Modeling the effects of space travel on the cardiovascular system using a bio-mimetic *in vitro* hagfish protein model of the myocardium

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Abstract

Long-term space travel is a harsh experience for the human body. Astronauts exposed to the effects of space travel often experience bone and muscle loss, as well as an increased risk of diseases, including heart disease[1].

While the human heart has shown an impressive ability to adapt to spaceflight, many of the long-term effects on cardiovascular health are unknown [2]. To plan for missions requiring longer flight durations, understanding these effects is necessary to assess the risk to the astronauts undertaking these missions. However, evaluating these risks is difficult due to the lack of a sufficiently accurate model. While animal models and studies of returned astronauts can be informative, these methods also have significant limitations.

The development of an accurate *in vitro* model of the myocardium would be a beneficial tool to further investigate the underlying cellular mechanisms affecting cardiac health during spaceflight. This project seeks to create and validate an accurate three-dimensional model of the myocardium using novel bio-mimetic hagfish proteins. Hagfish protein threads that mimic the properties of the myocardium and support cardiomyocyte cell culture were produced. Upon further validation, this model will be exposed to an artificial spaceflight environment incorporating simultaneous microgravity and radiation to investigate the effects of spaceflight on the myocardium.

I. INTRODUCTION

Cardiovascular disease is one of the most common causes of death worldwide. In 2019, ischemic heart disease alone accounted for 16% of deaths globally [3]. While progress in the field of medicine has decreased the fatality of cardiovascular diseases, there are still countless unanswered questions about how these diseases progress and more importantly, how to stop them. An accurate model of the cellular mechanisms that occur during the progression of these diseases would help researchers advance toward their cures. As many of these conditions affect the heart wall, this tissue makes an effective target for *in vitro* modeling. The heart wall consists of the endocardium - the lining of the chambers of the heart, the pericardium - the outer protective layer of the heart wall, and the myocardium - the muscle of the heart, and the tissue most affected by many cardiovascular diseases (**Fig. 1** [4]).

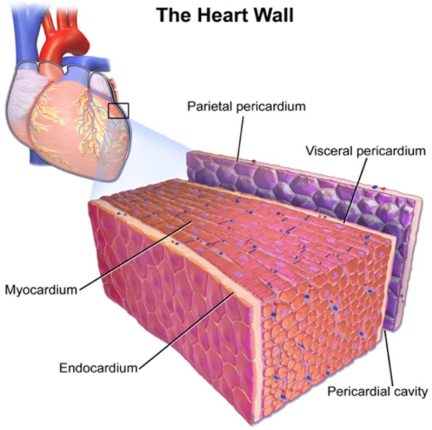

Figure 1: Schematic of the heart wall [4].

One cardiovascular disease that affects astronauts is cardiac atrophy. Cardiac atrophy is caused by a decrease in muscle mass in the heart and increased ventricular stiffness [5]. Cardiac atrophy also affects individuals experiencing bedrest, malnutrition, and HIV [6]. This disease is especially prevalent among cancer patients, who experience both cardiac unloading due to bedrest and cardiac damage from radiation and chemotherapeutic agents [7]. One study showed that in individuals diagnosed with breast cancer, cardiovascular disease competed with breast cancer as the leading cause of death [8]. While some treatments are available for those affected by this disease, this disease can be difficult to cure and prognoses are often poor [9].

Many of the cellular mechanisms involved in cardiac atrophy remain unknown due to the lack of a sufficient model [10]. Traditionally, researchers utilize 2D *in vitro* models, animal models, or *ex vivo* models. While each of these models has contributed to the understanding of cardiovascular diseases, they also each have significant drawbacks. 2D models, while cheap and accessible, do not represent the complex behavior of heart tissue in the body. Animal models and *ex vivo* studies may be more mimetic, but they are also significantly costlier and ethically complicated. Our mimetic 3D model of this tissue would provide the complexity needed to study these diseases, while also remaining cheap and straightforward to produce.

While there are existing *in vitro* models of the myocardium, such as micropatterned or bio-printed models, they often lack one or more of the essential properties of the myocardium. Myocardium models should mimic the geometric and mechanical properties of cardiac myofibers while supporting the healthy growth of cardiomyocyte cell culture. Additionally, a model with tunable properties has the potential to model not only healthy tissue but diseased tissue as well. Creating a model demonstrating all these properties, however, has its challenges.

