Artificial cells: Prospects for biotechnology.

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Summary

A variety of techniques can now be used to alter the genome of a cell. Although these techniques are very powerful, they also have limitations related to cost and efficiency of scale. Artificial cells designed for specific applications combine properties of biological systems such as nano-scale efficiency, self-organization and adaptability at relatively low cost. Individual components needed for such structures have already been developed, and now the main challenge is to integrate them in functional microscopic compartments. It will then become possible to design and construct communities of artificial cells that can perform different tasks related to therapeutic and diagnostic applications.

Designing an artificial “minimal cell”

Recent advances in molecular biology have created opportunities to modify living cells in ways that have significant applications in biotechnology. A variety of techniques can now be used to alter the genome of a cell by well-established approaches such as recombinant DNA, gene shuffling, and knock-out mutations. These techniques are most advanced for single-celled microorganisms but have also been successfully applied to multi-cellular species. Although genetic techniques are very powerful, they also have limitations related to cost and efficiency of scale. Furthermore, genetically modified cells require specialized nutrient media, the desired products of genetic manipulations may be toxic to the cells, and combining cellular and non-biological technologies is often quite difficult. Genetic engineering also poses significant safety and ethical issues that are particularly relevant in medical and environmental applications.

An alternative approach is to build simple, cell-like structures that are engineered for a specific application. A few such systems have been created so far, and many of the components needed to construct more complex artificial cells with biotechnological potential are already in place. We will first consider a theoretical minimal cell that would incorporate all properties of the living state, including growth and evolution. Such a minimal cell has never been constructed, but listing its
properties will illustrate how far we have to go before the claim of artificial cellular life can be made.

An ideal minimal cell would be expected to have the following properties:

1. An information-carrying polymer, such as a nucleic acid, must be synthesized by a template-directed polymerization that occurs in a membrane-bounded volume.
2. The monomers of the polymer must be provided externally, and transported across the membrane boundary to support the replication process. Also, other small molecules or ions needed for biosynthetic reactions must be delivered from the environment.
3. An external source of chemical energy must be available to drive the biosynthetic reactions.
4. A catalytic activity must be present that is linked to the replication process, so that variations in replication affect the rate or efficiency of the catalyzed reaction. Under such circumstances variations will change fitness of the system and lead to evolution. Compartmentalization of the replicating catalytic system within a membrane-bounded volume allows selection of variations, leading to "speciation" of the encapsulated genetic material.
5. The boundary membrane itself must be able to grow, either by accumulation of membrane-forming material from the environment or by conversion of precursor molecules into such material.
6. There must be a mechanism that allows the assembly to separate into two or more smaller structures during the growth process, and the smaller structures should contain the capabilities of the larger system.
7. Catalysis, replication and growth must be well regulated so that none of the processes lags behind or gets too far ahead of other processes in the cell.
8. The most complex version of a minimal cell, verging on being truly alive, would include a translation apparatus that could use sequence information in one kind of polymer to direct the synthesis of a second kind of polymer. In contemporary cells this is the ribosome, but simpler structures may be capable of similar functions.

If we were to attempt the construction of an idealized artificial cell with the properties listed above, what components are available to be incorporated into the molecular system? The simplest is the boundary membrane itself. Membranous vesicles composed of pure lipid bilayers have been in the laboratory repertoire for over thirty years.

The second component is a replicating molecular system. A variety of nucleic acid polymerases are commonly employed to catalyze the synthesis of DNA and RNA from template molecules, using nucleotide triphosphates as substrates.

The third component is a translation system, in which genetic information present in nucleic acids is used to direct the synthesis of specific proteins. Again, translation systems are readily available that use ribosomes and activated amino acids to produce peptides and larger proteins.

Finally, we have at our disposal a broad range of natural and engineered proteins that have been optimized for many catalytic, binding and structural functions. These proteins can be used to design artificial metabolisms inside vesicles.

