In-vitro Protein Synthesis in Semi-Permeable Artificial cells

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ABSTRACT: A novel cell free protein synthesis (CFPS) system utilizing Layer-by-Layer (LbL) polymer assembly was developed to reduce the operational cost of conventional CFPS. This yielded an encapsulated cell system, dubbed 'eCells', that successfully performs in vitro CFPS and allows cost effective incorporation of non-canonical amino acids into proteins. The use of eCells in CFPS circumvents the need for traditional cell lysate preparation and purification of amino acyl-tRNA synthetases (aaRS) while still retaining the small scale of an in vitro reaction. The reaction was shown to be scalable over a large range of reaction volume and the crowding environment in eCells confers a stabilizing effect on marginally stable proteins, such as the pyrrolysl tRNA synthetase (PylRS), providing a means for their application in in-vitro protein expression. Photocaged-cysteine (PCC) and Nε-(tert-butoxycarbonyl)-L-lysine (Boc-lysine) was incorporated into Peptidyl-prolyl cis-trans isomerase B (PpiB) using small amounts of ncAA with adequate yield of protein.

Introduction

Cell-Free protein synthesis (CFPS) is a versatile tool for functional and structural biology, allowing rapid and inexpensive production of proteins. The method was first introduced more than 50 years ago by Nirenberg and Matthaei, enabling them to decipher the genetic code.[1] Since then, remarkable improvements in reaction stability and protein yields have been achieved by carefully optimizing conditions under which the reactions are performed.[2] Combined with today's ease to commercially obtain kits for in-vitro protein synthesis, CFPS is increasingly used in many applications of basic research, biotechnology and health sciences.

Traditional CFPS is characterised by the use of a cell lysate that holds all the necessary components for energy production, transcription, translation and protein folding.[3-4] The lysate is a processed extract of the cytosolic component from a plurality of mono-clonal organisms and is used in conjunction with a buffer containing small molecule components essential to sustaining protein production. These include, but are not limited to, amino acids, nucleotides, an energy production system, co-factors and various salts. Finally, an appropriate DNA template is supplied which codes for the target protein to produce.

To achieve even higher reaction controllability of CFPS, Ueda et al fully reconstituted an in-vitro protein synthesis system from only ribosomes and essential elements of the *Escherichia coli* translation system.[5] In this PURE - "protein synthesis using recombinant elements" – system, all macromolecules required for protein synthesis (with the exception of ribosomes) are individually expressed, purified and then titered into a reaction mixture. While producing the PURE system is labor intensive and thus costly, this disadvantage is greatly offset by the abstinence of proteolytic and nucleolytic enzymes in the reaction mixture which can reduce protein product yield.

Two commonly used in-vitro protein synthesis systems are batch and dialysis CFPS. Batch CFPS is characterized by using small molecules coupled directly with transcription/translation components in a lysate as a one-pot mixture to generate protein. Dialysis mode CFPS is the same reaction mixture held within a regenerated cellulose membrane in an external equivalent of CFPS buffer. This regenerated cellulose membrane separates the 'inner' and 'outer' buffer. The CFPS reaction occurs within the permeable 10-15 kDA dialysis tubing which retains all macromolecules necessary for translation, while smaller molecules can diffuse between the inner and outer buffer. The speed and ease with which CFPS can be performed and the full control over its reaction conditions make it possible to use CFPS where *in-vivo* protein expression cannot be applied. Toxins, biopharmaceutical proteins and membrane proteins have been shown to be successfully expressed in CFPS and proteins were produced *in-situ* for high-throughput, arrayed analyses. [6] [7] [8] [9]

Although CFPS has become a viable alternative to traditional *in vivo* expression systems, it requires development. Major limiting factors are intense energy costs, expensive reagents and lower reaction yields.[10] Furthermore, standard *Escherichia coli* lysates are 20 times more dilute than the cytosolic compartment of the cell.[3] This decreases rates of elongation, translation and protein expression, and further affects stability of enzymes in the lysate. Compartmentalized systems where macromolecular translation/transcription components are confined have been developed to emulate *in vivo* systems.

