Producing a melanin-rich topical from genes present in *Cryptococcus neoformans* Fungi to prevent cell irradiation in astronauts

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Exposure to cosmic radiation in astronauts is known to damage cells as some of the nitrogenous bases that make up DNA can get removed through ionization. This can cause an increased risk of degenerative diseases, such as cancer. A fungus discovered near the Chernobyl power plant, known as *Cryptococcus neoformans*, thrives in a highly radiative environment because it over-expresses melanin. The melanin absorbs this radiation to facilitate a process analogous to photosynthesis known as radiosynthesis. We propose producing a topical shield that is rich in the protein responsible for the fungi's survival. We will do this by exploiting the TYR gene from the *C. neoformans* for melanin production via a reconstituted cell-free system with *Escherichia coli* parts, as melanin is a pigment that produces a visible product. Through a process known as in vitro translation, we will harness TYR inside in vitro biochemical reactions. Astronauts will then be able to use the topical to protect themselves during space travel and protect their cells from the damaging effects of ionization. Our design produces melanin directly through a cell-free system using genes from the fungus instead of having to extract the protein from *C. neoformans*. This approach allows for a more efficient production of melanin that will act as a topical shield.

Keywords: *Cryptococcus neoformans*, melanin, ionizing cosmic radiation, TYR gene, fungi

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Watch a video introduction by the authors at https://youtu.be/vR3rk4KXsjw

Background

Astronauts are exposed to about 300 millisieverts of radiation per year on the International Space Station. The risk of carcinogenesis begins at about 100 mSv per year (Niiler, 2021), as the ionizing radiation can damage DNA direction and its interaction with charged particles removing nitrogenous bases. It can also cause lesions in the DNA, such as the breaking of strands, chromosome aberration, and the formation of a micronucleus (Pariset et al., 2020). These mutations cause radiation-induced cancers, the most notable being Leukemia, Carcinoma, and Sarcoma (Niiler, 2021). Although some organisms on Earth have methods to repair DNA damage, these processes are hindered by ionizing radiation, especially when mixed with the micro-gravitational environment of space. Space radiation refers to galactic cosmic rays, which consist of protons released from large solar particle events, making their energy so high that it is difficult to shield against using conventional methods, and even with the current methods used, such as with lead, or depleted thorium, astronauts, are still suffering from lasting effects and need more protection (Monreo-Villanueva et al., 2017).

Fungi discovered near Chernobyl, known as *C. neoformans*, undergo radiosynthesis by participating in energy transduction and electron transfer processes. The electrons move from one molecule to another, transfering energy to allow the fungi to withstand stress caused by ionizing radiation (Khajo et al., 2011). Though the byproducts of radiosynthesis remain unknown, research has shown that ionizing radiation changes the electronic properties of melanin. The process increases electron spin, which increases the electron transfer properties, causing the growth of the fungus. Though *C. neoformans* is a pathogen, and melanin is an indicator of its virulence (Alp,2010), we are still able to use the fungus for this product. This is what allows expressing melanin through a cell-free system to be even more beneficial as it will allow us to guarantee the purest form of melanin (Tinafar et al., 2019) using only the genes present in *C. neoformans*, not the virulent melanin itself. Using this method will ensure that we do not risk any kind of virulence or toxicity within our product that may come with extracting the melanin directly.

Studies have shown that radiosynthesis predates photosynthesis since melanin pigments are found in all biological kingdoms and are much easier to synthesize than chlorophyll pigments (Casadevall et al., 2017). There is ongoing research on how to use fungi to protect astronauts from cosmic radiation. However, most of these iterations use melanin extracted directly from the fungi (Tran, 2019).

The advantage of using a cell-free system is that it is a simple process that is unlikely to get complicated by external factors that can come with involving other organisms. It allows direct control of transcription, translation, and metabolism (Lu, 2017). Since we are able to synthesize Cryptococcus melanin through a cell-free system (Chatterjee et al., 2018), it would be in our best interest to go with the simplest option system for our design. Also, since we only have one target protein - melanin - it would not be necessary for us to use a complicated system for translation. With a simpler system and without worrying about plasmid assembly and cell transformation, we can test multiple gene sequences at once. On the other hand, using a cell-free system can also come with disadvantages, like any protein expression method. One of the main disadvantages of working with a cell-free system is that it can give a low protein yield, which could hinder the production of our product. However, when working with a virulent fungus, it would be beneficial to take a more cautious approach when trying to produce a present associated with the virulence of the organism. In addition, in order to catalyze the reaction and increase the yield as much as possible, we can add enzymes such as tyrosinase externally to our topical (MedlinePlus, 2022).

