Astropharmacy Phase I, Final Report

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Summary

Disease is an inherent part of being alive, and thus disease prevention, diagnosis and treatment is critical to human space missions. Pharmaceuticals are used to diagnose, treat, cure, or prevent disease, but suffer from lack of stability on Earth and even more so in the space environment. As NASA embarks on a new era in space exploration beyond Low Earth Orbit, the need to provide effective pharmaceuticals in space must be addressed. What if small quantities of pharmaceuticals could be made in space, on site, on demand? One class of drugs poses the greatest challenge: small protein (peptide) drugs. These drugs, even with refrigeration, have the shortest shelf-life (months), and therefore require a "production-on-demand" solution for long duration missions that may last years. The protein drugs—approximately 1/3 of all new drugs that come to market today—include some of the most important spaceflight countermeasures, such as filgrastim, a growth factor that can restore the bone marrow after radiation damage, and teriparatide, a peptide drug for preventing bone demineralization.

In Phase I we introduced our production-on-demand solution, which we call "Astropharmacy", a platform technology that involves the synthesis of drugs by a space-hardy spore-forming bacterium, *Bacillus subtilis*, together with a novel purification system that uses histidine tags for affinity purification. We engineered *B. subtilis* to produce the non-glycosylated drugs filgrastim and teriparatide, which we purified with a lightweight, small volume system adapted from standard laboratory protocols and enabled by judicious genetic engineering prior to launch.

1. Table of Contents

2. Quad chart

A flexible, personalized, on-demand Astropharmacy

Innovation

- An astropharmacy based on on-demand production of small quantities of protein-based pharmaceuticals. Microbes programmed on Earth for pharmaceutical synthesis during mission; potential for reprogramming or cell-free synthesis during the mission. Disease prevention, diagnosis, treatment
- Novel technology: Development of state-of-the-art genetic engineering and novel production system.
- This concept is for all human missions, but critical for missions >6 months without re-supply.
- Timeline: on Earth ~5 y, in space tech demo 3-5 y, implementation off planet mission dependant; regulatory

Technical Approach (major tasks, goals)

1. Raise TRL by synthesis and purification of biologics. Test purity and activity of biologics. Test and quantify parameters for production (time, temp, amount). Test parameters will be using *Bacillus subtilis* for the production chassis, and GCSF & Teriparatide as biologics.
2. Assess requirements for implementation in space (mass, etc.)
3. Identify key knowledge gaps & outline roadmap for technology development
4. Assess impact of technology for terrestrial applications (DoD applications, orphan drugs)

Potential & Benefits

- State of the art: bring drugs from Earth, improve packaging (TA 6.3). BUT..can't take everything, drugs degrade, unknown risks.
- Contribution to aerospace: Allows longer human missions, decreases astronaut health risks.
- Additional benefits: on-site, on-demand production of small quanitites of protein-based materials. Emphasis in space is small, customized production (unlike mass production on Earth). Hope for small-volume "orphan drugs" that are not profitable. Important for small groups of humans in prolonged isolation, esp in absence of refrigeration.

Evaluation Notes

3. Phase I Key Findings

We synthesized and purified therapeutic peptides G-CSF and teriparatide—which serve as relevant examples of pharmaceutical agents that crew members may require on long-duration missions.

Key findings drug production: There was an enormous difference in the secretion of drugs depending on the signal peptide used, which vindicates our approach of testing a variety of signal peptides. An unexpected key finding: the clone with the highest secretion activity showed mutations after multiple efforts, thus the highest production was toxic to the production cell, here, *B. subtilis*. Two solutions are to sacrifice high production to get a functional drug (approach used here), or produce the drug with a cell-free system which eliminates concerns about toxicity to the host cell, which we continue to explore. The temperature for optimal growth rate of the drug-producing *B. subtilis* is not the same as that for optimal drug production. Order-of-magnitude increase in the temperature stability of cell-free expression components has been achieved through combinatorial addition of sugars and polymers guided by Design-of-Experiment. Micro-glassification as an alternative to lyophilization of cell-free components has been demonstrated. The process is now being optimized to maximize expression on resuspension. We have studied cell-free synthesis of teriparatide and G-CSF, and the other protein therapeutics entolimod (potential radioprotective), and alfimeprase and reteplase (thrombolytics). Expression has been studied by RT-PCR. The role of disulphide bond enhancers in expression of the latter (9 disulphide bonds in reteplase cf. 2 in G-CSF) has been studied through separation on reducing and non-reducing gels.