The myocardium displays many unique properties that are difficult to replicate in an *in vitro* environment, namely the geometric and mechanical properties of cardiac myofibers. Cardiac myofibers have a diameter of approximately 10-20 microns [11]. These myofibers are small compared to skeletal myofibers, which range from 20-100 microns in diameter [12]. While previous research has shown that synthesis of natural fibers featuring such a small diameter is possible [13], these fibers lack many of the mechanical properties necessary to accurately model the myocardium. Cardiac myofibers demonstrate a very low stiffness, with an elastic modulus ranging from approximately 8-109 kPa in a healthy heart [14,15]. Many of the synthesized natural fibers have an elastic modulus in the MPa to GPa range, several orders of magnitude above *in vivo* conditions. Since the mechanical properties of the myocardium are integral to its function *in vivo,* they are an essential aspect of creating an accurate *in vitro* model. To achieve these properties, a biomaterial with equally unique properties must be used.

One group of proteins that may be able to replicate the properties of the myocardium is intermediate filaments. These proteins provide support within eukaryotic cytoskeletons, assist in the remodeling of extracellular matrices, and can be produced synthetically [16,17]. One type of intermediate filament protein is secreted by hagfish as proteinaceous threads as a defense mechanism [18]. These hagfish threads are composed of two proteins denoted α and γ, which are normally present in a 50:50 ratio. Recently, the Jones lab (collaborating

investigator) has shown that both proteins comprising the threads can be recombinantly expressed in *E. coli* and purified while maintaining desirable mechanical properties [19]. Basic properties of the proteins, such as the low elastic modulus of hagfish protein constructs and tunability of bulk material characteristics, suggest that they can be developed into a mimetic model of the myocardium.

In this study, recombinant hagfish intermediate filament protein fibers were developed and analyzed to replicate the myocardium *in vitro* focusing on replicating cardiac myofiber diameter and stiffness, and healthy cardiomyocyte cell support. The model was assessed for geometric and mechanical properties. The health of a human cardiomyocyte culture growing on hagfish threads was also evaluated using brightfield and fluorescent microscopy.

In addition to their unique mechanical properties, hagfish protein fibers are also well-suited for this application due to their tunability. The geometric and mechanical properties of the hagfish threads can be easily altered to fit the desired parameters such that hagfish protein threads will serve as a model of both healthy and diseased myocardium. By altering the threads to become thinner and stiffer, as seen in cardiac atrophy [5], this model can transition from modeling a healthy myocardium to a diseased one. These characteristics expand the application of hagfish protein threads for *in vitro* modeling of cardiovascular disease and enhance our understanding of why disease occurs and potential therapeutic targets.

This model will serve as a novel 3D high-throughput bio-mimetic *in vitro* model of the myocardium in both healthy and diseased states. The response of the cardiomyocyte cells to changing mechanical properties will be evaluated, providing valuable insight into the cellular mechanisms involved in myocardial disease, as well as an accessible platform for early-stage testing of therapeutics for diseases like cardiac atrophy. This model can also be used in artificial spaceflight environments to investigate the effects of microgravity and radiation on the heart. This model will advance our understanding of heart disease and improve upon traditional two-dimensional cell culture models (**Fig. 2A** [20]), helping us take more steps in the direction of curing cardiovascular diseases.

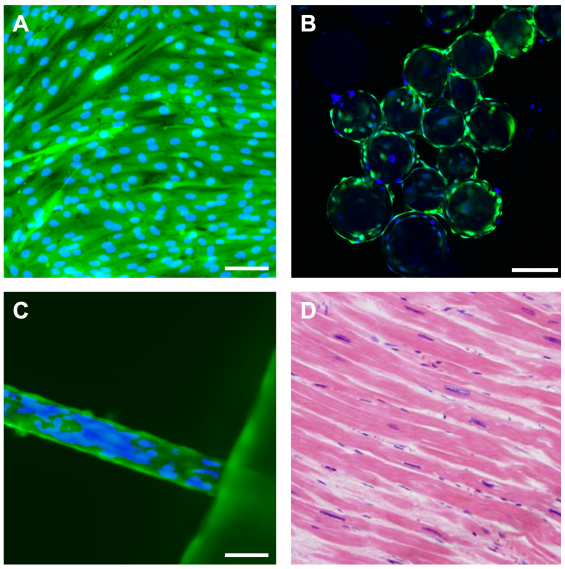

Figure 2: A) Fluorescent image of 2D h9c2 culture, B) Fluorescent image of microcarrier bead h9c2 culture, C) Fluorescent image of hagfish protein fiber h9c2 culture, D) Histology image of skeletal muscle [20].