Biomimetic confinements of lysate have been produced through various compartmentalization techniques. These methods include oil in water droplets,[11] coacervates,[12] lipid vesicles,[13] liposomes[14] and polymersomes.[2] These compartmentalized systems can be utilized to house genetic information and biomolecules related to transcription and translation. Liposomes are a common compartmentalization technique which can emulate biochemical reactions in a controlled

in vitro environment. They are small vesicles comprising of a lipid bilayer and are often used as a cell model. For example, in 2009 Swartz et al synthesized functional aquaporin Z in synthetic liposomes with CFPS. They reported a 40 times increase in yield of functional aquaporin and into the membrane correctly inserted aquaporins over standard *in vivo* methodology. The aquaporin loaded liposomes were able to act as a simple cell model and allowed permeability measurements for a variety of biologically interesting molecules.[9]

One of the unique characteristics of living cells is their shielding boundary that separates their strongly homeostatic inside from the varying extracellular environment. This membrane-based compartmentalization of molecules is required to perform basic and complex functions.[15] Gram-negative bacteria have a highly negatively charged outer membrane due to the anionic moieties of lipopolysaccharide.[16] We hypothesized that the physical properties of *Escherichia* coli's outer membrane allow cell surface modification to turn bacterial cells into semi-permeable containers for in-vitro protein synthesis.

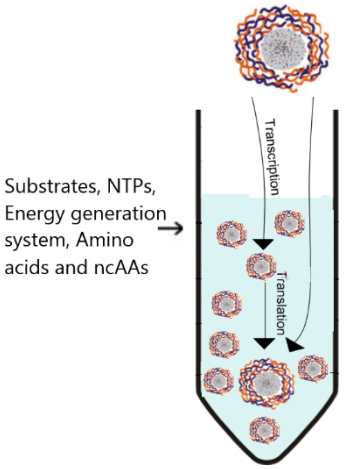

Figure 1: A schematic of *in vitro* protein synthesis using capsules produced through LbL assembly. All substrates required for protein synthesis are present in the CFPS buffer and can freely diffuse into the capsule.

Bio-polyelectrolytes have been used extensively to modify the surface of cells, especially *E. coli*. Layer-by-layer polymer assembly (LbL) relies on the adsorption of long chains of polyelectrolytes on a surface, to various levels of thickness, and operates through electrostatic interactions.[17] When a negatively charged surface is exposed to a cationic based polymer, the net charge of the surface changes due to adhesion and overcompensation by the polyion.[18] Iterations with polyelectrolytes of alternating net-charge can be repeated until a desired thickness of polymer is achieved.[19] Chitosan and alginate are commonly used bio-polymers for LbL deposition. Chitosan is a form of deacetylated chitin, which has a positively charged amino group in its monomer unit.[20] Conversely, alginic acid, often derived from brown algae, consists of anionic monomer units.[21] LbL assembly for cell surface modification has been applied as early as 2006 for bio-recognition.[22] Chitosan and alginate were found to be biocompatible and able to be adsorbed onto the cell surface without affecting cell viability. To confirm deposition of each layer of polyelectrolyte, zeta potential measurement was used, showing the charge alternation with successive deposition of cationic polymer and anionic polymer. It has been shown that visual confirmation of deposition of polyelectrolytes is possible. Imaging of these coated bacteria using TEM shows nanometer range deposition of polyelectrolytes, encapsulating them.

Cell encapsulation with chitosan and alginate was also employed for library screening of detergent stable mutants of G coupled protein receptors.[23] Plückthun et al applied two layers of the polyelectrolytes onto the outer membrane of *E. coli* cells which expressed a library of G coupled protein receptor mutants linked to sfGFP to test retention.[24] The native *E. coli* membranes and peptidoglycan layer were then dissolved using detergents, and microcapsules were selected using fluorescence activated cell sorting (FACS). The collected 1% capsules with the highest fluorescence also contained highly detergent stable protein mutants.

Here we demonstrate that the concept of LbL encapsulation of bacterial cells can be extended to generate semi-permeable artificial cells with femto-liter volume which are apt for *in vitro* protein synthesis. By LbL deposition of alternate charged polyions onto the cell surface, rigid shells are formed that trap all components of the bacteria. High internal pressure, between 3-5 atmospheres, exists within *E. coli* cells and its structure is stabilized by a highly crosslinked peptidoglycan layer.[25] Only a small number of these crosslinks need to be broken for the plasma membrane to be ruptured and cause hypotonic lysis. We use a hydrolytic enzyme to lyse the native cell wall of the bacteria, leaving a layered and semi-permeable capsule surrounding all constituents of the cell. We refer to these polyelectrolyte encapsulated cells with the cell wall lysed as eCells.

