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A PROTOCELL DESIGN FOR BIOACCUMULATION APPLICATIONS

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ABSTRACT

This article provides a specific example of recombinant cell and protocell technology, moving from what is presently known to suggesting how novel application of existing methodologies could be utilized to design a complex synthetic system in form of a self-sufficient light empowered protocell. A practical application of protocells using a primary example of desalination in water treatment is given, followed by a more general approach to bioaccumulation and bio-diagnostics, outlining the possibilities associated with applications of protocells. The key hypothesis is that the inside-negative electrochemical membrane potential generated by Cl^- pump activity via halorhodopsin could also be utilized to drive the accumulation of cations into a protocell. Thus, the functional expression of halorhodopsin could energize proton-coupled uptake of substances or metals through a selective cotransport channel for a number of applications in biotechnology, molecular medicine, and water biotechnology.

Keywords

Protocells, membrane potential, polymersomes, bioaccumulation, molecular medicine.

INTRODUCTION

Between 4.0-3.5 billion years ago the first cell on Earth emerged. This first cell existed only a brief moment, and represented the beginning of life as we know it [Altermann et al., 2003]. Shortly after the abiogenesis this original cell split in two, and these two split again, and during a short geological time scale Earth was populated by unicellular organisms. That was the beginning of the history of life on this planet, and thus the beginning of the history of biology itself.

Synthetic biology reflects the view that the best way to investigate the accuracy and limits of current biological knowledge and phenomena is to modify or engineer a different artificial version of a complex biological system and compare its functions with theoretical expectations [Solé et al., 2007]. Efforts to understand the origin of life have resulted in the research in protocells. Research in protocells is a bottom-up form of synthetic biology that is synthesizing novel kinds of living systems from components that have never been alive [Rasmussen et al., 2009].

Designing the first bottom-up protocell will herald the first time science have synthesized life from wholly nonliving materials. Advances in protocell research are gaining public awareness, and this awareness will increase as work on protocells proceeds from the research to the development stage [Mark et al., 2009]. There is much ground to be explored before autonomous protocells become a reality. As well as fundamental construction issues, design will focus on maintaining compatibility—whether by separation of processes or tailoring of reagents. But when we do gain the ability to create protocells this could have profound impacts on many people's view of life.

While having vast importance in their own right, protocells also offers great promise in biotechnology, where they offer a valuable simplification compared to the use of for instance recombinant bacteria, which might ease public acceptance. Carefully selected parts of metabolic pathways can hypothetically be utilized to create purpose-built protocells for a variety of applications with highly specific functions.

The ability of bacteria to degrade a range of organic compounds has been utilized in waste processing, bioremediation and the production of chemicals to mention just a few examples [Ishige et al., 2005]. Their wide applicability could also be utilized by harvesting and expressing membrane transport proteins from i.e. cyanobacteria into the synthetic membranes of protocells. A number of membrane proteins have been successfully integrated into GUVs of a size appropriate for a protocell, including potassium channels [Aimon et al., 2011], and bacteriorhodopsin [Girard et al., 2004].

In this article a practical application of protocells using a primary example of desalination in water treatment, followed by a more general approach to bioaccumulation and bio-diagnostics will be discussed, exemplifying the possibilities associated with applications of self-sufficient protocells, many of which have relevance to other biotechnologies.

RECOMBINANT CELLS AS DESALINATION RESERVOURS

Progress in recombinant biology techniques in the 1990s heralded new approaches enabling exploration of biological systems in ways never seen before. The power to clone, modify and express a huge array of proteins was extended even further in synthetic biology

by the capacity to reconstitute recombinant macromolecules into cellular systems [Barla et al., 2013]. The first and most basic example in this paper is a desalination process consisting of the creation of a low-salt biological reservoir system within a water environment that can function as a protocell ion exchanger.

Cyanobacteria are a photosynthetic organism capable of growing phototrophically due to oxygenic photosynthesis, utilizing photosynthetically active radiation as their main source of energy, and heterotrophically at the expense of diminished carbon sources in the dark [Miller et al., 1984; Nogales et al., 2012]. Cyanobacteria can be cultivated under outdoor conditions and a potential biological utility of cyanobacteria for desalination with some success was the reclamation of salt-affected agricultural soils in India [Singh, 2010] and the Soviet Union [Gollerbach et al., 1956] using endemic strains, leading to subsequent cultivation of horticultural crops.