II. PROGRESS

The first goal of this project was to develop a method of synthesizing and preparing hagfish protein threads for assembly and cell culture without compromising the geometric and mechanical properties of the threads.

The hagfish protein threads are synthesized by completely solubilizing hagfish protein in a formic acid solution. The solubilized

protein solution is then extruded through a needle syringe into a water bath. Upon extrusion, the formic acid is dissolved into the bath, leaving a hydrogel-like protein thread structure. The geometric and mechanical properties of the threads are controlled primarily by the gauge of the needle used during extrusion and the protein concentration respectively. In previous applications, these threads were made at a 20% w/v concentration and extruded through a 26-gauge needle, which has an inner diameter of 260 μm. These conditions create threads with a diameter of approximately 150 μm and an elastic modulus of 400 kPa. While these conditions show an improvement in the geometric and mechanical properties of other myocardial models, they do not meet the ranges seen *in vivo* (10-20 μm, 8-109 kPa).

To create a protein thread with properties more similar to *in vivo* conditions, the needle gauge and protein concentration used in the synthesis process were decreased. Several needle gauges were tested, including 26G, 30G, 32G, and 34G, with inner diameters of 260 μm, 159 μm, 108 μm, and 51 μm respectively. While there is most certainly a lower limit for the diameter of these threads after which they become too fragile, this limit was not reached as each of these threads was able to be handled without breaking. **Table 1** below shows the resulting diameter of the protein threads at each of these gauges.

Table 1 Average thread diameter of protein fibers extruded through needles of various gauges. (10% w/v)

Needle Gauge	Average Protein Thread Diameter (μm)
26 G	120.1
30 G	61.4
32 G	43.5
34 G	Data Forthcoming

As is seen in the table above, the protein thread diameter decreases fairly proportionally with the gauge of the needle used for extrusion. The protein threads extruded through a 34 G needle are expected to have the closest diameter to *in vivo* conditions.

The next step in optimizing the protein thread characteristics was changing the protein concentration. Previous studies have found that decreasing the protein concentration decreases the elastic modulus of the fiber, however, no studies were conducted on concentrations lower that 10%. To determine the ideal protein concentration for this application, threads at 10%, 7.5%, and 5% protein concentrations were synthesized. While the 5% protein concentration solution did form threads, these threads were too fragile to be handled and were deemed unusable for this application and were not assessed. **Table 2** below shows the average elastic modulus for the 10% and 7.5% protein threads.

Table 2 Mechanical properties for hagfish protein threads at 10% and 7.5% w/v protein concentration. All threads were extruded through a 34 G needle. All data is preliminary.

	Elastic modulus (kPa)
10% w/v	105.78
7.5% w/v	41.46

As seen above the mechanical properties of these threads are within the expected range as compared to *in vivo* values. These mechanical properties are highly unique and display the potential of this biomaterial an *in vitro* modeling application.

Now that the ideal procedure for synthesizing a hagfish protein thread mimicking the myocardium *in vivo* had been established, these threads were prepared for cell culture. The main obstacle in this preparation was the handling of the threads. When the threads are stored in a liquid, they can retain their mechanical properties, but they are quite difficult to organize for cell culture. The protein threads can be dried, but this often results in a severe increase in elastic modulus, up to the MPa range, several orders of magnitude above *in vivo* conditions. To prepare the threads for cell culture, they needed to be dried without affecting their mechanical properties. One potential solution was lyophilization. By freezing the threads in water and removing the ice through sublimation, the threads would be completely dried, however, it was unknown how this process would affect the mechanical properties of the thread.

To determine this effect, the elastic modulus of the thread was measured under three conditions. The first batch consisted of threads that were never dried. The second batch consisted of threads that were lyophilized after being frozen in a water bath and rehydrated. The third batch consisted of threads that were lyophilized without a water bath and rehydrated. The results show that the third batch of threads increased in brittleness even after rehydration, however, the first and second batches showed no significant differences. This confirmed that lyophilization could be used to dry the threads without compromising the mechanical properties. This process, however, introduced a new obstacle.