An important feature of these semi-permeable artificial cells is that the entire macromolecular repertoire of a living cell is present in approximately the same composition, concentration and volume, which presents an advantage over lysate based *in-vitro* expression. Enzymes that are associated with the membrane are generally lost through lysate preparation and as a result, metabolic pathways can be more fragmented in traditional CFPS.

The ancillary pathways which assist protein translation are hard to quantify in the S30 extract. In particular, the PURE system consists of reconstituted proteins directly related to protein transcription/translation, individually purified and used for protein expression.[5] Within the PURE system there is a low level of protein expression indicative of a complex network of processes required for protein production. Encapsulated protein synthesis holds the entire repertoire of protein elements of an *in-vivo* system and after lysis they are contained in a polyelectrolyte capsule. This expanded repertoire allows for the function and use of multi-step enzymatic pathways that require proteins associated with the membrane or other proteins lost during the preparation of the cell lysate. These capsules could provide added efficiency to *in vitro* systems that utilise membrane proteins in multi-step enzymatic cascades, either for energy-generation or for a specific by-product

Materials and methods

Polyelectrolytes

Polyelectrolytes were purchased from Sigma; low molecular weight chitosan (448869, 50,000-190,000 Da) and sodium alginate.

CFPS protocol

The protocol for CFPS was adapted from Noireux and Caschera's hexametaphosphate(HMP)/ maltodextrin phosphate recycling system [26]. The HMP/maltodextrin CFPS buffer contains 0.9mM of UTP and CTP, 50mM HEPES, 1.5mM GTP, 1.5mM ATP, 0.68uM folinic acid, 0.64mM cAMP, 1.7mM DTT, 3.5mM of amino acid mix, KGlu 60mM, MgGlu 6mM, 2% v/v PEG-8000, 5mM CoA, 35mM Maltodextrin, 30mM 3-PGA, 1.2 mM HMP and 10mM NAD. Cyclic HMP was linearised at 100 ^{o}C for 5 minutes and then used after cooling.

CFPS using a creatine phosphate as energy source was used to express Peptidyl-prolyl cis-trans isomerase B (PpiB) containing photocaged cysteine (PCC), as higher protein yields are obtained with this more efficient ATP regeneration system. The creatine phosphate CFPS buffer contains 0.9mM of UTP and CTP, 50mM HEPES, 1.5mM GTP, 1.5mM ATP, 0.68uM folinic acid, 0.64mM cAMP, 1.7mM DTT, 3.5mM of amino acid mix, KGlu 60mM, MgGlu 6mM, 2% v/v PEG-8000, 250ug/ml of creatine kinase, 80mM of creatine phosphate. Roche complete mini™ PI inhibitor was added to CFPS buffer after being dissolved in 10mL at 10% v/v. IPTG, and, where required, non-canonical amino acids were at 1mM concentration when added to the final buffer. Photocaged cysteine (PCC) was an exception, and a higher amino acid concentration of 3.5mM (or 33mg in 30mL) in creatine phosphate CFPS buffer was used.

Encapsulation procedure

Xjb cells were used and induced for endolysin production at the beginning of inoculation with a final concentration of 3mM arabinose. The cells were grown to OD 0.6 and washed three times with PBS-E (Phosphate buffered saline 1mM EDTA) pH 7.4 and resuspended in 0.25mg/ml of Chitosan in PBS-E solution with vigorous shaking for 20 minutes. The cell pellet was washed with PBS-E pH 6.0 three times to remove excess chitosan and then resuspended in 0.25mg/ml of alginate PBS-E solution and subjected to vigorous shaking for 20 minutes. The cells were then washed 3 times with PBS-E pH 6.0, resuspended in PBS-E pH 7.4 and directly stored at -80^{o} C in 1 ml aliquots in PBS-E buffer. For larger expression, the entire capsule pellet was frozen and then used.

eCells with CFPS protocol

The frozen aliquots of encapsulated cells were thawed, spun down and the pellet immersed in CFPS buffer. CFPS for each experiment was conducted at 37^{o}C in an induction shaker at 180rpm. Fluorescence readings were taken every 1.5 hours for 12 hours and a final endpoint reading at 24 hours later using a SpectraMax fluorescence reader, with SoftMax pro as the measurement software. Protein expression was conducted with 500 µl of CFPS buffer using the HMP and maltodextrin method of energy generation with the addition of protease inhibitor. Each of the fluorescence readings were of *100 µl of CFPS immersed encapsulated cells.*