As a line of defense, cyanobacteria employ a range of Na^+ export proteins in their cell membrane, where the activation of Na^+-export mechanisms such as Na^+/H^+-antiporters and Na^+/ATPases results in the reduction of the sodium concentration [Nitschmann et al., 1992]. The hydrolysis of ATP by ATPase is the driving force for Na^+ transport and the rate of hydrolysis is controlled by ion potential, i.e. the lower the potential, the higher the activity [Wiangnon et al., 2007].

Naturally, if the utilization of ATP is blocked, either by itself for some reason or is deliberately manipulated through genetic or environmental means, the export of Na^+ would come to a halt. As soon as active Na^+ export has come to a halt, Na^+ ions will begin to diffuse from the region of higher concentration to a region of lower concentration until equilibrium with the external medium is reached. Diffusion is thermodynamically favorable until equilibrium is reached and additional extraction of Na^+ from the medium will then demand an energy source. If desalination of water is the objective, then it is necessary to prevent renewal of Na^+ export, and the energy-harvesting system employed during this step should not utilize ATP as an intermediate.

Potential candidates for ATP-independent light-powered biological units could be halorhodopsin (Hr) proteins from *Natronomonas pharaonis* (pHr), that is, inward-directed Cl^- pumps [Lanyi, 1990]. Halorhodopsin uses light energy to import the chloride ion into the cell against the membrane potential. The Hr from *Natronomonas pharaonis* has been cloned and functionally expressed in heterologous systems such as *E. coli* [Hohenfeld et al.., 1999].

As will be explained in more detail in the next section, the idea is to introduce such a light empowered halorhodopsin pump into the membrane. The electrochemical potential that emerges across the recombinant membrane due to anion transport could be used to energize proton-coupled uptake of Na^+ through a selective cotransport protein.

This approach could hypothetically create a recombinant cyanobacteria strain, using novel methods [Heidom et al., 2011; Minas et al., 2015], where the resulting light empowered NaCl accumulator bypasses the endogenous energy metabolism and should hypothetically remain operational despite the increasing intracellular Na^+ levels inhibiting other metabolic functions of the recombinant bacteria.

Expression of pHr in such a cyanobacterial culture should remove both Cl^- and Na^+ from their surrounding water environment and thus becoming a living vessel for bioaccumulation. However, excess accumulation of NaCl in natural and presumably recombinant cells threatens to lead to destabilization of membranes and proteins [Huflejt et al., 1990] and a cease of photoautotrophic growth [Bhargava et al., 2003].

Further, modifying existing organisms this way still holds some inherent limitations; the degree of control by re-directing living cells still holds many variables and attempts to integrate new biological functions are often unsuccessful. While optimization can be accomplished to some degree through metabolic engineering of organisms, e.g., by evolution and selection [Smith et al., 2011] host organism modification is not easily done [Fischer et al., 2008], and a number of issues associated with modifying existing organisms exists.

A top down approach could be attempted to simplify the recombinant cyanobacteria in order to make it a better design. However, theoretically, a protocell designed bottom up offer here a much simpler solution. The degree of control over protocell design far exceeds that which can be obtained by re-directing living cells, which will be discussed shortly.

THE MEMBRANE POTENTIAL

As explained, once active Na^+ export has come to a halt, Na^+ ions will begin to diffuse from the region of higher concentration to a region of lower concentration down a concentration gradient until equilibrium with the external medium is reached. The electrical potential difference that counterbalances diffusion due to the concentration difference is called the equilibrium potential for that specific ion. Further extraction of Na^+ from the medium will demand an energy source.

An energy source is vital in any cell, synthetic or living. The latter utilize biochemical respiratory pathways and the electron transfer chain to produce energy currency such as ATP to drive reactions. In the most elementary protocell design, all of the required small molecule components can be provided for biosynthesis, with ATP provided as an energy source [Noireaux et al., 2004]. These elementary designs eliminate the demand for energy generation and

although impressive in their own right, are unlikely to be the wanted end designs due to the demand for continual renewal of all molecule components. In order to prevent renewal of Na^+ export, the energy-harvesting system used during this step should therefore not use ATP as an intermediate.