It is clear that the individual pieces of molecular machinery are available, but what is lacking is their incorporation into a supramolecular structure in which all of the components function as an integrated system. We will now go on to describe each of the components in greater detail, then discuss the prospects for establishing such a system.
Creating membrane boundaries.

Preparing lipid bilayer vesicles (liposomes) is a mature field, since they are already being used by the pharmaceutical industry as drug delivery agents and in certain cosmetic preparations. Lipid vesicles were first prepared in the 1960s. As technology progressed, specific terms came to be associated with the physical and chemical properties of various preparations. The original liposomes produced by Bangham and co-workers are now referred to as multi-lamellar vesicles (MLV) due to the fact that most vesicle were composed of hundreds of concentric lipid bilayers. Because the multilamellar character is often a limitation, efforts immediately began to produce more homogeneous preparations, and it was found that small unilamellar vesicles (SUV) could be prepared by sonication of MLV preparations, having dimensions in the range of 25 nm - 100 nm. Large unilamellar vesicles (LUV) ranging from 100 nm to 1 μm diameter were first prepared in the mid-1970s and are standard preparations today. The most common preparation method is extrusion through polycarbonate filters.

Which of these preparations might be most appropriate for artificial cells? The smallest forms of cellular life are approximately 0.2 - 0.5 μm in diameter, so LUVs in principle could contain a sufficient number of macromolecules to form artificial cellular systems. These are convenient for a number of reasons, the most important being that they are readily prepared and reasonably stable.

Transport across cell walls

A vesicle is just an envelope for an artificial cellular system. To turn it into a functional unit there must be mechanisms for exchange of nutrients, waste products, regulatory molecules, macromolecules and ions between the vesicle and its environment. The internal volume of a typical liposome with a diameter of 0.2 μm is only 4x10^-18 liters, which means that in a 1.0 mM substrate concentration only 2400 substrate molecules might be captured with a given enzyme activity. These would be entirely used up in less than a second at typical enzymatic reaction rates, so transmembrane transport is essential.

While many small, neutral molecules simply permeate through vesicle walls by a form of diffusion, lipid bilayers present substantial permeability barriers to charged, zwitterionic and macromolecular species. To some extent their permeation properties can be regulated through manipulating the width and composition of the bilayer. For instance, by choosing short-chain lipids it is possible to prepare liposomes that allow smaller molecules to permeate but retain macromolecules. However, to achieve efficient, controlled and selective transport an assisted mechanism is necessary. This is provided by either carrier molecules or transmembrane protein channels and pumps. Channels and carriers mediate passive transport (along the concentration gradient) whereas pumps use energy to transport species against the gradient.

Although an ion-conducting channel in a cell membrane can be quite complex, the transmembrane portion is often relatively simple, consisting of a bundle of α-helices or a barrel of β-strands but occasionally in the form of nanotubes. Moreover, many channels retain their functions even if a large fraction of the protein is removed. This relative simplicity can be exploited
to design simple, synthetic channels or re-engineer natural ones so that they have the desired transport properties. This is done through manipulations of the amino acid sequence along the pore, helix orientation and the diameter of the transmembrane cavity, thereby providing fine-tuning of electrostatic and steric interaction between the solute and the channel. Probably most common design is based on four to six helices arranged about a central axis. A particularly simple example is the neutral peptide, Ac-(Leu-Ser-Ser-Leu-Leu-Ser-Leu)3CONH₂, which forms a voltage-gated channel. Addition of a negative or positive charge in the mouth of the channel respectively increases or eliminates selectivity towards cations. In contrast, a tetrameric channel formed by 25-residue fragments of the M2 protein from the human Influenza virus, also folded into α-helices, does not transport alkali ions but instead conducts protons at remarkably high rates. This channel is shown in Fig. 1. Proton transport presumably proceeds through a chain of properly aligned water molecules filling the transmembrane cavity. Selectivity is provided by a gate made of four histidine residues, one from each of the channel-forming helices, which are sufficiently large to occlude the lumen. These histidines also actively participate in proton transport.

