Radioprotectant Ceria Nanoparticles Drug Delivery System to Reduce Reactive Oxygen Species Levels and Mitigate Spaceflight Osteopenia

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RADIOPROTECTANT CERIA NANOPARTICLES DRUG DELIVERY SYSTEM TO REDUCE REACTIVE OXYGEN SPECIES LEVELS AND MITIGATE SPACEFLIGHT OSTEOOPENIA

by

BALAASHWIN BABU
B.S. University of Central Florida, 2020

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Sciences in the NanoScience Technology Center in the College of Graduate Studies at the University of Central Florida Orlando, Florida

Spring Term
2023

Major Professor: Sudipta Seal
ABSTRACT

In the extreme environment of space, radiation induced health issues is a major concern. The ability to induce higher levels of free radicals and reactive oxidative species (ROS) are factors to be addressed. Ceria nanoparticles (CNPs) can provide a means to scavenge ROS through regenerative redox coupling that occurs on the surface and acts as a radioprotectant. Additionally, the defect levels in CNP can be categorized by the Ce$^{3+}$/Ce$^{4+}$ ratio with a high ratio indicating superoxide scavenging, a radical species with high oxidative potential. However, engineering a specialized Ce$^{3+}$ NP often requires a harsh reducing agent.

We hypothesized a wet-chemical synthesis utilizing both reducing and non-reducing sugars to engineer stable CNPs with high Ce$^{3+}$ state. This sustainable process uses simple sugars to ensure high biocompatibility while maintaining the specific vacancy density found in Ce$^{3+}$ dominant CNP. X-Ray photoelectron spectroscopy (XPS) show that the reducing sugars allows for nearly 70/30 Ce$^{3+}$/Ce$^{4+}$ ratio compared to non-reducing sugars. This high Ce$^{3+}$/Ce$^{4+}$ ratio of CNPs can be useful in scavenging ROS in space radiation applications.

In addition, typical drug molecules utilize biopolymers which can increase ROS under the ionizing radiation environments found in space. To improve the drug viability, we hypothesized CNPs conjugated to drugs as a viable solution. Current results indicate high Ce$^{3+}$ structural confirmation of ceria nanoparticles using a wet-chemical synthesis. The synthesized nanoparticles were then conjugated with risedronate, a third-generation bisphosphonate. Microwave radiation studies indicated that CNP was able to be used as a radioprotectant as it prevented changes in drug chemistry as detected by UV/Vis spectra after exposure to microwave radiation. Finally, in vitro human mesenchymal stem cell results from alkaline phosphatase (ALP) and Alizarin red S (ARS)
assays, suggest potential for the conjugates to differentiate cells. The mechanism for radioprotective ability can be attributed to the high Ce\textsuperscript{3+} state.
This thesis is dedicated to the Babu family: Akshay, my mother and my father who inspire me to be my best every day. Also special thanks to Camryn for her love and support.
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<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EMF</td>
<td>electromagnetic field</td>
</tr>
<tr>
<td>GCR</td>
<td>galactic cosmic rays</td>
</tr>
<tr>
<td>IR</td>
<td>infrared ray</td>
</tr>
<tr>
<td>IRn</td>
<td>ionizing radiation</td>
</tr>
<tr>
<td>LFNIR</td>
<td>low frequency near infrared ray</td>
</tr>
<tr>
<td>mSv</td>
<td>milli-sievert</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (NAD) + hydrogen (H)</td>
</tr>
<tr>
<td>NIR</td>
<td>non-ionizing radiation</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SPE</td>
<td>solar particle events</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet ray</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible light</td>
</tr>
<tr>
<td>X-ray</td>
<td>x-ray</td>
</tr>
<tr>
<td>α particle</td>
<td>alpha particle</td>
</tr>
<tr>
<td>β particle</td>
<td>beta particle</td>
</tr>
<tr>
<td>γ</td>
<td>gamma rays</td>
</tr>
</tbody>
</table>
CHAPTER ONE: INTRODUCTION