Systems level

By exploiting the gene responsible for creating the fungi's melanin, we can create a topical shield for astronauts that will reduce the effects of cell ionization. Since melanin is a naturally occurring pigment in the skin, there should not be any extreme risks with topical applications (Cleveland Clinic, 2022). However, we should still be careful and continue to monitor how the topical behaves on human skin since there is a lot about the melanin pigment and radiosynthesis that remains unknown (Dadachova & Casadevall, 2008). In addition, before we test on human skin, we will first test on *Saccharomyces cerevisiae* yeast, as those cells will mimic mammalian skin. (Parapouli et al., 2020). This topical will absorb the radiation and limit the probability of astronauts developing high-risk oncogenes or genes that can cause cancer, mutated by substances or external environments. After expressing the TYR gene from the *C. neoformans* fungi for melanin production and radiosynthesis via a cell-free system, we will be able to produce the topical rich in melanin protein. We plan on using a reconstituted cell-free system with *E. coli* parts. Our product would benefit more from a reconstituted cell-free system than a lysate because we want to focus on just achieving the melanin protein for our product in its purest form possible (Tuckey et

al., 2014). A reconstituted system will also allow for a simpler process, as we anticipate the need to repeat the synthesis several times in order to achieve a reasonable enough yield. We feel that involving a bacterial lysate (Huang et al., 2018) will be unnecessary considering the formulation of our product. The topical should replicate the same results as the fungi, where it will absorb radiation, minimizing DNA damage. We plan to overexpress the TYR gene using BioBits, a reconstituted cell-free protein expression kit (Huang et al., 2018), and apply the melanin to a petri dish containing *S. cerevisiae* yeast to observe its effects on cell growth in various radiative environments (Parapouli et al., 2020).

As shown by research done on melanized *C. neoformans*, irradiated melanin had a 4-fold increase in its ability to reduce NADH in comparison to non-irradiated melanin (Khajo et al., 2011). The XTT/MTT assays showed an increase in metabolic activity and a change in electron spin, which all caused the *C. neoformans* cells that were exposed to radiation to grow significantly faster than those which were not, as shown by higher CFUs (Khajo et al., 2011).

With typical sunscreen, experts recommend that you apply at least 1 ounce for the entire body (or 2 milligrams per square centimeter of skin) for maximum protection (Taylor & Diffey, 2002); therefore, we need at least 1 ounce of a topical highly concentrated with melanin in order to protect a person for a few hours. However, since ionizing radiation is five times as strong as UV rays (Karam, 2003), one should use at least 2 ounces of the highly concentrated topical, and they should reapply every few hours. Since there is already technology in the space station that protects against cosmic radiation (Tran, 2019), they do not need 5 ounces of the topical at a time, but should still ensure that they use an adequate amount to evenly coat the entire body before putting on other space gear. Melanin is a stable protein, especially at temperatures below 25 °C (Dolinska et al., 2020), and melanogenesis, the synthesis of melanin, is catalyzed by a variety of different enzymes that can be added to our cell-free system externally. Though it may take longer to get a large amount of the product, a reconstituted cell-free system will ensure a pure target protein, which will be more valuable to us when compared to concentration. Further, since the International Space Station (ISS) is kept at 22 °C (NASA, 2018), the topical should have no issues when stored in the ISS.

Device level

The main component of our device would be the TYR gene, which plays a major role in the production of melanin. This is because the TYR gene codes for the enzyme tyrosinase, located in melanocytes, or cells specialized for producing the melanin pigment (MedlinePlus, 2022), and it has shown promising effects when added topically as it is currently being tested in doses for vitiligo treatment using the *C. neoformans* fungus (Aruna et al. 2020). In addition, TYR gene has been previously tested in a cell-free system (He et al., 2015), and does not need anything specific to function directly. Most experiments involving the expression of the TYR gene have been successful, and the improvement that our iteration makes is that we specifically use the melanin from a virulent fungus. Even though our gene extraction site is virulent, the expressed protein will not be pathogenic.