Key findings drug purification: To circumvent the requirement of external pumps for microfluidics, we designed three different astropharmacy devices that utilize syringes as a means of propagating fluid flow. These devices consist of a custom syringe with built-in purification, a purification column attachment for standard syringes, and a credit-card-sized microfluidics chip, which is designed to fit a standard syringe to promote fluid flow. Through simulated fluid-flow modeling of the microfluidics devices, we observe that the pressure of a syringe is comparable to the external pump systems used in most polydimethylsiloxane (PDMS) microfluidics systems. This significantly paved the way for future prototyping of a device that 1) offers potential for a longer shelf life 2) provides the crew with on-demand, on-site pharmaceutical manufacturing, 3) decreases the capacity required of medicines and 4) allows for pre-packing on Earth.

Key findings of testing purified biologic for purity and activity. The purity of the samples were tested by sequencing the constructs, and by SDS-PAGE and Western blot using standard procedures. Activity assays are also key. We have established a second teriparatide activity assay recording cAMP release from SaOS-2 cells.

Key findings for implementation in space: Spores produced from *B. subtilis* engineered to produce the drugs, dried on paper for a week, produced the drugs as well as the fresh cells.

Key findings for implementation on Earth, with focus on DoD. Reduction of the resources needed for protein-drug storage is a major driver of DoD's *Biologically-derived Medicines on Demand* (Bio-MOD) initiative. Virtual and in-person meetings with several DoD personnel tasked with medical futures, facilitated by Dr. Kristen DeWilde, Major, USAF, MC, SFS who at the time was stationed in the Pentagon. While this work was of great interest to them in general, one application seemed at the time to be intriguing: the on-demand production of antidotes to toxins.

Clearly, different parts of DoD will have different priorities, and the advantage of a generalized platform is that it can be adapted. We learned that DoD had not thought beyond miniaturization to the size of a car, whereas we are aiming for systems closer to the size of a wallet.

4. Background

4.1 The problem

When taken ill, it is a quick trip to the pharmacy that puts you on the road to recovery...unless you are in space. For short missions, it is feasible for an astronaut to carry a few general medicines. 'Med Kits' on the ISS, for example, include small supplies of painkillers, antibiotics, antiemetics, etc. If needed, an ISS medical emergency can be dealt with by aborting the mission and returning to Earth. Reliance on pharmaceuticals is especially important in space since invasive procedures cannot be performed during flight (Blue et al., 2019).

Providing pharmaceuticals for a planetary mission, however, is currently not possible (Blue et al., 2019). First, the shelf-life of many pharmaceuticals is limited, with a further decrease in life-time because of the space environment (Kast et al., 2017). Of the medications flown on ISS, 87% have labeled shelf lives of < 24 months. An important class of therapeutic, the biopharmaceutical or 'biologic', plays a critical role in treating many of the medical conditions and emergencies that astronauts are known to, or could likely, face. These protein-based drugs, approved by the FDA, are now used in the clinic to treat embolisms, hemorrhages, renal stone formation, bone loss, infection, thrombotic complications, etc. Unfortunately, biologics degrade in 6 months, even with refrigeration. To compound the problem, as the period of time away from Earth-bound pharmacies increases, the necessary range of medications of a rising variety of dosage and delivery forms increases, increasing up-mass. An ESA report (Berry et al., 2002) calculates the probability of a medical trauma is 0.06 per person per year. Our inability to store, transport and deliver pharmacologically-active biomolecules such as vaccines, antibodies, and other medications will limit long-term human missions as recognized in TA 6.3, "Human Health & Performance", and TX06.3.7 Transformative Health and Performance Concepts in the 2020 NASA Technology Taxonomy, and DRA5.0 7.1.3 "Medical Care". The Human Research Roadmap identifies "Risk of Ineffective or Toxic Medications Due to Long Term Storage" as a major risk. The use of medicines in space flight also has to change, since the way the body processes them is altered (Williams, 2003), as is their efficacy (Eyal, 2020). If we are to meet mission objectives of long-duration human missions including the exploration of Mars, and beyond, a new paradigm of pharmaceutical provision is required. A true paradigm shift would be the on-demand production of drugs, which is the goal of our proposed "Astropharmacy."