An alternative and more sustainable design would require pathways for independent and efficient energy generation essentially designing an autonomous protocell capable of utilizing an easily available energy source such as sunlight to support function. Such a synthetic design would require a straightforward metabolic pathway to harvest the light energy into a usable form of energy.

Proposed candidates for ATP-independent light-powered biological units could be halorhodopsin (Hr) proteins. Hr is an inward-directed Cl^- pump [Mukohata et al., 1981; Lanyi, 1990]. Hr, functioning as a Cl^- pump, has the ability to transport Cl^- against an electrochemical gradient, and can generate an inside-negative membrane potential to support ATP synthesis [Rudiger et al. 1997]. Hr, a member of archaeal rhodopsins, occurs ubiquitously in highly salt-tolerant halobacteria and contains all-trans-retinal as a chromophore and uses light energy to mediate primarily the chloride ion into the cell [Váró, 2000]. Absorption of a photon with a defined optimal wavelength (X_{max}) induces trans-cis isomerization of retinal, which triggers a catalytic photocycle of conformational changes in the chromophore protein, resulting in the net transport of one chloride per photon toward the cytosol [Kouyama et al., 2010].

An effective implementation strategy would be to segregate the energy-generating pathway in an artificial protoorganelle, basically mimicking the mitochondria in eukaryotic cells. In mitochondria the formation of a proton gradient is an important phase in energy harvesting and conversion. Although such procedures appear difficult, there is precedent in the form of synthetic systems for ATP production. For example artificial chloroplasts utilizing ATP synthase and bacteriorhodopsin can be designed from a limited number of components [Choi et al., 2005]. Through a sophisticated synthetic biology approach, artificial proton pumps were engineered from modified cytochrome c and cytochrome c oxidase in artificial polymerosomes [Hvasanov et al., 2013]. These proton pumps generated an artificial proton gradient across the polymerosome membrane, which could hypothetically be harnessed for energy production. This could hypothetically be linked to the formation of ATP required in protocells to form the basis of a straightforward light driven energy source.

The halorhodopsin from *Natronomonas pharaonis* (pHr) has been cloned and functionally expressed in systems such as *Xenopus laevis* oocytes [Gradinaru et al., 2008]. This expression resulted in a light-dependent Cl^--inward current and thus a considerable negative shift in the membrane potential (~−400 mV), indicating that pHr is a highly active Cl^- pump [Seki et al., 2007]. The opportunity to manipulate the membrane potential through pHr in conjunction with light energized cation channels has been utilized as a tool in optogenetics, gaining control of action potentials in neurons [Fenno et al., 2011; Zhang et al., 2011].

With the results from Hvasanov et al., 2013, Mukohata et al., 1981, and Zhang et al., 2011 in mind, a hypothesis can be put forward, that the inside-negative electrochemical membrane potential (V_m) generated by Cl^- pump activity via pHr could also underpin specialized accumulation of cations, positively charged substances, in protocells. Thus, the functional expression of pHr could energize proton-coupled uptake of a range of nutrients such as Ca^{2+}, Mg^{2+}, K^+, Fe^{2+}, or metals such as Na^+, Cd^{2+}, Ni^{2+} for bioaccumulation.

Expression of the electrogenic anion transport via pHr in a recombinant bacteria or a protocell should remove both Cl^- and Na^+ from the surrounding water environment and thus provide a methodology for desalination. The K_m values of pHr for chloride uptake have been reported to be approximately 25 mm for chloride [Duschl et al., 1990], which are in an optimal range for the present objective. To enhance the speed of Na^+ accumulation, the conductance of the membrane could be enhanced by coexpression of Na^+-selective and voltage-dependent channels along with pHr. Such proteins could be NaChBac, a transmembrane-spanning protein cloned from *Bacillus halodurans,* the first functionally characterized bacterial voltage-gated Na^+-selective channel [Ren et al., 2001] or the voltage-dependent Na^+ channel Na_VPZ from *Paracoccus zeaxanthinifaciens* [Koishi et al., 2004].

A SYNTHETIC LIGHT DRIVEN PROTOCELL

The previous discussion could be applied to recombinant bacteria, where a top down approach could be attempted to simplify the recombinant cyanobacteria in order to make it a better design.