Using a cell-free system like BioBits provides a simpler way to express the TYR gene (Lu, 2017), as opposed to cell culture. Since our final objective is to produce a topical, BioBits could also allow protein production without the need for purification from cells. We are using BioBits, not cell lysate, which is reconstituted and addresses any health issues and regulations regarding use as a topical. The qPCR allows us to analyze RNA transcription from BioBits. Since melanin is an observable protein, we can visualize expression based on the brown/black color (Chatterjee et al., 2018) it produces, as addressed in Figure 1. Concentration is not measurable; only the factor of expression is. A tube that does not change color could mean the protein did not fold correctly, DNA was not transcribed, or the RNA was not translated (Taylor et al., 2017). In this situation, we would use qPCR to sort out any issues, especially because in

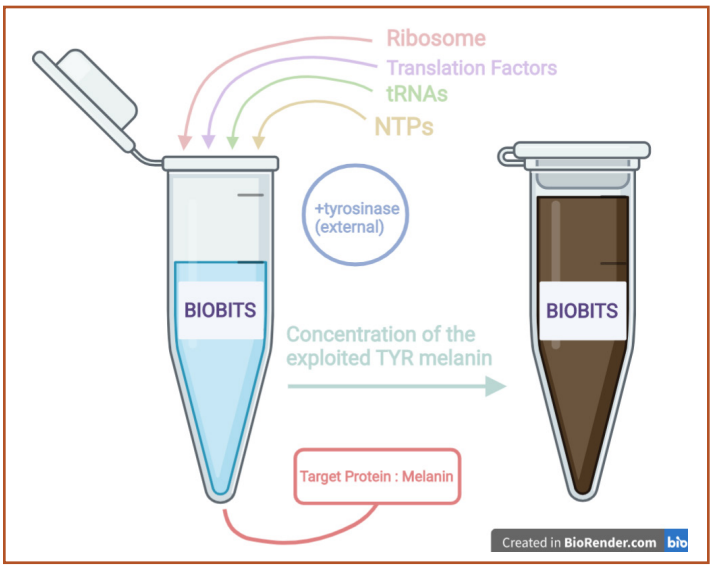

Figure 1: *Melanin is being expressed through BioBits, a cell-free protein kit. In order to achieve the quickest and purest protein product, tyrosinase is added externally. Since melanin is an observable protein, we can visualize expression based on the color it produces (Chatterjee et al., 2018). Figure created in BioRender.com.*

space, there are bound to be issues. If the tube does change color, we will focus on the protein, as the RNA has already been correctly translated. Suppose the tube did change color and the melanin is being expressed, in that case we will need to know the following aspects of the RNA: (i) how much RNA is being translated, (ii) how stable this RNA is before it is degraded, (iii) if there is a percentage of RNA not being translated, and (iv) seeing how much RNA is necessary for our desired melanin production. Considering all these aspects is necessary to produce the most effective topical solution.

Parts level

We have chosen a vector designed for *E. coli* to express the melanin protein; however, we plan to use it in a cell-free system. Therefore, we propose expressing the protein via in vitro transcription and translation (Figure 2). We have chosen to use an *E. coli* vector design for our cell-free system because it is a relatively simple form of translational bacteria for our vector (Iyer et al., 2013).

We have chosen a T7 promoter placed at the 1-17 position to drive the high-level transcription of downstream genes. It will also clone the repressor, LacI, to introduce a negative regulatory element (Sigma-Aldrich, 2022). The T7 promoter is also shown to increase transcription yield, and since our product is cell-free and requires a lot of a single protein, it is important that we have a high protein yield to make our topical truly melanin-rich. Experiments involving cell-free expression of a protein with E. coli have found that they could synthesize between 0.5 and 1 mg/ml of active protein when the cell-free system is using T7 RNA polymerase (Shin & Noireaux, 2010). While it can be said that cell-free systems produce lower amounts of protein and would therefore be much more costly in terms of production, actual extraction is more costly considering the cautions needed when dealing with a virulent fungus.

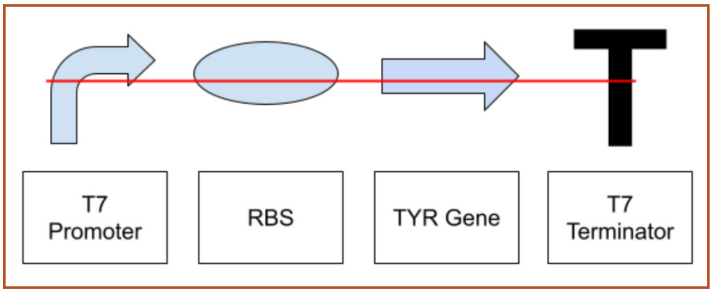

Figure 2: *The genetic parts that we will insert into our cell-free system for melanin expression. In addition, a plasmid vector would also contain ampicillin, pBR322 ori, and Rop, after the T7 terminator. The red line indicates that it is all one component.*

