Development of a Nano-Encapsulation Platform with a Novel Antioxidant Indigoidine for the Mitigation of Space Radiation

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Abstract: **Space includes many hazards to the human species, one of the most detrimental to us is the biological damage from radiation. Loading an astronaut's immune system with free radical scavenging molecules as a potential shield from this damage is one method to prevent biologically relevant damage that has only been slightly explored. Unfortunately, the pharmacological potential of many antioxidants is severely restricted because of their low solubility/absorption. Here, I characterize a powerful delivery system that can deliver many different hydrophobic antioxidants to the cell, Increasing their bioavailability. The novel antioxidant Indigoidine has been explored in this delivery system. It has been successfully encapsulated and shown as an increased colloidal suspension in aqueous solution thus, increasing the bioavailability to the human body.**

1. INTRODUCTION

As we venture further into space with longer times spent outside of the earth's protective electromagnetic field, our astronauts are exposed to larger amounts of radiation. If we want to venture to mars on a long space flight mission, we need to mitigate the damage caused by radiation. Once radiation enters the human body it's energy can be transferred into free radical oxygen species. This can lead to severe damage such as cancer-causing DNA breakage, protein denaturation, and even cell death.[1]

The SI unit of a radiation dose equivalent is the Sievert (Sv) which is defined as the biological effect of radiation-induced genetic damage and potential of cancer. The average typical dose on for a human on earth is about 0.0036 Sv/year[2], which is several orders of magnitude smaller compared to the exposure of what a career astronaut can be exposed to. (Greater than 0.05 Sv/year[2] for those who work in spaceflight)

Also, it is crucial that astronauts are in peak performance while in space and their brains need to be fully functioning. This ionizing radiation can severely impact an astronaut's central nervous system causing short- and long-term effects. Short-term memory, concentration, and fatigue can impact an astronaut's ability to work, and long-term effects such as cancer and dementia are current detriments of being exposed to large amounts of radiation.[3]

The first step in designing an effective biological mitigation strategy for the damage caused by radiation on the nervous system is by developing a baseline for the damage. This damage is currently being understood by the cohort in my lab using our brain organoid model.

In the past our preliminary results of the brain organoid exposed to microgravity has shown sustained damage, separate from the damage caused by radiation. Currently we are analyzing the data of brain organoids exposed to gamma radiation and hard work has gone into establishing a baseline of exposed damage. Once we have developed the baseline damage, we can begin to understand how to prevent it.

Sufficient remedies to the damage from ionizing radiation have yet to be discovered. Currently there is no internal mechanism of preventing the damage of radiation in the human body. Large sheets of lead block radiation but, they are bulky and can interfere with an astronaut's activities during their mission. The CDC only has released remedies for radiation as "emergency use"[4] all of which are far from ideal and are in a preliminary state. One method that has yet to be tested is the preventative measure of loading an astronaut's central nervous system with free radical scavenging antioxidants. This is the remedy I hope to explore during my graduate studies.

Unfortunately, most antioxidants of interest inherently have a low bioavailability due to their hydrophobicity. This inherent property can be overcame using nano-encapsulation of the antioxidants with solubilizing agents called Pluronics.

2. BACKGROUND

Pluronics or poloxamers are a class of synthetic tri-block copolymers. They are composed of three blocks of poly (ethylene oxide) (PEO) and poly(propylene oxide) (PPO) arranged PEO-PPO-PEO. The two PEO groups are hydrophilic while the PPO group in the middle is hydrophobic. The lengths of each of the polymer blocks can be customized giving many different existing poloxamers with different thermodynamic properties. The ratio of the number of hydrophilic PEO monomers to the number of lipophilic PPO monomers determines the hydrophilic/lipophilic balance (HLB) of the poloxamer. Here we look at two different poloxamers consisting of different HLBs F127(22)[5] and F68 (29)[6]. The chemical structures of F127 and F68 with their respective polymeric ratios can be seen in Figure 1.