Another class of transmembrane channels is based on cyclic peptides, which adopt flat ring structures. They stack to form hollow cylindrical nanotubes stabilized by backbone-backbone hydrogen bonding interactions. The rings are formed either by alternating D- and L-α-amino acids or by homochiral β-amino acids. Because no side chains project into the lumen of the channel their specificity is manipulated primarily by changing the diameter of the cavity rather than the identity of residues forming the ring. Tubular channels are stable, well-oriented in the bilayer and can be designed to transport solutes that markedly differ in size, ranging from protons to glucose.

Lipid bilayers can be made accessible to even larger solutes through incorporation of a natural channel, α-hemolysin or its genetically engineered mutants. For instance, the hemolysin channel can transport not just individual solutes such as nucleotide triphosphates, but even linear polymers as large as single stranded nucleic acids.

Despite considerable advances, designing efficient and selective membrane channels remains a challenge. First, the channels must be stable and assume correct orientation and stoichiometry. This is not a simple task, especially for self-assembling structures, because single units often do not partition into membranes. They gain stability in the transmembrane orientation only through associations. The free energy of helix-helix association was measured for the transmembrane fragment of the glycophorin A dimer and was found to be 9 kcal mol⁻¹ in detergent micelles. Single point mutations can change this free energy by 1-3 kcal mol⁻¹. One strategy to avoid difficulties associated with insufficient stability and improper orientation and stoichiometry is to covalently attach helices at specific positions to a template.

A second challenge is to regulate channel activity. This can be done by including triggers, that turn the channel on or off once, or switches, which modulate channel activity in response to a signal. Triggers and switches can be either chemical or physical, where the stimulus is provided by light, pH or voltage. For example, pH-sensitive channels that can be conveniently coupled to light-driven energy generation have been constructed. For engineered α-hemolysin channels both biochemical and physical triggers have been constructed. Despite these successes, controlling channel activity in liposome preparations still remains a difficult task.
Supplying energy to the liposome

A source of chemical energy is required to drive metabolic reactions inside an artificial cell. Energy can be supplied from the environment in the form of ATP molecules, but this mechanism is possible only if the membrane bilayer provides transient defects that allow ATP to permeate into the vesicle. This is possible under certain conditions but permeation rates are slow, ranging around a few nucleotides per second in dimyristoylphosphatidylcholine liposomes.

An alternative approach is to mimic the energy transduction process employed by all living cells, in which light or chemical energy from the environment is converted to a transmembrane proton gradient, which is subsequently used in the production of ATP from ADP and phosphate. Probably the simplest and the most robust artificial system for the continuous, light-driven generation of ATP consists of two membrane proteins: bacteriorhodopsin, which works as a proton pump, and ATP synthase (ATPase), which couples the flow of protons through the enzyme to the phosphorylation of ADP. If the thermophilic ATPase is used the system is very stable and can function for several months. The rates of continuous ATP production in such a system at saturating light intensity are 0.2-0.8 nmol of ATP min⁻¹ mg⁻¹, but rates can be improved by optimizing conditions for the insertion of the proteins into liposomes in the correct orientation and coupling between the components. Other ways to generate transmembrane proton gradients include a light-induced electron transfer along a redox chain consisting of carotene, porphyrin and naphthoquinone linked so that they span the membrane.

Macromolecules inside the liposome

Any artificial cell-like system is expected to encapsulate metabolic functions catalyzed by macromolecules. Although considerable progress has been made toward developing lipid vesicles containing a variety of macromolecules (see Fig. 2), the task of assembling an encapsulated system capable of catalysts presents several formidable difficulties.

First, the encapsulation must occur without damaging the catalytic activity of the macromolecule. An early approach to this problem is the dehydration-rehydration method in which proteins can be efficiently encapsulated without loss of activity. The method is relatively gentle, in that it relies on dehydration to drive a fusion event in which liposome membranes "sandwich" the macromolecule. Another early method is the freeze-thaw technique, which also depends on a kind of dehydration (ice formation) leading to membrane fusion and encapsulation. A more recent approach is simply to inject the molecules into sufficiently large liposomes. The latter is limited to only a few vesicles and could not be used for an industrial scale preparation.

