Advancing Space Flight Medical Care Through On-demand Protein Therapeutic Production Capabilities

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Emily Long Zhao, Recipient

Brigham Young University
Department of Chemical Engineering
Bradley C. Bundy, Advisor

Abstract –

Cell-free protein synthesis (CFPS) is an in vitro protein synthesis system that provides a uniquely adaptable platform for protein production. In recent years, lyophilization of CFPS have expanded the capabilities of this system by improving reagent stability outside of cold-chain storage. This project sought to utilize lyophilized CFPS reagents to develop a convenient, shelf-stable, on-demand therapeutic protein production platform. The therapeutic protein production potential of this novel platform was demonstrated by the successful expression of an FDA approved therapeutic protein. With the advancement of shelf-stable, on-demand protein therapeutic production platforms comes the potential to provide point-of-care medical treatment for space-flight crews during long duration missions.

Introduction

As the dream of long-term space exploration becomes a reality, so do the unique health risks associated with space flight. Missions requiring long-duration habitation beyond low-earth orbit pose significant challenges to the health and well-being of the crew. Successful medical interventions, as well as biomedical countermeasures to prevent or mitigate high-risk conditions, are contingent on the therapeutics available to the crew. Some of the most

effective treatments for cancer, genetic diseases, neurological disorders, and radiation syndromes. However, the availability of protein therapeutics is limited by their low stability under high-levels of radiation and long term storage [1].

This project sought to overcome the challenges of protein therapeutic availability during long-term space flight by engineering a lyophilized cell-free protein synthesis (CFPS) system capable of producing protein therapeutics on demand and thus provide life-saving treatments to space flight crews.

Relevance to NASA Aims

This work focuses on supporting Strategic Goal 2 of NASA's 2018 Strategic Plan which is to "Extend Human Presence Deeper into Space and to the Moon for Sustainable Long-term Exploration and Utilization" [2]. In particular, this proposal addresses the specific challenges [illegible] Technology Roadmaps: pharmaceutical stability under space flight conditions (TA 6.3.2), effective treatment options for high-risk medical conditions for long-duration health (TA 6.5.2) [3]. These challenges can be addressed through on-demand production of protein therapeutics during long-term space flight.

Protein therapeutic availability on board spacecraft can improve treatment options and countermeasures during interplanetary missions against cancer, genetic diseases, neurological disorders, and radiation syndromes (TA 6.5.2.4, 6.5.2.2, 6.5.2.1). However, the availability of these powerful therapeutics during missions is limited by their long-term stability under space-flight conditions and the difficulty of determining the therapeutics one will need prior to take-off. On-demand protein therapeutic production will eliminate the need to store protein therapeutics long-term while also improving the versatility of in-flight biomedical countermeasures and treatments

Cell-free Protein Synthesis

The production of proteins requires biological processes as conducted by specialized cell machinery. The traditional method of protein manufacturing produces proteins within living cell cultures, or *in vivo*. Cell-based production platforms are dependent on the growth and replication of living cells and therefore require time intensive production, purification and protein isolation steps. Protein therapeutics are conventionally purffied and stored in aqueous or dried form in low temperature storage until administration. However, stability of these isolated proteins are limited over time and elevated temperatures. Cell-free systems are an emerging production platform that provides an alternative to the complicated manufacturing process. Protein therapeutic stability limitations can also be overcome by eliminating the need to store proteins on board spacecraft and instead produce them on-demand through CFPS.

On-demand protein synthesis can enable crews to produce protein therapeutics as the need arises. Protein therapeutics are most commonly expressed in vivo; however, cellular systems are not particularly effective in the space environment due to cell destruction under ionizing radiation [4]. These in vivo systems also require a significant amount of equipment and complex purification steps. An alternative approach is through *in vitr*o cell-free expression. CFPS systems make it possible to synthesize proteins outside of a cell by combining the DNA of the protein of interest with emancipated cellular machinery in a reaction vessel (Figure 3). Cell-free expression has been demonstrated on a wide array of therapeutic proteins including antibodies, vaccines, and cancer drugs [5]. CFPS technology facilitates rapid product purification with minimal equipment and provides an efficient protein production platform with high protein yields in as little as an hour which is an