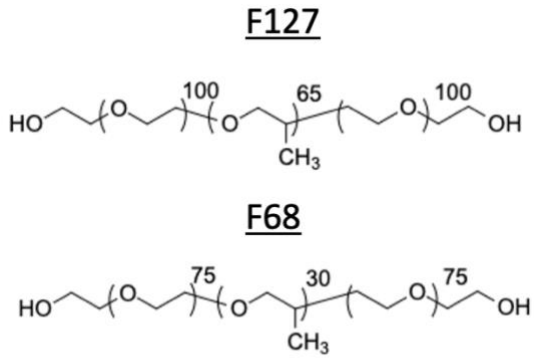

Figure 1: Pluronic F127(Top) andF68(Bottom) and their respective chemical structures. F127 is generally more hydrophobic compared to F68.

These were chosen not only for their high hydrophilicity but also their excellent biocompatibility (other solubilizing agents are more detrimental to the cell compared to Pluronics). These synthetic polymers have many unique properties but most importantly is their thermoreversible process of forming macromolecular structures called micelles. In concentrations above the critical micelle concentration, and

given temperature requirements, the free poloxamer (known as a unimer) forms micelles with a hydrophilic shell and a hydrophobic core. These micelles are highly ordered structures ranging from 10-100nm.

Polymeric micelles are thermodynamically self-assembled nanostructures, formed by a natural phenomenon known as the hydrophobic effect. The hydrophobic effect is caused by the process of water hydrogen bonding to the surfaces of molecules and with itself. This overall process weakly bonds to the surface of hydrophobic materials exposing the hydrophilic tails, thus encapsulating the material. **Micelles can encapsulate around our hydrophobic antioxidants and help deliver them to the cell.**

This is an important feature as many free radical scavenging molecules suffer from poor bioavailability. This platform can increase the aqueous solubility and therefore make the hydrophobic antioxidant bioavailable to the cell. Micelles also offer a high affinity to cell membranes, being able to deliver their cargo into the cell when the micelle integrates with the cell membrane.[7] It has also been shown that micelles can integrate with the blood brain barrier[7,8] , a highly fatty barrier that protects the brain. A vital component when developing delivery devices for the immune system.

3. MICELLE VEHICLE CHARACTERIZATION

With the understanding of the poloxamer delivery platform, we can begin to characterize and quantify key parameters relevant to the delivery to the brain.

The characterization of the F127 and F68 micelles for antioxidant delivery is as follows. First, the critical micelle temperature (CMT) was characterized. The temperature dependence of micelle formation can be seen in Figure 2.

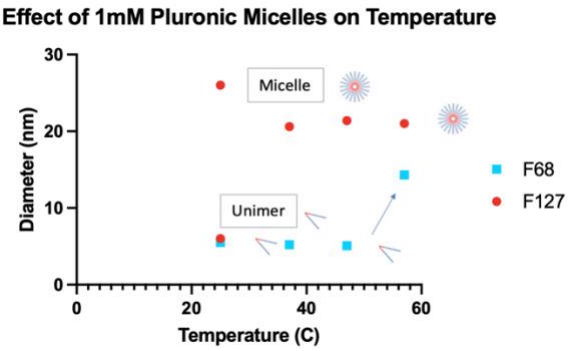

Figure 2: Micelle and unimer particle size of F127(red) and F68(blue). F127 forms micelles from temperatures 25-57C while F68 only forms micelles above ~50C.

found in F127 and F68 using a constant 1mM concentration for each. F68 does not form micelles until heated past ~50C, while F127 is shown to form micelles ranging from room temperature to above 50C. For our application of replicating the human brain, we will need our delivery vehicle to form at 37C. Thus, we can conclude that F127 is a better nanocarrier compared to F68. F68 is still capable at dissolving our antioxidants of interest and in the past has shown to be less cytotoxic compared to F127 so it will still be considered as a treatment in the upcoming studies. This finding is also supported by the literature showing that the F68 micelle only forms above 50C[9].

Other critical parameters to consider are the critical micelle concentration (CMC) and the upper micelle saturation concentration (UMSC). These two concentrations were determined with dynamic light scattering (DLS) for F127 and can be seen in Figure 2 on a log scale. Dynamic light scattering is a technique that primarily measures the Brownian motion of nanoparticles/macromolecules in solution. The angle at which scattered light changes is

proportional to the size of the particle and this information is then used to approximate the size of our micelles. This is a robust technique that relays the size and size distributions of nanoparticles.