To initiate the protein synthesis of melanin, we have a ribosome binding site (RBS) at the 76-80 position to recruit the ribosome to the mRNA and to align the ribosome with the start codon, ultimately initiating the protein synthesis of *E. coli* from the transcription of the TYR gene. This is also when the enzyme tyrosinase will begin to convert the amino acid tyrosine into the dopaquinone compound (MedlinePlus, 2022). It will ensure the accuracy and the efficiency of translating the TYR gene into a melanin protein as it will position the genetic code correctly to properly ensure translation (ThermoFisher, n.d). Further, to increase the melanin formation and stimulate the conversion of tyrosine to dopaquinone (Slominski et al., 2011), we can add the enzyme tyrosinase externally into our topical.

The six tandem histidine tag at the 91-108 position will facilitate the purification of the tagged melanin by a nickel or cobalt matrix. It will also allow us to selectively extract the melanin protein in order to create our topical.

The T7 terminator at the 1763-1809 position will allow transcription termination of RNA transcribed by bacteriophage T7 RNA polymerase. T7 RNA polymerase is a very active enzyme, synthesizing RNA at a much faster rate than *E. coli* RNA polymerase (Tabor, 2001), it is important to have a T7 terminator that can terminate transcription just as fast as the T7 promoter can start transcription in order to prevent the transcription from circumnavigating the genetic code.

The pBR322 origin of replication in the 3261-3849 position will facilitate the replication of the TYR genetic code. It will regulate the low-copy plasmid number when the Rop protein is present and the medium-copy plasmid when Rop is absent. The Rop protein at the 4276-4467 position will result in a low and controlled copy number of the plasmid.

Safety

Suppose we plan to carry out the experiment ourselves, we will use an Ultraviolet lightbox to see the effects of non-ionizing radiation on yeast cells with the TYR topical. We will take the necessary precautions to be safe from this radiation, such as wearing a face shield, goggles, gloves, and clothes that completely cover the skin to limit exposure time (McLeod, 2019).

However, astronauts are exposed to ionizing radiation, not non-ionizing like UV Radiation. Experiments ionizing radiation poses a significant danger if not in a licensed setting, which we do not have access to, as it is a high form of energy that must be strictly controlled. Nevertheless, using non-ionizing radiation does not

affect the results of our experiment. Suppose the yeast cells died in the UV light with the TYR topical, that means that the exploited melanin cannot be an effective form of protection against ionizing radiation, and we should not move forward in our experiments.

If this experiment were done on the International Space Station, then there would not be an added risk for us to worry about because radiation is already there. If we could access t ionizing radiation on Earth, we would need to take even more precautions than using a UV lightbox. Such as maintaining a reasonable distance from the radiation source, implementing a shield between the source and the people present, and wearing lead vests, lead gloves, and lead safety goggles. (United States Department of Labor, 2004). Monitoring the concentration of radiation present and the amount of exposure time will also be necessary to ensure minimized effects on those present. (United States Department of Labor, 2004)

Additionally, a cell-free system ensures the safety of our users because using an organism can have its own set of risks when changed into a topical form, as virulence and reactions with the body remain in question. In addition, since the product is going directly on the skin and is not used as a shield on space equipment, it is vital that we do not use the *C. neoformans* directly as melanin is an indicator of its virulence.

Further, another risk comes with the fact that the byproducts of radiosynthesis remain unknown, as the discoveries about the properties of *C. neoformans* are quite recent. Questions remain on how long the protective properties will last when applied to the skin and how often the product needs to be reapplied. After testing it on non-human eukaryotic cells, we should move on to human skin cells under a "space-like" environment and observe the topical's protective properties in set increments of time. To ensure safety, consumers should patch test the product as they would with any topical to reduce the risk of any reaction with the skin.

Discussions

The increasing interest in space travel throughout the years has allowed for significant developments in the technology created to protect those exposed to space complications, yet one hazard still poses a great threat to an astronaut's health. Ionizing radiation damages DNA structure and repair abilities, increasing the likelihood of degenerative diseases.