Due to their short shelf-life and easier route to synthesis, our Astropharmacy will begin with the production of non-glycosylated biologics. Being proteins, they can be synthesized by cells such as bacteria. Further, the machinery that makes these proteins can be extracted from the cell, and the proteins made in the proverbial test-tube. This "cell-free expression", where protein is manufactured according to instructions written in strands of DNA, can produce within hours a biopharmaceutical on-site, on-demand. Want a different drug? Use a different DNA sequence. This is the core of our Astropharmacy: the rapid on-site on-demand production of protein-based therapeutics. This technology will pave the way to a future production of a wide array of drugs, from glycosylated biologics to those requiring more complex biosytheses.

4.2 The innovation: On demand pharmaceutical production

Now imagine that when a small (~1 mg/mL) quantity of a pharmaceutical is needed, an astronaut (or robot) would activate the Astropharmacy (Fig. 1). Small bladders (1) pre-loaded with dried spores of Bacillus subtilis genetically engineered to produce the biologic with chromogenic markers for visual monitoring, a TEV protease site to remove the his tag (2), a his tag for purification (2) and a secretion tag to eliminate the need for cell lysis and simplify purification. Sterile culture medium is injected into the bladder (1). The spore germinate and produce and secrete the biologic with tags into the medium. Bacteria to make TEV protease would be loaded in a second bladder. After incubation of a few hours (depending on the host organism and temperature, armpit temperature should work for most production platforms in an emergency), the cells can be syringe filtered and injected into the Ni column where only the his-tagged biologic and protease will bind. They will then be eluted, combined to allow the protease to cut the protein and remove the tags and markers. As a reference, ThermoFisher's Ni resin has a capacity to bind up to 60 mg of 6xHis-tagged protein per milliliter of resin.[1] The required dose for two biologics of interest in space is far lower (Teriparatide, 30 µg, and G-CSF, 200 µg). The combined biologic/protease solution will be run through a second Ni column. The eluent should only have the untagged biologic. To further ensure purity and suspension of biologic in injection buffer, the resulting solution will be run through a size chromatography column, also loaded in a syringe (3). If any adjustments to the buffer or concentration need to be made, they can be done (4). Finally, the biologic is ready to inject (5). We anticipate that the entire production system should take under 24 h. It is anticipated that the purity of the system will be ensured in ground-based studies. However, the gold standard is usually HPLC, which could be done in flight. A pharmaceutical should be 98% or more pure, with no more than 1% of any single contaminant.