Protein purification and Plasmid constructs

The gene of monomeric neon-green Green fluorescent protein (nGFP) in a plasmid with CloDF13 origin of replication and amber stop codon interrupted at position 13 was used to test the incorporation of non-canonical amino acids. 'Wild type' (WT) neon-green monomeric GFP was expressed from a pET vector with pBR322 origin of replication. A derivative Methanococcus jannaschii Tyrosyl aminoacyl-tRNA synthetase (aaRS) and $tRNA_{sup}$ pair was used for incorporation of para-acetyl-phenylalanine(PaF).[27] This aaRS/$tRNA_{sup}$ pair is under a GlnS promoter in a plasmid with pUC origin of replication. The same construct with Methanosarcina mazei Pyrrolysl tRNA aaRS/$tRNA_{sup}$ was used to incorporate N^{ε}-(tert-butoxycarbonyl)-L-lysine (boc-lysine) into both nGFP and PpiB, at positions A13TAG and A147TAG respectively.

For protein purification, 1 ml His GraviTrap TALON columns (GE Healthcare) were used for PPiB, which was expressed in an encapsulated pellet from 500ml of culture, 3mls of CFPS to induce expression and had approximately 0.5mg/ml of boc-lysine incorporated PPiB in 500µl. For photocaged cysteine incorporation, cells were cultured in 1L and then encapsulated. The volume of CFPS buffer was 30mL and the protein yield 0.65mg. Protein concentration was determined using the Thermo Scientific Nanodrop™ One/Onec Microvolume UV spectrophotometer.

Mass Spectrometry analysis

High resolution electro spray ionization mass spectrometry was performed in positive mode on an Orbitrap Elite mass spectrometer and on Orbitrap Fusion™ Tribrid™ mass spectrometer coupled with an UltiMate 3000 UHPLC (Thermo Scientific, USA). Samples were injected into the mass analyzer via an Agilent ZORBAX SB-C3 Rapid Resolution HT Threaded Column, using an acetonitrile gradient (10–85 %) and 0.1 % formic acid. Mass spectra were deconvoluted and processed using the Xcalibur software package.

Results

Diffusion of WT nGFP

An expression plasmid with WT mNeonGreenGFP as reporter gene was expressed in the Xjb cell line and eCells prepared as described in the method section. Based on this 26.9 kDa monomeric form of GFP, the passive diffusion of macromolecules from the encapsulation can be monitored by fluorescence measurements. The two layers of polyelectrolyte were hypothesized to be able to retain macromolecules while being permeable to small molecules.

Figure 2 shows the amount of nGFP remaining in encapsulated cells over the time course of 24 hours. The main diffusion of nGFP into the surrounding solution occurs over the course of six hours, and of the 100% of nGFP present at the beginning of the diffusion experiment, approximately 10% are present 24 hours later. The fact that substantial nGFP diffusion from the eCells is observed substantiates that the bacterial cell wall is lysed in the eCell preparation, as nGFP is not able to escape from living cells with intact membranes. The half-retention time of 26.9 kDa nGFP in the encapsulations is 3.46 hours modelled by a fluorescence change due to passive diffusion.

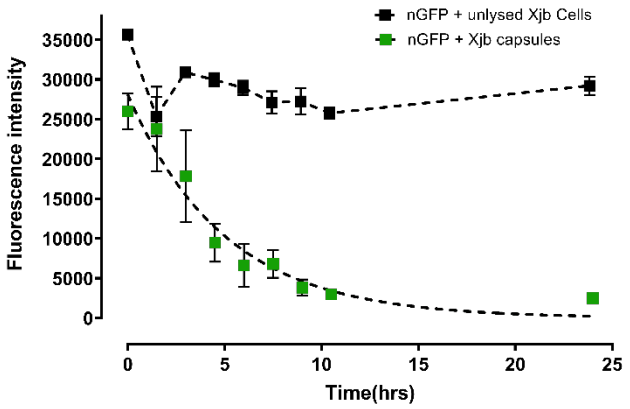

Figure 2: Induced, encapsulated and lysed Xjb(DE3)* with WT nGFP in PBS-E buffer in comparison to live Xjb Cells with nGFP expressed both done in triplicate. The relative diffusion of GFP from an encapsulated system over the course of 24hrs, with the half-point of GFP diffusion at approximately 4 hours. The nGFP fluorescence of the live culture remains static throughout the experiment. Experiments were done in triplicate; error bars are added to graph.