When the threads are dried via lyophilization, they become extremely fragile until they are rehydrated. Additionally, the threads retain the shape in which they are dried, meaning that to prepare threads for cell culture before rehydration, they must already be organized prior to lyophilization. As this was a new requirement in this process, a new process of winding the threads during extrusion had to be designed. Finding a way to wind the threads without creating disturbances in the extrusion water bath and without removing the threads from the extrusion bath was difficult. But using new parts that were custom-designed and 3-D printed to fit the extrusion machine, the threads were organized before drying. Using both this new winding mechanism and lyophilization, the hagfish protein threads were prepared for cell culture by winding them around the chassis designed by *Clegg et al* [20], sterilizing using UV, and decreasing hydrophobicity using oxygen plasma (**Fig. 3)**

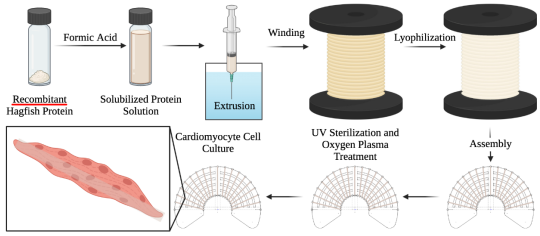

Figure 3: Hagfish protein synthetization and preparation procedure.

The next step in verifying hagfish protein threads as a mimetic *in vitro* model of the myocardium was determining their compatibility with cardiomyocytes. After being prepared for cell culture, the threads were seeded with h9c2 rat cardiomyocytes. The cells grew for up to 12 days and then were assessed for morphology, shown in **Fig. 4**.

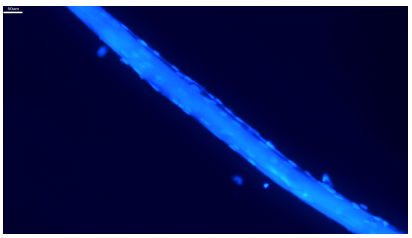

Figure 4. Nuclear stain of h9c2 cells on hagfish protein threads.

As seen in **Fig 4.**, when compared to a two-dimensional control (**Fig 2.A**), the cells grown on the protein threads show more similar morphology to *in vivo* conditions. These initial results show promising potential for the hagfish threads to fully support h9c2 cell culture.

III. ONGOING WORK

As this project continues, the ability of the hagfish protein threads to support cardiomyocyte cell culture will be further assessed using protein expression and contractile force analyses. H9c2 cells grown on hagfish threads will be assessed for F-actin, Cardiac Myosin Heavy Chain, Cardiac Troponin T, and Sacromeric Alpha Actinin and compared to 2-D controls. The expression of these proteins will provide information on the contractile, regulatory, and structural states of the cells and determine if the protein threads can support mimetic cell culture. Additionally, the force produced by contracting cells on the threads will be measured and compared to literature values.

Once these threads have been established as a suitable *in vitro* model of the myocardium, cardiomyocyte cell cultures on hagfish threads will be exposed to artificial spaceflight conditions to investigate the effects of these conditions on myocardial tissue.

The artificial spaceflight will be simulated with artificial microgravity and/or radiation. These spaceflight conditions will be tuned to simulate various spaceflight conditions, such as a year on the international space station, or a journey to Mars.

The artificial microgravity conditions will be simulated using a random positioning machine, which utilizes an algorithm that reorients the cells faster than the biological response to gravity, creating artificial microgravity on the cellular level. The cosmic radiation will be simulated using a gamma radiation source, dosed according to the conditions being replicated. The artificial microgravity and radiation conditions can be used separately or simultaneously. After exposure, the cell culture can be assessed for morphology, viability, protein expression, and contractile force to determine the effect of these conditions on the cells. The hagfish protein threads will provide a more mimetic environment, reducing the variables that could introduce error between *in vitro* and *in vivo* conditions.

The results from this project will help inform future space travel planning on the risks to the cardiovascular health of the astronauts on these missions, provide potential treatment targets, and further the understanding of the effects of space travel on the human body.

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