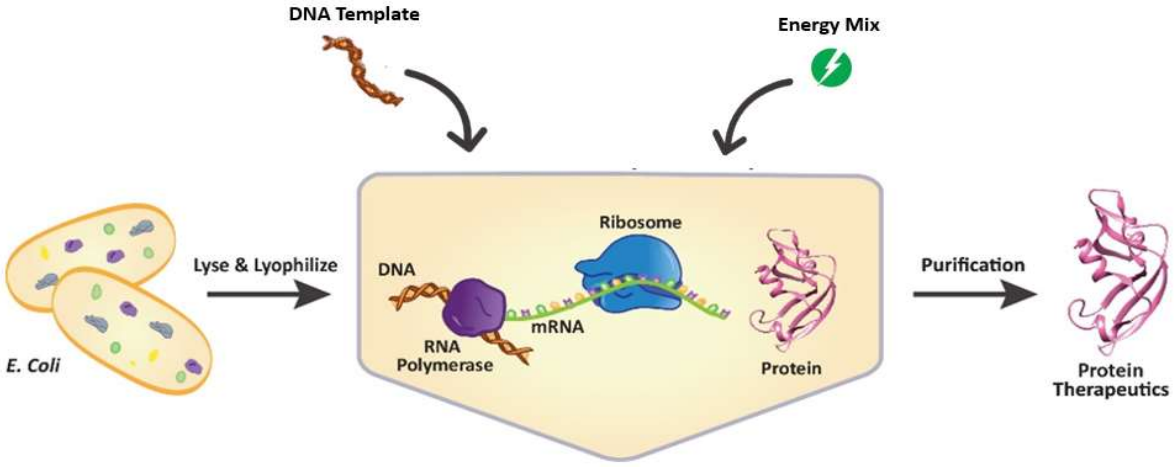

Figure 1: Illustration of CFPS: E. Coli cells are lysed for components to be used in CFPS system for the production of protein therapeutics or other biocatalysts.

order of magnitude faster than in vivo production [6].

Many cell types have been utilized for CFPS reactions; however, this project will use CFPS reagents harvested from the bacteria *E. coli* [5]. *E. coli* lysates are desirable for this work due to the robustness of *E. coli* machinery to harsh conditions and their rapid high-yielding capability [7, 8]. Currently, *E. coli* is the most cost-effect method to produce many complex proteins including insulin and antibodies [9]. While *E. coli*-based machinery may lack some machinery needed for post-translational modifications required by certain proteins, current work in the field is engineering this ability into the *E. coli*-based system [10]. Regardless, optimizing *E. coli*-based CFPS will provide important reference data applicable to other cell-free systems.

Lyophilization

CFPS provides rapid recombinant protein production; however, the major components of a cell free system must be stored in aqueous solutions below freezing [9]. To ensure protein synthesis viability, the CFPS solutions are most commonly maintained at -80°C, requiring low temperature freezers. To overcome this drawback, lyophilization can be used to preserve CFPS systems. Lyophilization, or freeze-drying, is a commonly used preservation technique. We can utilize this technique on our CFPS systems because they do not require living cells. A CFPS system can therefore be created from lyophilized, shelf-stable reagents (Figure 4) [11]. These ready-made powdered CFPS systems are capable of high-density, thermostable storage with the proven capacity to produce protein therapeutics [12]. An example of a stored, small-scale lyophilized CFPS system is shown in Figure 2.

Initial lyophilization work using freeze-dried cell extracts and separate powdered energy systems demonstrated that the lyophilized systems retain viability over a 60-day period at room temperatures [11] and in follow on work has demonstrated retained activity after 1 year when stored at 4°C. In comparison, all activity was lost in the non-lyophilized sample [12].

Cryoprotectant additives can be considered for even greater retention of CFPS activity over longer storage periods. Initial studies using cryoprotectant have shown promise for prolonged storage [13].

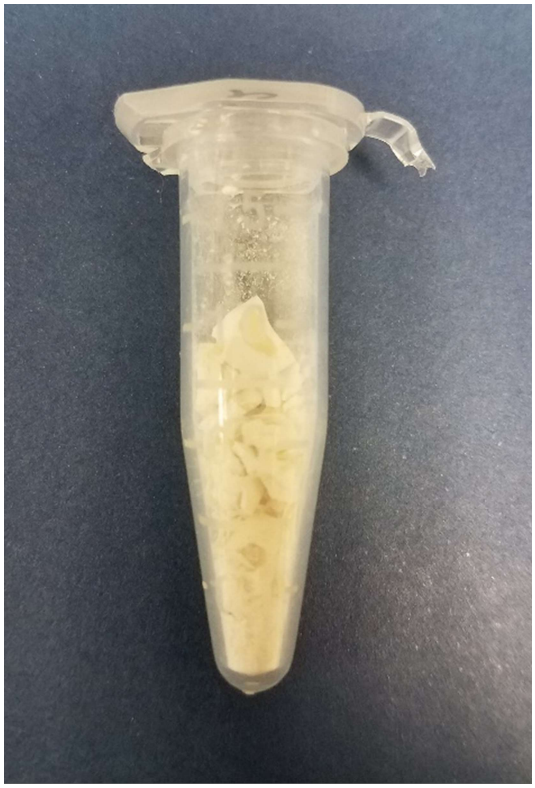

Figure 2: Image of Lyophilized CFPS system stored in test tube 6 months at room temperature

As mentioned, previous iterations of lyophilized CFPS have required the cell extract and energy mix to be stored separately. One reason for this may be the hydrophobic nature of some of the components of the energy mix which may decrease dry storage. Separate storage of the cell extract and energy mix limits the convenience of the current lyophilized CFPS stems. A one-pot CFPS platform which only requires rehydration of water and addition of DNA template would provide a straightforward, user-friendly platform. To this aim this project sought to optimize cryoprotectant additives to enhance

preservation, especially with the addition of hydrophobic energy mix.