Based on this observation we hypothesized that other larger biomolecules with varying physio-chemical properties are also likely held within the polyelectrolyte shell, and protein components for transcription and translation remain functionally active to sustain *in vitro* protein synthesis. To test this hypothesis, we performed cell-free protein synthesis using eCells from *E. coli*.

Encapsulated Cell-Free protein synthesis

Figure 3 shows the cell-free protein synthesis of nGFP in eCells with and without external supplement of nucleotides, amino acids and small molecules necessary for ATP production. As expected, eCells which did not have these small molecules externally supplied but contained 1 mM IPTG to induce transcription, showed no significant *in vitro* production of nGFP. The lack of nGFP production indicates complete depolarization of the cells and thus no residual cell activity remains. In difference, eCells in cell-free protein synthesis buffer produce nGFP over 20 hours, validating that transcription and translation components are effectively retained in eCells and are active over a long time.

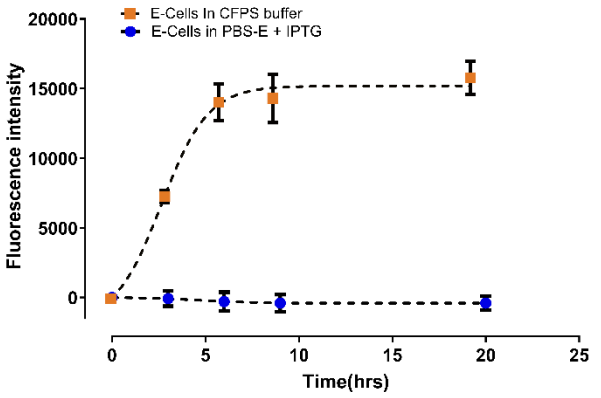

Figure 3: *In vitro* protein production of WT nGFP in encapsulated and lysed eCells using HMP/ Maltodextrin cell free protein synthesis mode. A substantial production of GFP in this method, exceeding that of the background control of PBS-E IPTG, indicating that *in vitro* protein synthesis occurred. All fluorescence expression was done in triplicate.

ncAA incorporation

Next, we illustrated that eCells also are apt to produce proteins containing non-canonical amino acids (ncAA) when the ncAA is externally supplied. Using a *M. jannaschii* suppressor tRNA and tyrosyl tRNA synthetase mutant pair to selectively encode the amber stop codon for p-acetyl-L-phenylalanine (PaF), the nGFP gene in a pCDF plasmid with amber interruption at amino acid Leu13 was expressed to produce a fluorescent reporter.

Results shown in Figure 4 confirm the ability for ncAAs to be incorporated into an encapsulated CFPS system. As there was no tRNA synthetase or tRNA supplemented within the CFPS reaction, it highlights the flexibility of the capsules as the orthogonal tRNA/tRNA synthetase pair is expressed during cell preparation.

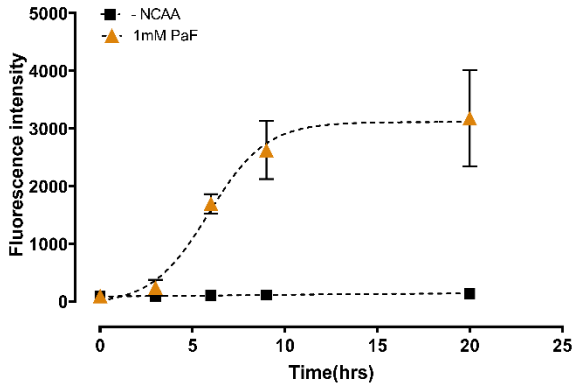

Figure 4: Fluorescence intensity using nGFPamb and a previously engineered Mj. TyrRS/tRNAsup system. The reported fidelity of incorporation using this tRNAsup/tRNA synthetase pair is 99.8%

We then explored if the cell-like environment of macromolecules in the eCells is beneficial for stability and activity of enzymes. Using an established example of pyrrolysl-tRNA synthetase (PylRS) to incorporate a non-canonical amino acid (ncAA) we demonstrate the ease in which a traditionally difficult ncAA incorporation can be achieved using encapsulated CFPS. The experiment is based on the work by Yokoyama *et* . who previously engineered a *Methanosarcina mazei* PylRS/$tRNA_{sup}$ pair for N^{ε}-(tert-butoxycarbonyl)-L-lysine (boc-lysine) incorporation into proteins.[28]