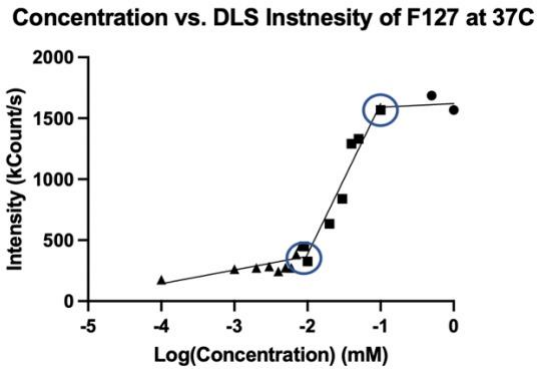

Figure 3: DLS Intensity vs Concentration of F127 at 37C. The intensity is correlated with the number of micelles formed in solution. The CMC (circled left) and the UMSC (circled right) were determined.

The CMC can be shown in Figure 3 and is the intersection between the purple and green data (circled left). The CMC is important to consider because micelles will not form at any concentration below that point. We want the maximum number of micelles to form with a minimum amount of Pluronic added. To achieve this, we also we need to understand the UMSC. The UMSC can be seen in Figure 2 and is the intersection between the green and red data(circled right). This concentration minimizes the amount of Pluronic added while adding as many micelles as possible to deliver our chosen antioxidant to the cell and. **The UMSC will be the working concentration of Pluronic F127 for the organoid studies subjected to radiation**. With this data we can determine a working concentration of F127 being between 0.001-0.01mM and gives us a baseline concentration for upcoming cytotoxicity studies.

The Pluronic F127 vehicle has also been characterized under atomic force microscopy. These preliminary results of the F127 micelle can be seen in Figure 4. This method of characterizing micelles can lead to measuring micelle size, morphology, and agglomeration.

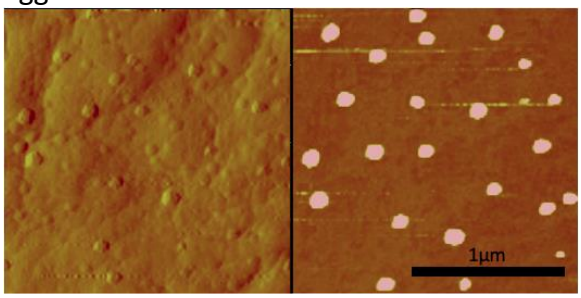

Figure 4: AFM image of the F127 micelle dried at a 1mM concentration at 37C. Micelle nanoparticles can be seen with an average diameter of 75nm.

Atomic force microscopy uses a cantilever to scan the surface of the sample, resulting in a clean image of the topographical surface on a nanometer scale. This is smaller than what the visible wavelengths of light can elicit. The average diameter of these particles is ~75nm in diameter. This is about 3x larger than what is measured hydrodynamically by DLS(~25nm). The measured samples that have been dried and are measured statically and not hydrodynamically in solution; this could lead to the larger particle size shown. These AFM results could also be showing some artifact produced by the instrument which could also be an explanation of the larger static particle size.

Another important characteristic to measure is the cytotoxicity. Like most molecules even poloxamers have a toxic cell concentration. Before assessing the more *in vivo* method of brain organoids, an appropriate *in vitro* cytotoxicity screening must be performed. In this study we perform viability testing on an L929 mouse fibroblast cell line. We selected this cell line because fibroblasts are found in connective tissue around the body and is the preferred cell line

of the MTT assay. They also have been used widely to study general cell cytotoxicity, including the impact of other different nanomaterials.

Cytotoxicity will be assessed with the MTT assay. MTT is a metabolic activity marker that produces a purple dye proportional to the number of viable cells. The purple dye can be measured spectrophotometrically to quantify the cell viability using the following equation:

$$\%Viability = \frac{Absorbance\ of\ Treatment}{Absorbance\ of\ Control} * 100$$

Once the cell viabilities have been calculated we can then evaluate the cell viability of the F127 vehicle the CMC and the UMSC. The cytotoxicity results for both Pluronic F68 and F127 can be seen in Figure 5.