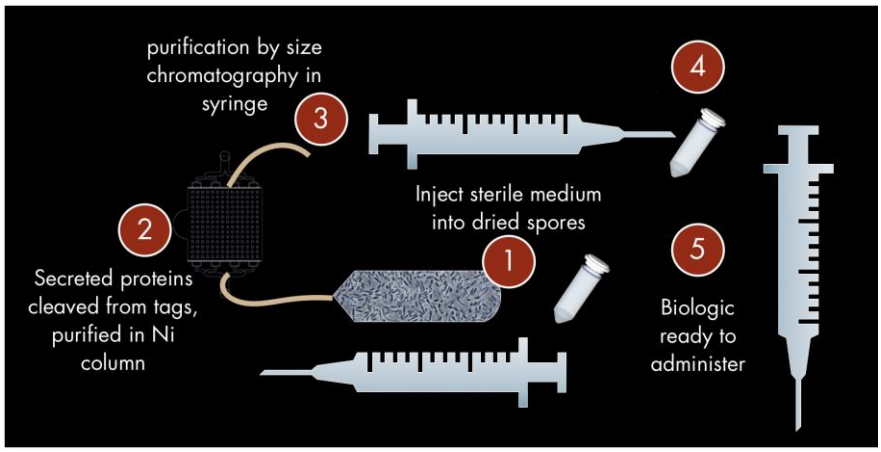

Fig. 1. Microfluidics-based Astropharmacy production system. The microfluidics in step 2 might be able to be replaced with a syringe-based system. It should be possible to use human thumb pressure to operate the columns, thus eliminating the need for a syringe pump, although the rate of flow of a syringe pump would be more reliable. In the lab, some of these columns are used with centrifugation. A small, heated/cooled centrifuge[2] could provide an alternative to some of the columns as well as provide heating during (1) and chilling during the remaining steps.

[1] https://www.thermofisher.com/order/catalog/product/88224

[2] For example, Eppendorf Refrigerated/Heated Centrifuge Model 5702RH, which has an adjustable temperature range of –9°C to 42°C, allowing heating, chilling, and centrifuging in the same unit with dimensions of 38 × 58 × 26 cm, maximum power consumption of 380 W, 36 kg base unit, and capacity up to 400 mL allowing scale up to several doses produced at once.

As a more versatile, but less robust system, a cell-free production system could be used instead of pre-programmed cells. To activate, we would add template DNA, and an energy mix to a dried cell-free powder that is stable at room temperature, incubate the mixture for an hour, and the material would be produced. A similar purification system would be used to the cellular production system. The flexibility of the system comes from adding DNA templates immediately before the addition of the energy mix. We envision that DNA synthesis could be done in space creating a "telepharmacy", or pre-launch on Earth. If we succeed, astronauts can make and test personalized drug therapies during space travel. This cornerstone of a space health systems marks progress towards self-sufficiency off-planet.

4.3 Credible and Reasonable

All of the steps outlined are adapted from standard bioengineering lab procedures. We have altered protocols to enable miniaturization, ease of use, and the ability to store precursors for years without refrigeration. The key is the extreme stability of *B. subtilis* spores, with survival of nearly 6 years in space during LDEF (Horneck, 1993). The EuCROPIS PowerCell payload (Rothschild, PI; McCutcheon et al., 2016) has demonstrated the ability of B. subtilis spores to be dried in microfluidic flight hardware for >3 years at room temperature, activated in flight with the addition of LB, with subsequent growth (flight in progress). The mass of the spores is negligible and takes up little room, so if spores are dried on 5 x 5 mm squares of the equivalent of 20 lb copy paper, 2520 samples could be transported to space in <5 g.

Recent advances in cell-free expression technology have enabled the rapid synthesis of recombinant proteins, utilizing bacterial, rabbit reticulocyte, and human derived lysate systems (Noireaux, 2010), allowing the production of proteins including antibodies at location. Commercial cell-free systems such as the PURExpress¨ Synthesis Kit (NEB) and the Expressway™ Mini Cell-Free Expression System (ThermoFisher)[3], both derived from Escherichia coli, can produce mg quantities protein/mL, the equivalent of >30 doses of vaccine. Cell-free protein expression system, containing all the necessary transcription-translation enzymes, small molecule substrates and DNA for the construction of genetic circuits, can be lyophilized on filter paper (Pardee et al., 2014; 2016). The paper, prepared with protein expression mixture and DNA, can be stored at room temperature and used, after rehydration, with the same results as using freshly prepared cell-free protein expression mixture in liquid reaction or in vesicles. The field of stable, dried cell-free systems is being driven by the need for diagnostics and other paper-based devices. An example is Phil Williams (University Nottingham) 'microglassification' approach to storage by synthesizing microparticles of solid (non-crystalline, glassified) protein.