Results show a clear difference of fluorescence with 1mM boc-lysine and without the ncAA, indicating that there was efficient incorporation of boc-lysine in eCell CFPS. A small amount of amber suppression with canonical amino acids is also observed in the control experiment omitting boc-lysine in the reaction buffer, which is also previously reported in the literature. The present mutation A→G in the tRNAsup acceptor stem decreases ncAA suppression efficiency by 42% and increases canonical amino acid suppression.[29] In difference, this is not seen when PaF was incorporated into nGFP, as the Mj. TyrRS/$tRNA_{sup}$ pair has a reported high fidelity for ncAA incorporation of 99.8% and very low tendency for mis-incorporation.[27]

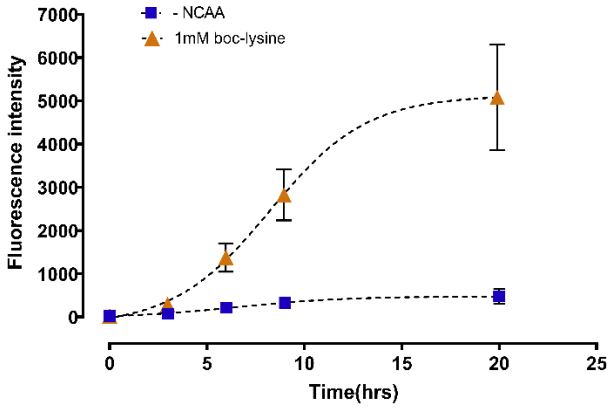

Figure 5: Fluorescence intensity using nGFPamb using Nε-(tert-butoxycarbonyl)-L-lysine and PylRS/tRNAsup system. The A-G change on the acceptor stem of tRNA allows for nAA suppression of the negative control.

To confirm that ncAA incorporation occurred successfully, a large scale encapsulation and eCell CFPS was conducted in 3ml of MD/HMP CFPS buffer, with A147amber PPiB as reporter protein. Full length PPiB expressed in high yield (0.5mg; Figure 6A) with modest requirement of the ncAA; only 0.74 mg of boc-lysine was supplemented to the eCell CFPS reaction.

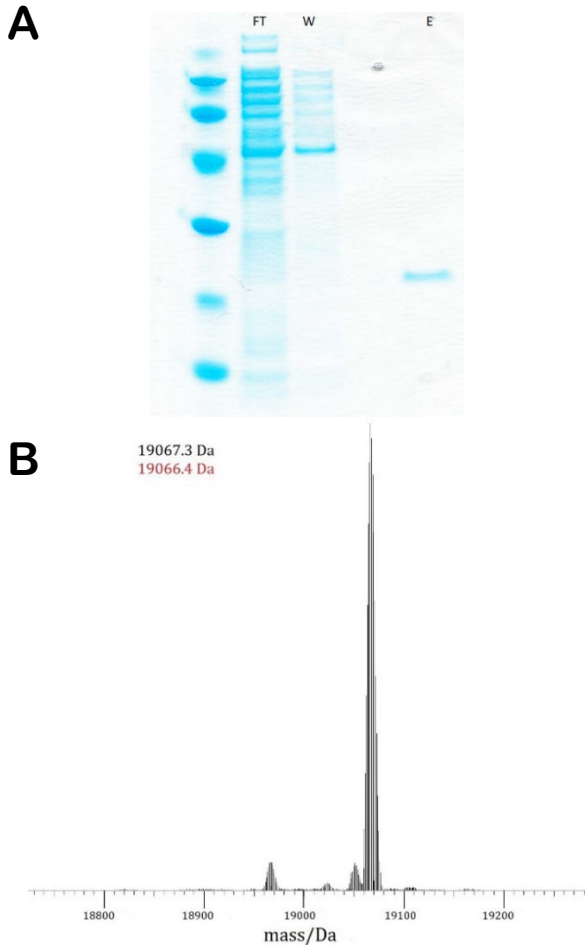

Figure 6: A.) SDS-PAGE gel of different fractions during the purification of PPiB, showing flow through (FT) wash (W) and elution (E) B.) Whole protein mass spectrum of Boc-lysine incorporated PPiB at position 147. Expected mass 19067.3 Da. Other peaks are related to natural amino acid incorporation.