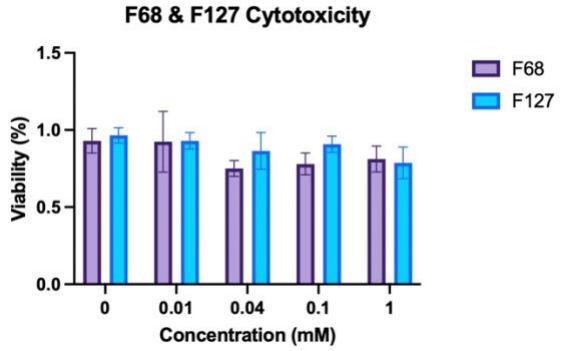

Figure 5: Cytotoxicity (%) of L929 fibroblasts cells at different Pluronic F127 & F68 concentrations.

The UMSC and CMC for F127 both fall in the range of 0.01-0.1mM. These values are very comparable to the control showing almost no cell cytotoxicity. However, when the concentration of F127 is increased past 0.5mM you start to see an effect that the Pluronic has on cell viability. At the concentration of 1mM you start to see a statistically significant effect of the F127 concentration on cell viability. But at the working concentrations at the UMSC there is not a statistical difference when compared to the control. This shows that the F127 and F68 delivery devices have a high biocompatibility making them viable for our more complex organoid studies.

4. LOADED INDIGOIDINE CHARACTERIZATION

After the development of the blank nanoencapsulation platform, I investigated a novel antioxidant uniquely studied at USU - Indigoidine (IND) and its loading effects in the F127 micelle. Indigoidine (named after its unique blue color) is a natural blue dye with antioxidant and antimicrobial properties. Its chemical structure can be seen in Figure 6.

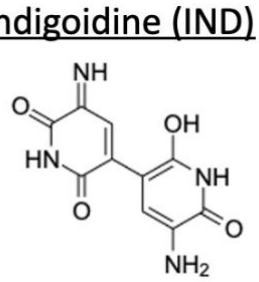

Figure 6: Chemical structure of Indigoidine, an inherently super-hydrophobic molecule and potent antioxidant.

The supply of this molecule is in very short supply due to the production being from microorganisms in culture[10]. It is a superhydrophobic molecule when initially dissolved in water. It has almost no change in its absorbance spectra compared to distilled water and settles out of solution in seconds to minutes. Indigoidine is postulated to have great antioxidative properties based on the two-pyridone ring structure with carbonyl and amino groups allowing it to reduce free radicals with a free hydrogen.[11] Increasing the bioavailability of the compound without modifying the

chemical structure, may pave the road to better understand its biological activities.

Indigoidine loaded micelles were constructed using the thin film hydration method. In short, both Pluronic and indigoidine were dissolved in a like solvent tetrahydrofuran. Enough Pluronic F127 was added to keep the concentration at the UMSC, with different ratios of indigoidine by weight. Once a homogenous solution was reached the solution was then roto-vapped to remove all the tetrahydrofuran. Once a thin film was formed, it was then hydrated in PBS and filtered to remove any unincorporated indigoidine. A table of the results for the encapsulation of indigoidine in F127 can be seen in Figure 7.

IND: Pluronic Ratio (w/w)	Encapsulation Efficiency (%)	LOADING AMOUNT (mg/ml)	Size (nm)
0.5: 1	24.78	0.045	35.7
0.4: 1	34.53	0.030	26.8
0.3: 1	55.02	0.036	25.6
0.2: 1	61.89	0.056	26.7
0.1: 1	80.24	0.025	25.9

Figure 7: Table of particle size and encapsulation efficiency of different indigoidine ratios to constant Pluronic concentrations

The encapsulation efficiencies were calculated with the following equation.

$$EE\% = \frac{Weight\ of\ IND\ in\ Micelles}{Weight\ of\ IND\ used\ in\ Preperation} * 100$$

The amount of IND in micelles was found spectrophotometrically at λ_{max} = 615nm and using a standard curve in DMSO. The size of the micelle did not change significantly with an indigoidine loaded micelle compared to the blank micelle, while the encapsulation efficiency varied widely. This shows that at the higher concentrations most of the unincorporated indigoidine was filtered out. The average loading of the amount of indigoidine was a concentration of 0.0384mg/mL or 0.155mM. This is several orders of magnitude larger than what is estimated to be for free indigoidine in water (estimated to be in the Nano-Pico Molar range). Indigoidine can be seen suspended with Pluronic F127 qualitatively in Figure 8.