4.4 The Aerospace Architecture Concept

The ultimate vision of the proposed Astropharmacy could be applied to any long-term human mission, but will focus on a "long-stay Mars mission" Drake (2009; 2014) since the importance increases with time and distance. Note some steps have multiple approaches.

4.5 Mission Context

The proposed Astropharmacy would equip astronauts with the autonomy to manufacture pharmaceuticals for their own health system. This approach aligns with the self-sufficiency goals outlined in the Mars DRM 5.0 and addenda. Pharmaceuticals are for medical care (7.1.3; Drake, 2009) and as countermeasures to tissue atrophy in reduced-gravity (TA 6.3.2) and radiation

[3] https://assets.fishersci.com/TFS-Assets/LSG/manuals/expressway_milligram_man.pdf

exposure (TA 6.5.2; Singh et al., 2015). The Mars to Earth 3-20 min communication delay and multiyear mission scenarios necessitate on-site responses. An on-demand Astropharmacy will ensure the health and safety of crewed missions of >6 m without a quick abort.

4.6 Wider Benefits of the Study

4.6.1 Benefits to NASA and the Aerospace Community

Limited shelf-life and inability to transport unlimited pharmaceuticals creates risks to astronaut health and thus limits long-term human missions. This study focuses on a solution where the pharmaceuticals are produced on-demand with limited upmass. While we focus on pharmaceutical applications, this work is applicable whenever small amounts of biologically-produced materials are needed on demand. For example, the repair of small-scale rips could be fixed with biological glues. The ability to produce non-biologic drugs and other materials through multi-step cell-free systems will be important in space as not all systems can be predicted based on terrestrial tests. Large-scale manufacturing of proteins from previously engineered B. subtilis fits with long-term plans for synthetic biology in space (e.g., Rothschild, 2016). Here we examine how to put the production and purification steps into practice.

4.6.2 Wider Benefits of the Study

The ability to produce small quantities of on demand pharmaceuticals with minimal power, mass and storage demands should be of enormous benefit in field situations where time is of the essence or resupply impossible. Such situations include remote areas including the Antarctic, and extended submarine deployments. The Air Force has already expressed interest, although they have fewer constraints on long-term stability. This approach could make orphan drugs[4] for terrestrial applications more affordable. Second, the ability to produce small quantities of non-drug materials on demand could prove useful. Third, cell-free systems are increasingly being used to prototype genetic pathways and circuits in synthetic biology; a more stable system would be broadly useful. For workforce development, we anticipate including a masters student to conduct some of the research. This work already engaged the 2019 Brown Stanford Princeton iGEM (international Genetically Engineered Machine competition) team, some of whose members wish to continue to develop this work under the auspices of a NIAC.

4.6.3 Workforce development, public engagement.

Human drugs are a relatable subject, so not surprisingly this topic has generated a lot of press attention, including articles describing this project in The Medicine Maker,[5] IFL Science,[6] NeoLife,[7] and the Smithsonian's Air and Space Magazine.[8]

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[4] "The Orphan Drug Designation Program provides orphan status to drugs and biologics which are defined as those intended for the safe and effective treatment, diagnosis or prevention of rare diseases/disorders that affect fewer than 200,000 people in the U.S., or that affect more than 200,000 persons but are not expected to recover the costs of developing and marketing a treatment drug." https://www.fda.gov/about-fda/office-clinical-policy-and-programs/ office-orphan-products-development

[5] https://themedicinemaker.com/manufacture/the-astropharmacy-concept

[6] https://www.iflscience.com/astropharmacy-and-a-telescope-on-the-moon-among-new-concepts-selected-for-nasa-funding-55653

[7] https://neo.life/2021/01/to-boldly-go-where-no-pharma-has-gone-before/

[8] https://airandspace.si.edu/air-and-space-quarterly/summer-2022/there-doctor-house

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6. Draft publications

Two manuscripts have been prepared from this work, and are included here.