Space exploration has gained new traction in recent years and an influx of private business ventures has fostered a broader interest in space flight and technology. The global space economy will drive further research and exploration as demand for space-related mining, \textit{in situ} resource utilization, colonization, and discovery are projected to increase in the future (Henderson et al., 2012). Market value for space-related exploration will undoubtedly increase in future years as technology improves to allow for advancements in space travel. The current market value for space-related ventures is valued at USD 423.8 billion and is projected to increase to USD 1 trillion by the year 2040 (Vernikos, 2008). This high market value will span across multiple industries including the health sector as space-related health and medicine will be an important component of human advancement (Denis et al., 2020). In fact, the Human Research Program, within the US National Aerospace Association’s (NASA) mission directorate program explores human space flight capabilities as it is integral to the organization’s mission to expand space exploration beyond low earth orbit (LEO) (Alwood et al., 2017). They, for instance, have grouped the health risks of a possible Mars mission into 5 categories, including distance from Earth, gravity fields, hostile/closed environments, isolation and confinement, and space radiation. As stated by NASA, one of the most significant challenges requiring considerable resources and effort will be enabling humans to survive the space environment for extended durations (Aglietti, 2020).

On Earth, humans are typically not exposed to high doses of ionizing radiation, radiation of high enough energy to induce damaging electron displacement from atoms and molecules, but this drastically changes past LEO environments. Recent data shows that
astronauts are subjected to radiation at doses of 50-2000 milli-Sievert (mSv) which is equivalent to nearly 150-6000 chest X-rays (J. C. Chancellor et al., 2014). The two most common types of ionizing space radiation include solar particle events (SPE) and galactic cosmic rays (GCR) with GCR being the most abundant and high energy form of space radiation (Onorato et al., 2020a). GCRs pose an especially high threat toward human health due to their ability to penetrate spacecraft materials as well as interacting, in some portion, with such materials leading to the evolution of harmful secondary radiation (e.g., beta particles, X-rays, gamma rays, and other waves varying in energy and frequency). This secondary radiation produced through nuclear fission reactions between GCRs and metal shielding materials can increase the radiation dose dealt by astronauts (Dobynde et al., 2021). The persistent, substantial danger from space radiation exposure will be especially pronounced when traveling in long duration space journeys and could lead to diseases such as carcinogenesis, CNS problems, acute radiation syndrome and degeneration of tissues (J. C. Chancellor et al., 2014). The specific pathway that contributes to these ailments has not yet been fully studied in space but theoretical knowledge from biological effects associated with radiotherapy patients show a parallel between the two distinct, but similar conditions. Although these factors have an unclear mechanism of action, oxidative stress and gene-level damage induced by radiation are commonly considered the basis of radiation exposure-mediated sicknesses. These conditions are also representative of the two categories for ionizing radiation effects; namely, direct and indirect, respectively.

Direct and indirect ionizing radiation effects occur through molecular absorption of radiation or through interaction with an intermediary radical oxidative species (ROS) formed from radiation (Riley, 1994). Direct effects can occur as disruption in polymeric organic structures such as nucleic acids by breaking structural bonds, single and double-strand breaks,
without any intermediary steps as well as lead to chemical modifications such as the formation of cross-links (e.g., inter/intra strand-strand, protein-strand) (Desouky et al., 2015). This process can even disrupt polymeric based microsphere drug excipients (non-active part of drug responsible for tissue absorption) leading to the production of free radicals by therapeutic agents (Blue et al., 2019). Additionally, direct lipid peroxidation can occur which can initiate a cascading chain reaction leading to further increases in free radicals (Ayala et al., 2014). Indirect ionizing radiation effect often involves radiolysis of water molecules. Generally, hydroxyl radicals are formed and quickly reduced through cellular metabolic processes to form either hydrogen peroxide or superoxide, leading to DNA damage and eventual cytotoxic effects (Riley, 1994). Further complicating the issue of space radiation damage, the production of ROS is exacerbated by both microgravity and radiation. Oxidative stress is a disruption in the oxygen metabolism mechanism that is inherent to proper cell functioning. Without proper metabolic respiratory mechanisms, the human body will not be able to fight off pathogens, will increase occurrence of cardiovascular disease (CVD), neurodegenerative diseases, and would increase ageing (Maritim et al., 2003). When the balance of oxidation mechanism to antioxidants is disrupted, oxidative stress occurs and causes conditions that result in cytotoxicity in cells. Free radicals and reactive oxidative species are often used interchangeably; the main creation of free radicals within the body is associated with the creation of radical oxygen molecules derived from molecular oxygen and water hydrolysis products (Liochev, 2013). By addressing the generation of reactive oxidative species, a proper biological solution to space radiation can be developed. Typical NASA solutions to address radiation effects calls for reduction of exposure time within the environment. However, as space exploration reaches new depths, preventing astronauts from being exposed to extraterrestrial radiation will be a lost cause. Recent trends in research reflect using radioprotectants
to mitigate biological effects from radiation exposure (Figure 1). Drugs such as amifostine can provide a viable solution to prevent the generation of oxidative species. The drug works as a radioprotectant, providing significant protection against radiation effects by scavenging reactive oxygen species (Hosseinimehr, 2007). However, logistical complications such as drug efficacy, daily dosing requirements and molecular chemical transformation from the radiation itself compounded with the toxicity create obstacles for this solution (Meerman et al., 2021). Additionally, the drug is only able to treat lower doses (0.7-6 Gy) of radiation toxicity, as determined based on results from cancer therapy applications (Crook et al., 2021).