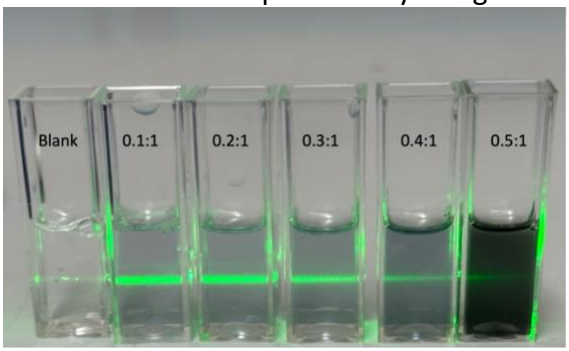

Figure 8: Tyndall effect of Indigoidine suspended at different concentrations (w/w). The laser is refracted off the encapsulated particles while it is absorbed in the highest concentration.

The Tyndall effect can be seen with a green laser shining through each cuvette with an increasing concentration. The Tyndall effect is qualitative way to measure the characteristics of a disperse or colloidal system.[12] Each system has a different capacity, when illuminated unilaterally, of scattering light in all directions. The beam of light is scattering through the lower concentrations and absorbed at the highest concentration. This enables the beam of light to become visible by illuminating tiny particles. **The solubility of IND in water has been drastically improved with the addition of F127 micelles.**

5. FUTURE WORK

During the last year, we completed several major milestones in developing methods to mitigate the damage of space radiation. This can be split into two parts.

This first is developing the baseline for radiation damage. Bailey McFarland (Another PhD student in my lab) is currently developing the baseline for this brain model and the damage caused by radiation.

Recently we have received a new Instrument, a confocal microscope. This new microscope has powerful fluorescence capabilities and is allowing us to preferentially stain certain cellular components in the brain organoid and visualize the baseline damage from radiation. He is currently gathering this data and setting the baseline for this radiation damage which has yet to be published. Because of his NASA fellowship he was able to use NASA Ames' source of gamma radiation on this batch of organoids. This allowed us to continue evaluating the damage caused to the organoids despite the problems we had ran into acquiring a source of gamma radiation on our own.

Once the data has been analyzed we plan to implement encapsulated indigoidine into the cultures and see if there is a reduction in the radiation induced damage. We are currently working with the college to get our own source of gamma radiation. In the meantime, I hope to develop and characterize more antioxidant remedies from already well-known antioxidants. Antioxidants of interest include the widely encapsulated curcumin, mesobiliverdin, and the well-known potent antioxidant vitamin-C.

I also hope to expand on our understanding of the free radical stress to the cell. The antioxidant capacity of our antioxidants have been measured by chemical means in the past and it is well known that they work. I hope to do this by introducing new methods of measuring the response of free radicals in cell culture to our lab.

In conclusion, the F127 micelle has been characterized for the delivery of hydrophobic antioxidants to the cell. With the preliminary cytotoxicity studies done we are now ready to move to understanding the biological effects of free radicals and their damage to the cell, and if our unique antioxidant indigoidine will show an inhibitory effect *in vitro*.

6. ACKNOWLEDGMENTS

I would like to thank Dr. Jixun Zhan, and his student Jie Ren for producing and isolating my supply of indigoidine. I would also like the thank Bailey McFarland and Chen Cheng for their work on establishing the baseline damage of microgravity and radiation on the brain organoid model. Also, I would like to thank Dr. Yu Huang for serving as a trusted mentor and helping to steer my research in the correct direction. And ultimately, I would like to thank the Utah Space Grant Consortium for their support and fellowship throughout this project.

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