One complex enzyme system that has been demonstrated to function after encapsulation in liposomes is the T7 RNA polymerase. The enzyme uses all four nucleoside triphosphates to synthesize an RNA transcript from the DNA template. The substrates are supplied from the environment by permeation across the liposome membranes, and the system is "pumped" by raising and lowering the temperature through the phase transition of the DMPC liposomes at 23 degrees C. Under these conditions, the permeability coefficient for nucleoside triphosphates is 10⁻⁹ cm s⁻¹.
equivalent to approximately 5 NTP molecules per second entering a typical liposome. This is much below the saturating substrate concentration, so that the enzyme produces a variety of chain lengths of RNA until each newly synthesized strand falls off the active site. In order to confirm that transcription of a specific DNA sequence occurred, RT-PCR was used to amplify a specific strand interval defined by two primers. The results showed that specific sequences in the template are in fact transcribed by the polymerase into RNA. This is shown in Fig. 3.

A second complex system captured in liposomes was reported by Luisi and his collaborators who demonstrated that the PCR reaction could be carried out in liposomes. Because the substrate was also entrapped in this system, rather than supplied externally, only small amounts of product were produced. This group has also attempted to encapsulate ribosomes in liposomes and modest yields of a translation product directed by mRNA were detected.

Recently, the entire in vitro transcription/translation system and a library of genes linked to a substrate for the desired reaction have been captured in a different compartmentalized system - a water-in-oil emulsion. On average, one gene occupies a water droplet, and the genes are transcribed and translated within their compartments. Subsequently the newly synthesized proteins convert the substrate into a product. Then the emulsion is broken and genes linked to the product, but not to the unprocessed substrate, are enriched and amplified. If required they can be again linked to the substrate and subjected to the next round of selection. This process has been demonstrated by performing selection for DNA methylation.

Encapsulating a similar system inside liposomes would have several advantages compared to the water-in-oil emulsions. In particular, it would be possible to continuously supply substrates to the reaction compartments and therefore perform multiple-turnover selection rather than single-turnover catalysis, which occurs when the substrate is physically linked to the gene. This should markedly increase the efficiency of the selected catalyst. Furthermore, selection could be performed for a wide range of desirable reactions, which are not amenable to single-turnover catalysis. However, this procedure can be successful only if liposomes containing products can be separated from those that do not. This can be done, for example, if products, but not substrates, are fluorescent. Using micropipets and micromanipulation methods, it should be possible to isolate a single liposome containing several hundred RNA molecules produced by a transcription process that also incorporates fluorescent markers. RT-PCR could then be used to amplify the product, followed by further rounds of transcription and amplification until an RNA with a desired function has evolved. This kind of molecular ‘breeding’ has the potential to be more efficient than standard methods that involve trillions of RNA molecules simultaneously passing through a specific selective hurdle. Essentially the same strategy can be used in the recently developed multiple-turnover method for selecting novel proteins. This, in turn, would open a possibility for creating protein catalyzing technologically interesting chemical reactions that do not have equivalents in biological systems.

If capturing the transcription/translation system in liposomes was successful it could be readily extended to encapsulate “mini-genomes” yielding products that could not be obtained from single enzymatic reaction but instead required a metabolic pathway. This could be coupled to the energy supply, further enhancing the capability of the system. A schematic of a cell-like structure endowed with these properties is shown in Fig. 4. In addition, expression in such a system could be
regulated from the outside by including, for example, the Lac promoter in the mini-genome. Such extensions, however, would create a host of new issues associated with making all the elements work in concert. For example, accumulation of reaction products at high concentrations would result in product inhibition causing the reactions rates to be markedly reduced.

From what we have learned over the past decade, it is even possible that complex macromolecular systems can be encapsulated in membrane-bound structures that can grow and divide. For instance, Luisi and his colleagues have established several lipid model systems that have the capability to undergo a kind of growth process. Furthermore, Walde et al. used oleic acid vesicles to demonstrate an encapsulated reaction in which polynucleotide phosphorylase synthesized RNA from ADP. In the oleic acid system the lipid bilayer itself can be induced to "grow" under certain conditions, an essential property if the liposome is to become more than just a microscopic test tube.