At present, synthetic organisms are based on biological cells that have undergone modifications to selected metabolic pathways or have had new pathways integrated [Barla et al., 2013]. The potential benefits in modifying organisms this way is attractive. Metabolic processes, such as the ability to generate energy currency, already exist within host organisms and their endogenous biosynthetic pathways can be harnessed to produce precursors for synthetic chemistry.

However, despite these clear benefits there are certain inherent restrictions. Even in a seemly simple prokaryotic cell the regulatory network underlying substrate-product relationships is so complex that attempts to incorporate new functions are often unsuccessful. For example, modifying an organism's metabolism decreases its overall fitness due to a

buildup of inhibitory precursors [Pitera et al., 2007; Zhu et al., 2002] or due to the inherent toxicity of the preferred end products to the host cell. For example yeast cells designed to produce artemisinic are characterized by a noticeable increase in indicators of cellular stress responses [Ro et al., 2008].

As an alternative to the top-down approach, which aims to reduce natural biological systems, protocells could offer an alternative with their simplification of chassis. Designed from the bottom upwards, protocells comprise elementary systems containing only a minimal complement of components required to execute necessary cellular functions. So although the processes to maintain life obviously are essential for biological cells, many of these systems can be omitted or considerably simplified in protocell design, and pathways relating to function can be designed to optimize performance. Even with this reduced level of complexity, protocells might still perform a specified function as effectively as biological cells.

The first step will be to purify and express pHr in an appropriate host organism, such as an *E. coli* strain, to produce recombinant functional halorhodopsins along with highly selective ion transporters such as Na_VPZ. Integral membrane proteins are still considered as complicated with regards to their folding and function, which is dependent on an optimal membrane environment. Thus, there have until recently only been few examples of well characterized membrane proteins and methodologies for generating functional recombinant membrane proteins.

Progress during the last decade studying membrane protein function in a native membrane environment under controlled conditions has seen the emergence of effective techniques for integration of recombinant proteins into lipid membranes in vitro [Seddon et al., 2004]. It seems reasonable that these same methods could be adapted to the integration of surface transporters and receptors into protocell membranes. A number of membrane proteins have been successfully integrated into GUVs of a size appropriate for a protocell, such as potassium channels [Aimon et al., 2011], mechanosensitive channels [Folgering et al., 2004], a Ca^{2+} ATPase and bacteriorhodopsin [Girard et al., 2004].

A range of pHr proteins from a number of species have been characterized [Klare et al., 2008; Fu et al., 2012]. A appropriate choice could be halorhodopsin from *Natronomonas pharaonis* (pHr), that has been cloned and functionally expressed in heterologous systems such as *E. coli* [Hohenfeld et al., 1999].

The next step will be to integrate lipid patches containing the recombinant proteins, harvested from *E.coli,* into a synthetic cell membrane made of polymersomes. The membrane of polymersomes can be viewed as a reservoir system for both hydrophobic and amphiphilic molecules similar to cell membranes, which integrate cholesterol and membrane proteins. The folding and function of membrane proteins is known to be strongly influenced by the properties of the membrane in which they are integrated [Laganowsky et al., 2014; Curran et al., 1999], which are in turn determined by the lipid composition of the membrane [Cantor, 1999]. The choice of protocell membrane is therefore important to provide an environment suitable for membrane proteins incorporated into the protocell surface to function. It has been reported that membrane proteins such as OmpF, LamB and FhuA can be incorporated within the membrane of polymersomes while maintaining their functionality [Stoenescu et al., 2004].

The protocells made of polymersomes will be manufactured using a microfluidic platform. A miniature production and assembly line in the form of a self-contained microfluidic platform should be capable of producing protocells from purified components with the high efficiency and reproducibility that can be expected from microfluidics. The application of microfluidics in synthetic biology for design, component production and optimization has become an increasingly common practice [Huang et al., 2014].

It is well established that life possesses the unique ability to evolve in the face of natural selection pressures. Artificial protocells with their lack of replication machinery, do not posses this, as the ability to reproduce or divide is a condition for evolution to occur. In applied biotechnology the inability of protocells to evolve would be considered a merit, preventing undesired changes in the design. The downside is however that protocells cannot respond to artificial selection pressures to optimize performance. The optimal protocell must therefore be designed and pre-tested, it would be unrealistic to assume that simply integrating different components will produce an optimally functional system.