Current experiments use the melanin isolated directly from the fungus, which is then embedded into plastic polymers. It took so long for such experiments to occur because they are expensive, costing $400 to produce a gram (Cruickshank, 2019), proving the method inefficient. Expressing melanin in a cell-free system can allow for faster synthesis and is much more inexpensive (Lu, 2017). For a genetic code of the same size, our method of expressing the gene instead would be much more worthwhile, estimated to cost $250. Additionally, extracting the melanin from the *C. neoformans* would yield more costs as the fungus is virulent, and would require more protection and, therefore, more money to handle.

Next steps

Melanin is a pigment-protein, so observation of color should also indicate expression. Multiple experimental groups with *S. cerevisiae* yeast will be placed in a dish whose lid is coated with the TYR product at different production dilutions: pure melanin product, 1:2, 1:10, and no protective coating to observe protection limits (Figure 3). This yeast allows for the closest comparable model to mammalian cells, because of their similar biological natures (Parapouli et al., 2020). Additionally, the yeast allows for stronger reactions to conditions because of its simplified unicellular structure and ability to be easily manipulated (Parapouli et al., 2020). All groups will be exposed to a similar variable: some form of radiation that can be tested for the same period of time. Expecting the non-coated group to die, the rest will die or survive, as indicated by higher CFUs (Dadachova et al., 2007). This will provide data on the effects of TYR-based melanin protection on the growth and survival rate of eukaryotic cells, using yeast as a model. This will then allow us to determine the melanin concentration needed for the cream to be the most effective.

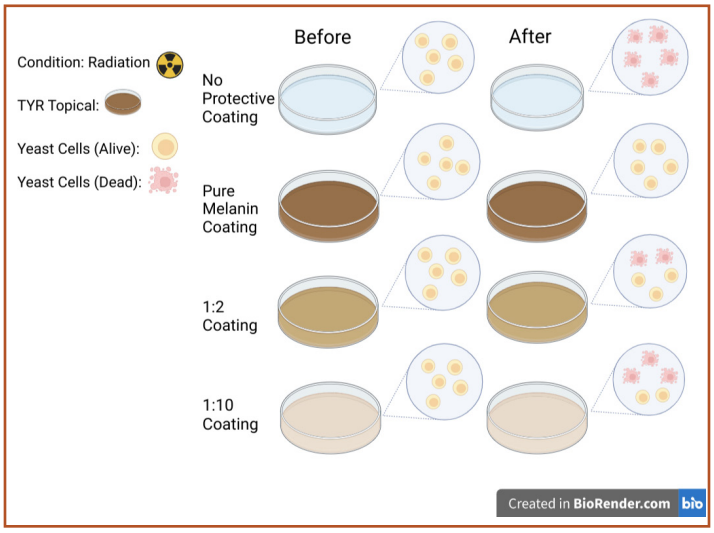

Figure 3: *TYR topical experiment on S. cerevisiae yeast at various dilutions. Figure created in BioRender.com.*

We have identified several extensions to our project. Firstly, we have thought of inserting our vector into an organism, particularly *E. coli*, since it is simple. It will allow us to observe if it is more lucrative to make it with an organism versus cell-free. Next, there are many ways we can formulate our topical. We have decided to make it purely melanin protein. However other ingredients such as different polymers or oils, could be considered to provide additional skin benefits in the harsh space environments.

It would be beneficial to continue testing this product on other organisms, such as plant life on the space station, as well as other equipment on the space station to see if it can be used as a shield on human skin and equipment. In addition, we may be able to learn more about radiosynthesis through testing. Since the process is analogous to photosynthesis, we know that chlorophyll is the pigment that allows plants to undergo photosynthesis, and melanin allows the *C. neoformans* and other radiotrophic fungi to undergo radiosynthesis. It is further known that both chlorophyll and melanin get their electrons through dissociating the water molecule; therefore, studying the properties of melanin through our system can allow us to learn more about radiosynthesis. It can also be beneficial to see if other radiotrophic fungi can be used to perform the same experiments.

Author contribution

All authors helped produce our overall concept through brainstorming. A.H, Z.H, and E.P contributed essential research regarding the functions of our variables: the behavior of the fungus and the genes responsible, the concept of radiosynthesis, the process of vitro translation with TYR, and the developments of oncogenes due to ionization. E.P created a thorough outline of our experiment and the application of our system. Z.H,E.P., and A.H, composed the manuscript, created figures, established the analysis and developed our design, and helped with the revision process. M.T helped with editing, and created the animations for our video.Z.H produced the video and the voiceover.

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