Convenient Downstream Purification

One of the most significant challenges to on-site, point-of-care production is purification efficiency and the risk of endotoxin exposure from incomplete purification of bacteria residue. Our lab recently developed an "endotoxin-free" CFPS system using an LPS-free cell strain as recently detailed [9]. By eliminating the source of toxic LPS, we have essentially eliminated this risk of endotoxin exposure due to therapeutic administration.

Experimental Methods

Data were collected on the effects of lyophilization methods on CFPS cell extract storage. Samples were were frozen in a -40°C ethanol bath (Just-A-Tilt Shell Freezer Chiller SF-4Az, FTS Systems, Warminster, PA). samples were then freeze dried (Flexidry MP, FTS Systems (Figure 3). After approximately 1.5 hours of freeze-drying, samples reached minimum weight suggesting samples were sufficiently dried.

Lyophilized CFPS systems were stored at elevated temperatures and tested periodically for protein synthesis activity. Activity was determined by measuring production levels of the fluorescent sfGFP protein. To demonstrate the capacity of this system to produce protein therapeutics, an FDA approved therapeutic was produced.

Figure 3: Freeze-drying apparatus used to lyophilize CFPS systems.

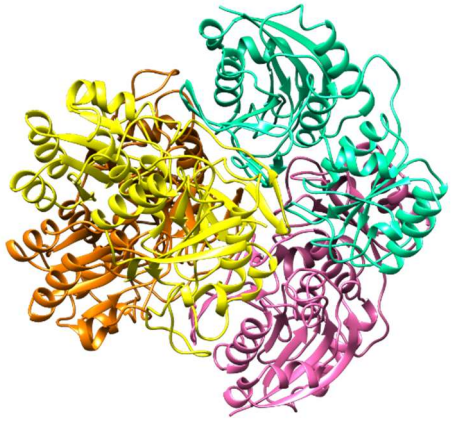

Figure 4: Protein ribbon diagram representative of an FDA approved protein therapeutic [14].

Results

This project constitutes the an important advancement of lyophilized CFPS system capable of producing yields appropriate for therapeutic doses. We have achieved an improved lyophilized CFPS system from those previously reported by our lab and others. This new system only requires rehydration with water and supplied with the DNA template of choice for on-demand CFPS protein production. This system was demonstrated to hold activity after storage at elevated temperatures. Therapeutic protein production was demonstrated.

A full disclosure of the results of this project are document in an article which is being prepared for submission to a peer-reviewed journal. The title of this article is "Thermostable Lyoprotectant-Enhanced Cell-free Protein Synthesis for On-demand Endotoxin-free Therapeutic Production" and it is anticipated that it will be published later in 2019.

Conclusion

Lyophilization, of cell-free systems creates rapid protein synthesis systems that can be created in mass and stored for later use. This ready-made powdered CFPS provides high density and non-standard temperature storage improving the overall cost, transportability, and convenience of a CFPS system

This work constitutes an important study for the design of lyophilized CFPS systems for prolonged storage by demonstrating the robust nature and therapeutic production capacity of a on-demand protein production platform. Significant progress has been made towards the development of practical on-demand CFPS systems for therapeutic protein production during long-term space flight.

Future Work

While a lyophilized, on-demand CFPS system has potential to provide life-saving protein therapeutics to space flight crews, the viability of this system under high-ionizing radiation exposure is unknown. Although the effects of radiation on DNA and *in vivo* biological processes have been extensively studied [4, 15, 16], little has been done to understand the impact on the individual biological molecules that make up the basic machinery of life. A follow-on project will constitute the first study of the effects of radiation on CFPS system machinery in combined and isolated format.

Despite the lack of research on radiation effects on this system, we expect to see decreased system activity due to the known degradation of macromolecular biological complexes by high-level radiation [15, 16]. To overcome this potential challenge, we will utilize protective additives. Antioxidants have been shown to protect biological systems from radiation induced oxidation events and NASA has reported polyethylene as a shield to high-ionizing radiation [17]. Both are commonly added to CFPS systems and do not inhibit its protein production activity [18]. A lyophilized system with improved radiation resistance will be capable of providing on-demand therapeutics and reduce the need to store protein therapeutics long-term while also improving the versatility of in-flight medical treatments.

Acknowledgements

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