**Coupling replication and ribozymes**

One potentially powerful approach to creating a minimal cell is to incorporate into vesicles a replicating activity based on ribozymes, which can act both as catalysts and carriers of genetic information. Several laboratories have made steady progress in this direction. In one laboratory model of a replicating RNA system, a reverse transcriptase first copies a DNA strand from a specific ribozyme, and a second polymerase makes multiple copies of the RNA from the DNA, thereby amplifying the original RNA strand thousands of times as the cycle is repeated. Importantly, the ribozyme itself can also evolve under these conditions when faced with a suitable selective pressure. For example, Beaudry and Joyce found that it was possible to produce a specific catalytic site on a ribozyme by continuously selecting for that site with biochemical hurdles, a kind of molecular breeding carried out in the test tube. Wilson and Szostak went on to show that a specific catalytic site could be selected from a mixture of trillions of random RNA sequences, similar to the kind of selection that would have occurred in a population of early molecules competing for a resource. Most recently, Bartel and co-workers have demonstrated that an artificially evolved ribozyme can in fact act as an RNA-dependent RNA polymerase. This opens the door towards creating a true, ribozome-based, self-reproducing system. However, several technical hurdles still remain which are related to the large size, low efficiency, poor fidelity and fast folding of the present polymerases.

**Biotechnological applications**

The simplest artificial cell based on ribozymes could provide new insight into the origin and early evolution of life and, in a long run, form the basis for developing complex, evolving, artificial cells with multiple functionalities. However, immediate practical applications of such a construct would be limited. From biotechnological perspective, it is more profitable to focus instead on cell-like structures that do not have the capability to self-replicate. They are, however, able to separate functional macromolecules from the environment, regulate the exchange of material across the membrane boundary, support metabolism, transduce environmental energy into chemical energy and synthesize a desired biosynthetic product.
Among many potential applications of artificial cell-like structures, one of the most promising is in pharmacology and medical diagnostics. In recent years, considerable progress has been made toward using liposomes as drug delivery vehicles. This technology can be extended by encapsulating a drug in an inactive form and providing the means to convert it into an active compound in response to an external signal. This signal could be, for example, light or compounds present in high concentration in the target tissue but not in other parts of the organism. If pH-sensitive liposomes containing a light-driven proton pump were used drug release could be triggered by their acidification upon illumination. This approach to the targeted drug delivery might considerably reduce toxic side effects and increase efficacy of the treatment.

Another class of applications is to use functionalized liposomes as biosensors. In their simplest form, these devices would contain a macromolecule that would respond to specific analytes by emitting a fluorescent signal upon binding the analyte. Recent advances in engineering fluorescing enzymes greatly improved their ability to work in stable, selective and reversible sensing devices. Enzymes, however, cannot be used in reversible devices that generate a sustained signal over longer periods of time because of substrate consumption. One strategy to avoid this difficulty is to use coenzyme-depleted enzymes (apo-enzymes) which still bind the substrate but are unable to catalyze its conversion to the products. For example, glucose oxidase is used to estimate glucose concentration in blood and urine samples. To prevent glucose oxidation, FAD cofactor required for the reaction is removed. Glucose levels can then be reversibly measured by monitoring fluorescence of a tryptophan residue that is sensitive to glucose binding. Sensitivity can be further improved by attaching to glucose oxidase a fluorescent label that is affected by glucose binding.

The capabilities of macromolecular biosensors can be greatly enhanced by encapsulation in liposomes. For example, instead of sensing an analyte directly it might be simpler to monitor the products of an enzymatic reaction for which this analyte serves as the substrate. This would be the case if a sensitive probe of the product but not the substrate were available. For such a system to work properly, all components should be co-located, which can be accomplished by enclosing them in liposomes. In another example, liposomes can provide the means to construct multi-functional biosensors. For instance, a single liposome might contain several components, each sensitive to a different analyte and capable of fluorescing at a different wave length.