Mass spectrometry confirmed the successful incorporation of of boc-lysine into PPiB. The major mass peak at 19066.4 Da corresponds to boc-lysine containing PPiB. The second, smaller peak at 18966.3 Da corresponds to amber codon suppression with glutamine. Using eCell CFPS, we demonstrate that also more chemically interesting, but in *in-vivo* protein expression highly costly ncAAs can be incorporated. Photocaged-cysteine (PCC) is cysteine with a photocage group covalently linked to the thiol group.[30] Site-specific incorporation of this amino acid was achieved using a suppressor tRNA from *M. mazei* and a *M. barkeri* pyrrolysyl-tRNA synthetase, with mutations to specifically recognize the ncAA. Due to difficulties in purification of the active tRNA synthetase, site-specific incorporation of PCC into proteins is not feasible *in vitro* and highlights the importance of eCells as a viable method for producing proteins with ncAA.

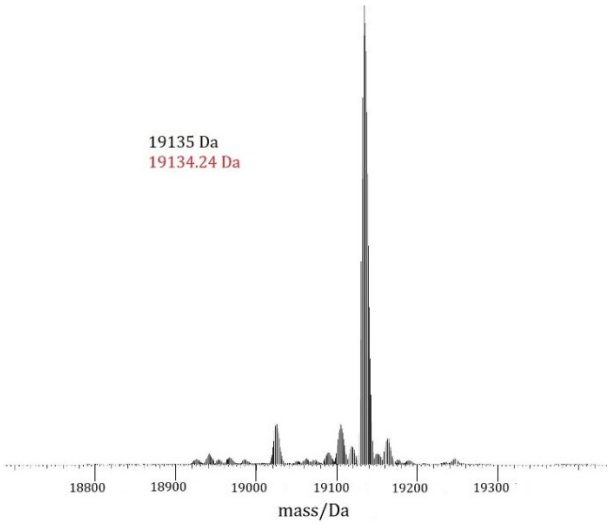

Figure 7: Mass spectrum of PpiB with photocaged-cysteine incorporated at position 147. The calculated mass is 19135 Da. Only a small amount of the protein is found to be decaged with a mass of 18941 Da.

PCC was confirmed to be incorporated in PPiB at position A147 with a yield of 0.65mg protein produced from a 30 ml eCell CFPS reaction. The expected mass is 19,135 Da with the expected mass of the decaged cysteine at 18941 Da. The successful incorporation of this PCC highlights the economical use of ncAA and the robustness of the system.

After a dual transformation of the plasmids with aaRS/tRNA pair and the reporter gene, CFPS can be started the same day as inoculation, without the need for the time-consuming purification step of the tRNA synthetase. Encapsulated CFPS provides a platform that can easily perform ncAA incorporation and streamlines *in vitro* expression of ncAA modified proteins.

Discussion:

CFPS is an advantageous technology which allows synthesis of proteins that are difficult to produce or have chemically interesting groups incorporated. Here we have developed the eCell technique where one can reliably control environmental factors and cost-effectively produce these specialized proteins.

The eCell system circumvents issues with enzymes that are difficult to purify and use *in vitro*, as it is the case with PylRS. The aaRS/tRNA$_{sup}$ purification process for PylRS is complex, which complicates ncAA incorporation. Many ncAAs that are incorporated with PylRS mutants are expensive to produce and so far could only be used in *in-vivo* protein expression. A previously described method to circumvent this complex purification was by creating a S30 extract transformed with the cognate aaRS/tRNAsup pair. A culture expressing the aaRS/tRNA¬sup was grown for ~13 hours and then the S30 extract was prepared. This was successful in a standard CFPS setting and allowed for ncAA incorporation in a PylRS system [31].

