuler, 2003; Rainina et al., 1996; Stenger et al., 2001). However the options that will be available for CLE components provide a much greater range of applications, storage and operational capacity. CBMS sensors could

- monitor activities in an area of interest such as detection and analysis of movement of animals or persons, vehicle emission detection, or environmental impact assessment;
- go where current macro-scaled technology cannot and significantly reduce risk of personnel exposure to toxic or hazardous environments;
- offer stealthy surveillance and reconnaissance potential, since these systems are chemically rather than electronically based, and thus do not radiate telltale signals unless interrogated or activated;

• be embedded into materials with the ability to be formed into a variety of shapes and features that provide natural camouflage for sensor networks;

• assist with rapid decision making based on real-time data gathered from multidimensional sensors;

• integrate bio-based constructs with mechanical devices to act as the central controller for autonomous movement and activity; and

• replace heavy, bulky equipment with ultra-lightweight sensors and systems for space applications.

27.4.2 Medical Applications

CLEs have a wide range of potential applications in medical diagnosis and treatment. Implants containing multifunctional CLEs could be designed to provide continuous monitoring of physiological biomarkers that indicate degradation in health or performance and rapidly modulate the effects with appropriate drugs or antibiotics before clinical symptoms develop. As a survival tool, CLEs can be designed to augment digestive capability, allowing enzymatic digestion of complex biomolecules such as cellulose (converting plant fibers to absorbable sugars).

In principle, CLEs could replace many treatments based on stem cells or gene therapy currently being evaluated. Instead of introducing a pluripotent stem cell and hoping the specific progeny develops in a timely fashion in the right place, the mature function could be designed into CLEs and implanted. Genes for producing insulin and clotting factors have been known for years and have been prime targets for both stem cell and gene therapy. CLEs could combine the production of these substances with the capacity to monitor the physiological status of the patient, making them an obvious target for this application (Sapir et al., 2005; Shi et al., 2005).

CLE implants could provide cytokine-based cell communication in a more cost effective manner than current treatments. Specific cytokine and chemokine cocktails could be engineered for immune system modulation for cancer treatments, tissue regeneration, and more. Incorporated into synthetic skin for burn patients, CLEs could activate on application and accelerate the integration of the material as well as producing adhesive, bacteriostatic, and immune-inducing compounds.

27.4.3 Other Applications

Much discussion has taken place over micro-scale and nano-scale motors. Such devices require integration into larger-scale platforms and present a challenge for control and functional support. The CLE platform has the potential to solve this problem by providing the capability for autonomous control of microelectromechanical devices. The size, durability, low energy requirements, and multifunctionality of CLEs provide advantages over other possible solutions.

CLEs also have some advantages in the area of biocomputing. The rate of advances in biomolecular computing is rapidly increasing. Functions such as inverters and AND gate toggle switches have been developed in *E. coli* from naturally occurring proteins (Feng et al., 2004; Weiss, 2001; Weiss et al., 2003; Yokobayashi et al., 2003). CLEs can be engineered to carry out any logic function that is possible in bacteria with two advantages: The simplicity of the CLE relative to natural bacteria reduces the chance of accidental interference between the genetic circuit and critical cellular functions, and CLEs will be able to survive better in environmental extremes than natural bacteria. In addition, the CLE membranes and input/output functions can be specifically designed to optimize bio-silico communication.

27.5 Conclusion

The concept of the cell-like entity is enticing because it uses the basic principles of living organisms (biomolecular reaction networks that couple genome to function) without actually qualifying as living. Thus, the CLE is on the boundary between nonliving and living. The technical challenges that remain are formidable, but recent research in the published literature has demonstrated the feasibility of the concept. Over the next several years, the big issues—synthesizing large artificial genomes, preparing defined transcription/translation systems, designing and fabricating encapsulating systems, and engineering functional modules—will be addressed and resolved. CLEs will not solve every technological problem, but they will provide unique options for solving a wide range of problems and very likely open up yet to be discovered application avenues.

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