Conclusions and outlook

Artificial cells designed for specific applications offer unprecedented opportunities for biotechnology because they allow us to combine properties of biological systems such as nanoscale efficiency, self-organization and adaptability for therapeutic and diagnostic applications. Many individual components needed for such structures have already been developed and many others are likely to be constructed in the near future. The main challenge now is to encapsulate them in a single cellular compartment and ensure that they will work in concert in a controlled manner. Once these tasks are accomplished it will become possible to construct communities of artificial cells that can self-organize to perform different tasks and even evolve in response to changes in the environment.
References


3 Hope, M. J. et al. (1985) Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size, distribution of trapped volume and ability to maintain a membrane potential Biochim. Biophys. Acta 812, 55-65


7 Lear, J. D. et al. (1997) Electrostatic effects on ion selectivity and rectification in designed ion channel peptides J. Am. Chem. Soc. 119, 3212-3217

8 Pinto, L.H. et al. (1997) A functionally defined model for the M2 proton channel of Influenza A virus suggests a mechanism for its ion selectivity Proc. Natl. Acad. Sci. USA 94, 11301-11306


16 Tuchscherer, G. et al. (1998) Protein design – on the threshold of functional properties Biopolymers 47, 63-73


19 Pitard, B. et al. (1996) ATP synthesis by the F₀F₁ ATP synthase from thermophilic Bacillus PS3 reconstituted into liposomes with bacteriorhodopsin. I. Factors defining the optimal reconstitution of ATP synthases with bacteriorhodopsin Eur. J. Biochem. 235, 769-778


**Figure Captions**

Fig. 1. The M2 proton channel in a hydrated phospholipid bilayer. The four $\alpha$-helices forming the channel are represented using the ribbon model. The membrane-forming lipids are shown as wires and water molecules are represented by CPK models (oxygen atoms are red and hydrogen atoms are white). The file of water molecules inside the channel is interrupted by histidine residues (also represented by CPK models) in the upper part of the channel. The structure of the system was generated from molecular dynamics computer simulations (Wilson, M. A. and Pohorille, A., unpublished).

Fig. 2. Phase and fluorescence micrographs of vesicles produced from n-dodecanoic acid n-dodecanol, 5:1 mole ratio, pH 8.0. The original vesicles formed by the acid-alcohol mix were first mixed in a 2:1 mass ratio with sonicated salmon testis DNA (~600 base pairs in length) then dried. A very dilute solution of acridine orange dye was then added and the vesicles were photographed by phase and fluorescence, original magnification 400 X. (These vesicles are on average 20 $\mu$m in diameter.) Note that phase-dark vesicles (A) contain fluorescent-stained DNA (B), while the phase-light vesicles do not take up dye and therefore do not contain significant amounts of DNA. The dark phase contrast is produced by the refractive index difference in those vesicles containing DNA.

Fig. 3. Fluorescence micrograph of a liposome preparation in which the encapsulated T7 RNA polymerase has synthesized RNA, using a DNA template captured with the enzyme activity and externally added nucleoside triphosphates (ATP, UTP, GTP, CTP). Some liposomes contain the presumed RNA, which is stained by ethidium bromide, while others are empty and do not manifest
the orange fluorescence characteristic of the dye. Synthesis of RNA by the encapsulated enzyme was confirmed by uptake of labeled UTP in RNA. Original magnification is 400 X. Vesicles range from 10 to 50 μm in diameter.

Fig. 4. A hypothetical cell-like structure. A gene is transcribed to RNA and translated to the protein with the aid of the encapsulated transcription/translation system (right side). Amino acids needed for translation are delivered from the environment through the transmembrane channel and activated (red square) by ATP (top). ATP is synthesized from ADP and phosphate by a light-driven bioenergetics system (left side) composed of bacteriorhodopsin (bR) and ATP synthase (ATPase). This system is described in more detail in the text.