eCells allow the use of PylRSs more easily as they combine advantages of *in vivo* and *in vitro* protein expression. The stabilizing effect in the eCells allow the use and storage of less stable proteins, as is the case with PylRS. This has been shown in eCells with the incorporation of two PylRS based ncAA into PPiB at adequate yields. The crowded environment in eCells have attributes similar to what is encountered in living cells and eCells are compartmentalized like single cells, rather than a whole lysate. Further, the entire protein repertoire is contained within these capsules which could allow the use of other cellular machinery beyond that of transcription and translation. eCells are also comparable to other *in vitro* CFPS systems with the numerous advantages that are associated with CFPS. The compartmentalization of proteins in eCells, which are permeable to small molecules, mimic features from dialysis CFPS. During CFPS eCells are immersed and resuspended in CFPS buffer. This allows higher translation yield, because phosphate, which in high concentration inhibit translation, can freely diffuse to the larger, outer reaction buffer. The chemical environment of the eCells can be altered to fit precisely the desired experimental conditions and reaction can be repeatedly scaled. CFPS in eCells is highly versatile and can be used with conventional and non-conventional energy generation systems such as CP and MD/HMP, which furthers its accessibility as a platform. Difficult and expensive ncAA, such as PCC, can be incorporated at a much lower cost than traditionally possible with *in vivo* methods. This is due to the large reduction of reaction volume, the inexpensive production of the eCells and the very modest requirement of ncAA. While the protein expression yield with eCell CFPS is markedly lower than what can be achieved with *in vivo* expression, overall costs are significantly reduced when expensive supplements are necessary. For example, *in vivo* expression yield for PPiB containing a single PCC was 4.5mg per liter cell culture, while the yield of the same protein when using eCell CFPS was 0.65mg. Considering the reaction volume in the eCell CFPS was only 30ml, the use of the ncAA is highly productive, with only 32mg of the amino acid consumed, rather than 314mg used in a 1L cell culture. Moreover, the screening capabilities of eCells is highlighted with the use of small amounts of both eCells and ncAA. Experiments with a fluorescent reporter used the equivalent of cells from 17mL of LB in 500uL of CFPS buffer. This coupled with approximately 100ug of ncAA being used for both, makes the system convenient for small scale CFPS. Because of this economic use of expensive supplements, we believe that eCells are a viable method to screen PylRS variants for their incorporation of hard to incorporate ncAAs. Cost is reduced further through the exclusion of dialysis tubing, continuous flow or other apparatus that are used to increase the reaction of CFPS. These apparatuses complicate CFPS and increases costs, restricting the technology to be applied in specialized laboratories only. In contrast, the eCell method is economical and relatively quick to perform when compared to the preparation of S30 or cell lysate for conventional CFPS.[32] Methods of lysate preparation are known to take an extensive amount of time and effort to prepare. The process requires cell breakage, washing with various buffers, centrifugation of large volumes at high speed, and also require expensive reagents. In contrast, eCell preparation can be completed within a timeframe of 2 hours and then used immediately after a single freeze thaw cycle. Inexpensive polyelectrolytes, such as chitosan and sodium alginate, are used for the LbL assembly and the encapsulated cells can be conveniently stored at -80^{o}C with no or little loss of function over time. To this end, the eCell technology provides a cost-effective alternative to conventional CFPS implementations, which is easily varied in size from laboratory scale screening experiments (as small as a single cell encapsulation) to pilot scale industrial processes of 100mL or more eCell CFPS reaction volume.

Conclusion:

A novel cell free protein synthesis (CFPS) system utilising Layer-by-Layer (LbL) polymer assembly was designed to generate an artificial cell that could reduce the operational cost of conventional CFPS. This yielded an artificial cell system that successfully performed *in vitro* CFPS and was used for ncAA incorporation into proteins.

eCells are a versatile platform that can be applied to numerous scientific fields. The expression of proteins that are unstable and are unable to be produced *in vivo* is a possibility with eCells. This could be further enhanced with the addition of stabilising additives in CFPS. In addition, there is significant potential with this technology in screening applications which require cell permeability or cannot be performed with live cells due to the toxicity of compounds. Therefore we believe that artificial cells like eCells carry outstanding potential for specialized CFPS, functional screening studies and cell-free metabolic engineering (CFME).

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Notes

The authors declare the following competing financial inter-

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ABBREVIATIONS

CFPS, Cell-Free Protein synthesis; PylRS, pyrrolysl amino acyl-tRNA synthetase; ncAA, non-canonical amino acids; S30, supernatant 30,000g; PCC, photocaged-cysteine; Ppib, peptidyl-prolyl cis-trans isomerase B; aaRS, amino acyl-tRNA synthetase; MD/HMP, maltodextrin/hexametaphosphate; CP, creatine phosphate; BocK, boc-lysine, Nε-(tert-butoxycarbonyl)-L-lysine; nGFP, neongreen-fluorescent protein; *E.coli, Escherichia coli;* LbL, Layer by layer assembly.

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