![Figure 1: Research on radioprotectants divided by years of publications. The pie chart shows a consistent increase in publications regarding radioprotectants from 1967 to 2022. The past decade contributed to nearly half of all research in the field and the past two decades resulted in over 75% of publications. The field of radioprotectants will continue to gain interest among the extensive scientific community based on this data (publication data was retrieved from the web of science, clarivate analytics)](image)

On the other hand, nanoparticles can be highly biocompatible due to their nanoscale physical dimensions and the potential for surface modifications or surface chemical reactions conferring bio-active functionalities. Among the nanomaterial formulations shown to have substantial bioactivity, particles showing surface chemical reactivities towards ROS species
have in particular shown high efficacy across a wide range of therapeutic applications (Li et al., 2012). Moreover, nanoparticles can also work as the encapsulant for drug formulations allowing further therapeutic modes of action. For instance, nanoparticle surface modification with organic radioprotectants, such as amifostine, as a nanomedicine formulation protect the human body subjected to the space environment (Theriot et al., 2010; Trajković et al., 2007). This can in turn amplify radioprotective effects by taking advantage of surface loading, stabilizing the organic component against degradation from the biological environment, while simultaneously conferring therapeutic action from the nanomaterial component (Schweitzer et al., 2010). Recent research from Coathup et al. revealed ionizing radiation’s influence on the skeletal system with substantial bone loss being a major factor. The research was conducted within the scope of radiotherapy to mediate protection of bone tissue from radiation induced damage, a common pathology observed among cancer survivors (Fei Wei et al., 2023). Mineral elements within hard bone tissue have a substantial radio-absorptive cross-section and, thereby, are highly sensitized to radiation exposure. Therefore, it is imperative to find a viable radioprotective solution for bone health to mitigate effects from space radiation (Curi et al., 2016). Utilizing nanomaterial research to develop a solution for space radiation can also enhance our understanding of terrestrial radiation applications. Focusing on skeletal tissue components and the potential for nanomaterials to protect these vulnerable biological structures will be important for future space, given the significant exposure towards hard radiation, as well as for terrestrial applications ("Structure and Function of Bone Marrow Hemopoiesis: Mechanisms of Response to Ionizing Radiation Exposure," 2002).

Among the current nanomaterials that have both radioprotectant nature as well as skeletal system applications, ceria nanoparticles (CNPs) present as a viable solution for space applications. The ability to scavenge reactive oxidative species due to the auto-regenerative
properties of cerium oxide is enhanced due to its nanostructure \cite{Loschen2007}. This catalytic function is present within the surface of the nanomaterial and serves an important biological characteristic of CNPs. Exploration of this function as well as its relation to bone differentiation properties is important as it can uncover specific biological processes that affect astronauts in space. Previous research conferred that a higher $^{3+}$ surface site of CNPs relates to higher osteogenic differentiation and mitigation of radiation-induced bone loss \cite{Wei2023}. This modulation of the surface chemistry can either be enhanced through redox chemistry introduced from the environment that regenerates Ce$^{3+}$ oxidation states or through creating surface defects that allow for higher oxygen vacancies.