It will therefore be necessary to tag the proteins with fluorescent LOV domains, since genetically encoded fluorescent proteins (FPs) based on the flavin-binding LOV domain provide an advantage over green fluorescent protein (GFP). Recombinant expression of LOV domains in *E. coli* is fast and easy to detect [Christie, 2012]. Then it will be possible to use a microfluidic fluorescence sorter to make distinctions between protocells that have positioned the proteins in the optimal orientation, and those that have not.

Different parameters such as concentrations, surfactants, flow rates etc. will then be systematically optimised through the application of a molecular evolution strategy recently pioneered. A synthetic organic reactor system using real-time in-line NMR spectroscopy can be utilized to self-optimize a reaction, exploiting feedback from the spectroscopic measurements. This real-time analytics enables the fast generation of data and information about the process studied and the fast optimization of the process,

allowing direct control of the reactor, reagent inputs, and process conditions [Sans et al., 2015].

Sensory function of a range of synthetic transport modules such as NaChBac, from *Bacillus halodurans* [Ren et al., 2001] or Na_VPZ from *Paracoccus zeaxanthinifaciens* [Koishi et al., 2004] will be tested by measuring pH profiles of the artificial cells in response to treatment with a range of chloride and sodium concentrations. The efficiency and functionality of the artificial cells will be benchmarked against in vivo bacterial systems such as the previously designed recombinant *E.coli*, functionally expressing identical synthetic membrane transport modules.

In conclusion, if the above methodologies are successful, then a synthetic system in form of a self-sufficient light empowered protocell has been designed.

DISCUSSION

Part of a rational design remit is to choose a minimal system that will function as required. Protocells with clearly defined synthetic applications, such as bioaccumulation or pharmaceutical delivery systems, could be designed as stripped-down models, incorporating essential biosynthetic and bioenergetic pathways onto a chassis, but omitting a number of other complex processes required for life.

This article provides a specific example of recombinant cell and protocell technology, moving from what is presently known to suggesting how novel application of existing methodologies could be applied to design a complex synthetic system in form of a light empowered protocell for bioaccumulation and bio-diagnostics. Protocells, with their polymersomes membranes serve a dual purpose, since firstly, they are in general more stable in the circulation than liposomes [Discher et al., 1999; Lee et al., 2011]. The possibility to load therapeutic molecules such as drugs, enzymes, peptides, and even DNA and RNA fragments into polymersomes has been highlighted for a number of applications, making them very desirable vesicles for a range of applications in drug delivery, biomedical imaging, diagnostics, and biotechnology [Lomas et al., 2007; Kim et al., 2010; Massignani et al., 2010].

Secondly, as discussed so far, the inside-negative electrochemical membrane potential generated by Cl^- pump activity via halorhodopsin could also be applied to drive the accumulation of cations, positively charged substances, into the protocell. Thus, the expression of halorhodopsin could energize proton-coupled uptake of not only substances such as Na^+, but also hypothetically Ca^{2+} (NaChBac selectivity can be converted from Na^+ to Ca^{2+} by replacing an amino acid adjacent to glutamatic acid in the putative pore domain by a negatively charged aspartate [Yue et al., 2002]), and along the same tangent Mg^{2+}, K^+, Fe^{2+}, or toxic metals such as Cd^{2+} (*Tolypothric tenuis* has demonstrated adsorption and removal of cadmium over wide pH and temperature ranges [Inthorn et al., 1996]), Ni^{2+}, or even a disease-related peptide through a selective cotransport protein such as for instance the highly expressing ion channel protein, NaChBac for bioaccumulation.

There is much ground to be explored before functional protocells enter the realm of reality. The discipline has still not yet progressed to the point where a fully functional self-replicating cell can be constructed; it is likely to be quite some time before a real autonomous synthetic cell can be designed, whereas the construction of simpler machines built for a array of synthetic or bio-delivery purposes is almost within reach.

Further, a process to separate the protocells from the environment will also be necessary, otherwise the substances or metals will eventually be returned to where they came. The future goal will be to develop a generic solution which should lead to the design of a protocell with potential applications to report on substances in water or body fluids and thereby open a field of varied opportunities for applications in molecular medicine, agriculture and environmental water treatment.

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