6.1 Manuscript 1. Introducing the Astropharmacy concept

Status: submitted to BioRxiv awaiting DOI number; to be submitted to journal March 2023

The "Astropharmacy": An On-Demand Peptide Drug Production System for Use on Earth and in Space[1]

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Keywords: Astropharmacy, on-demand drug synthesis, astronaut health, pharmaceuticals, space medicine, biologics. (Min.5-Max. 8)

1. Abstract

Many pharmaceuticals expire within two years of manufacture, after which potency and pharmacokinetics are no longer guaranteed. Though there are efforts to extend pharmaceutical shelf-life, the quality of pharmaceuticals vary based on manufacturing lot, storage conditions, and packaging. The spaceflight environment also affects the degradation of pharmaceuticals, though the extent remains unclear. With these facts in mind, the possibility of a multi-year crewed space exploration mission means that the pharmaceutical supply at launch is unlikely to remain viable for the duration of the mission. The logistics of re-supply are complicated on any space mission,

but re-supply is likely to be difficult or simply impossible on the longest duration space missions. A further challenge is the uncertainty of predicting the specific medication needs of the crew. Thus, a platform for on-demand drug synthesis is necessary to ensure the success of long-term space exploration missions, especially missions to Mars and beyond. Here we present the *Astropharmacy*, a peptide-drug production platform that uses genetically engineered bacteria (*e.g., Bacillus subtilis* or, as a proof of concept, *Escherichia coli*) or a cell-free system to produce drugs "on-demand." Teriparatide and human granulocyte colony-stimulating factor (hG-CSF) were chosen as target drugs for the purpose of demonstrating the technology because of their utility as countermeasures against complications from long-term spaceflight. Within a 24-hour period, a prototype version of the Astropharmacy was able to produce 6 dose-equivalents each of teriparatide and hG-CSF, using *E. coli*, and twenty dose-equivalents of teriparatide, using a cell-free system. Not only would this platform enable the production of drugs to meet specific astronaut needs, but it would have utility on Earth for production of drugs required to respond to bioterrorism events, for production of orphan drugs, and production of drugs in remote areas of the world where refrigeration is not available.

6.2 Manuscript 2. Engineering of *Bacillus subtilis* to produce drugs

Status: posted on BioRxiv, DOI 10.1101/2023.02.22.529550; to be submitted to journal, Feb. 2023 Please check for updated citation.

Bacillus subtilis engineered for aerospace medicine: a platform for off-planet production of pharmaceutical peptides

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Keywords: Astropharmacy, on-demand drug synthesis, astronaut health, pharmaceuticals, space medicine, biologics. (Min.5-Max. 8)

Abstract
Biologics, such as pharmaceutical peptides, have notoriously short shelf lives, insufficient for long-duration space flight missions to the Moon or Mars. To enable the sustainable presence of humans on the Moon or Mars, we must develop methods for on-site production of pharmaceutical

peptides in space, a concept we call Astropharmacy. Here, we present proof-of-concept for the first step needed: a low-mass system for pharmaceutical production designed to be stable in space. To demonstrate feasibility, we engineered strains of the space-hardy spore-forming bacterium, Bacillus subtilis, to secrete two pharmaceutical peptides important for astronaut health: teriparatide (an anabolic agent for combating osteoporosis) and filgrastim (an effective countermeasure for radiation-induced neutropenia). We found that the secretion peptides from the walM and yoqH genes of B. subtilis 168 worked well for secreting teriparatide and filgrastim, respectively. In consideration of the TRISH challenge to produce a dose equivalent in 24 hours, dried spores of our engineered strains were used to produce 1 dose equivalent of teriparatide from a 2 mL culture and 1 dose equivalent of filgrastim from 52 mL of culture in 24 hours. Further optimization of strain growth conditions, expression conditions, and promoter sequences should allow higher production rates to be achieved. These strains provide the template for future optimization efforts and address the first step in the Astropharmacy, capable of on-site production, purification, and processing of biopharmaceutical compounds in platforms amenable for use in space.