Creation of these enhanced CNPs can be accomplished through a myriad of syntheses but emphasis towards using sustainable chemicals and processes ensures high biological viability. The use of simple sugars such as glucose and fructose as a reducing agent in the synthesis of CNPs can replace harsh chemicals and complicated processes. However, the use of glucose and other sugars can possibly inhibit osteogenic differentiation \cite{Wang2019}. This is important to consider as sugar-based synthesis of CNPs could be useful in determining the specific factors that relate to increased skeletal system performance: redox. On the other hand, irradiated CNPs create surface defects that allow for the reduction of ceria ions from Ce$^{4+}$ to Ce$^{3+}$. This enables higher ROS scavenging as the material can transform highly oxidative super oxides to hydrogen peroxides \cite{Kumar2012}. Determining the surface defect nature or redox chemistry nature of CNPs relates to higher osteogenic differentiation can uncover the mechanisms that can be further evaluated for space applications.

This thesis will focus on the unique properties of ceria nanoparticles for their ability to scavenge ROS. Two approaches for creating high Ce$^{3+}$ state NPs is investigated to determine specific mechanisms responsible for osteogenic stem cell differentiation. The first approach
utilizes a sugar based synthesis that enables higher Ce$^{3+}$ % as the sugar works as a reducing agent. This created CNP will be compared to controls that do not include sugar within the synthesis. The second approach will uncover whether drug conjugation of bisphosphonates to CNPs will be resistant to microwave radiation. This study explores different methods to create drug-CNP formulations and subjugates these samples to microwave radiation to determine whether they cause toxicity to cells. Both approaches take into consideration the skeletal system applications and involve bone cell differentiation studies to investigate the potential for mitigating spaceflight osteopenia.
CHAPTER TWO: LITERATURE REVIEW

Few Sections Publisher: WIREs Nanomedicine & Nanobiotechnology 2023, [Accepted]. (Reuse Permission: Appendix A)

Space Radiation Overview

Space radiation greatly differs from radiation present on Earth, as it is largely composed of atoms in which the atomic nucleus remains (J. C. Chancellor et al., 2014). This phenomenon is observed due to the low pressure environment of space and speed of atomic species traversing at close to the speed of light, leading to the separation of significantly less dense electron clouds from the atomic system, leaving the positively charged nuclear core. The main components of space radiation particles include high-ionizing protons, electrons, and positrons (Figure 2). The high-mass, high-atomic-number, and high-energy nature of the particles pose various health risks to astronauts (Guan et al., 2004). Space radiation is composed of four kinds of ionizing radiation: Earth’s magnetic field particles, particles emitted from solar flares, galactic cosmic rays, and radiation belt particles enveloped in space around Earth1. The most harmful types of space radiation, such as solar flare particles and galactic cosmic rays, are widely prevalent in deep space. Solar flares are massive explosions that occur on the Sun’s surface and release high amounts of energy by releasing X-rays, gamma rays, and protons and electrons. Ionizing radiation induced by solar flares significantly harms astronauts regardless of their distance from the Sun (DeWitt & Benton, 2020). Galactic cosmic radiation is the most abundant radiation in space and majorly impacts astronauts and their space equipment1. Galactic cosmic rays (GCR) radiate high-energy protons and ions that originate outside the solar system but within the Milky Way galaxy1. The heavy, high-energy GCR ions are capable of easily ionizing other atoms upon interaction (Table 1). Relevant examples of such processes
can be observed as GCR ions pass through spacecraft and interact with the bodies of astronauts\(^1\). Chronic exposure to space radiation has been shown to weaken immune systems, increase viral activity, induce oxidative stress, and reduce bone mass (Dicello, 2003; Guan et al., 2004). These health deficits may potentially lead to host of human pathologies such as the development of cancer, damage to the central nervous system, and deleterious effects on musculoskeletal function.

NASA has studied the problem of radiation on biological and technological material systems for years and is considered, as an organization, to be an authority on radiation risks in continuous space flight. NASA has developed standards that all equipment intended for space operation must meet in an effort to protect astronauts. Additionally, they have conducted studies to show the effects of radiation, during a prolonged flight, will affect biological systems on board. NASA has also identified four primary space radiation risks for prolonged flight which include carcinogenesis, degenerative tissue effects, CNS decrements and acute radiation syndrome (Jeffery C Chancellor et al., 2014). Consideration of biological risks and factors are essential to the future of space exploration and a deeper understanding of radiation’s damaging effects. In anticipation for a future trip to Mars, NASA stated that astronauts will need to be protected from all potential sources of radiation more completely due to the lack of atmosphere and therefore the increased risk that radiation poses (Frazier, 2015). Knowledge of the effects from radiation on biological systems can lead a better-prepared solution to long-distance space travel.
Table 1: Summarizing the energy value, wavelength, and frequency of various radiation types including cosmic radiation. The variations in wavelength and energy value of each radiation should be considered to uncover the specific effects towards biological systems.

<table>
<thead>
<tr>
<th>Radiation Type</th>
<th>Wavelength</th>
<th>Energy Value</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactic Cosmic Rays</td>
<td>$10^{-14}$ m to $10^{-12}$ m</td>
<td>$10^{14}$ eV to $10^{20}$ eV</td>
<td>$300 \times 10^{22}$ Hz to $300 \times 10^{20}$ Hz</td>
</tr>
<tr>
<td>Solar Flare Particles</td>
<td><em>Wide Range</em></td>
<td>30 MeV to $10^{13}$ erg</td>
<td><em>Wide Range</em></td>
</tr>
<tr>
<td>Gamma Rays</td>
<td>$10^{-12}$ m to $10^{-10}$ m</td>
<td>$10^{8}$ eV to $10^{14}$ eV</td>
<td>$300 \times 10^{20}$ Hz to $300 \times 10^{18}$ Hz</td>
</tr>
<tr>
<td>X-Rays</td>
<td>$10^{-10}$ m to $10^{-9}$ m</td>
<td>$10^{8}$ eV to $10^{9}$ eV</td>
<td>$300 \times 10^{18}$ Hz to $300 \times 10^{17}$ Hz</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>$320 \times 10^{-9}$m to 400 x $10^{-9}$ m</td>
<td>3.1 eV to $10^{3}$ eV</td>
<td>0.94 x $10^{17}$ Hz to 0.75 x $10^{17}$ Hz</td>
</tr>
<tr>
<td>Infrared Light</td>
<td>$750 \times 10^{-9}$ m to $10^{-8}$ m</td>
<td>0.01 eV to 1.8 eV</td>
<td>0.40 x $10^{15}$ Hz to 300 x $10^{11}$ Hz</td>
</tr>
<tr>
<td>Microwaves</td>
<td>$10^{-3}$ m to 1 m</td>
<td>$10^{-3}$ eV</td>
<td>900 Hz to 2450 MHz</td>
</tr>
<tr>
<td>Radio Waves</td>
<td>$10^{-2}$ m to $10^{-1}$ m</td>
<td>$&lt;2 \times 10^{-24}$ J</td>
<td>30 GHz to 300 kHz</td>
</tr>
<tr>
<td>Alpha Particles</td>
<td>0.05 to 0.11 m</td>
<td>28.8 MeV</td>
<td><em>Wide Range</em></td>
</tr>
</tbody>
</table>

(Ahmed et al., 2020; Church, 1997; Day, 1955; Hall-Craggs, 1989; Har-Kedar & Bleeheen, 1976; Sankaran & Ehsani, 2014)

**Solar Flare Particle Radiation**

Solar flare events (SPE) emit various electromagnetic radiation particles such as X-rays, gamma rays, high-energy protons and electrons, alpha particles, helium, and minimal quantities of atoms with heavy nuclei (Foukal, 2008; Grieder, 2010; Haigh, 2003). SPE occurs when accumulated energy is released from active solar regions’ magnetic fields and characterized by the release of highly accelerated protons from the Sun (Chen et al., 2020). Radiation particles from solar flares have energies greater than 30 mega-electron volts (MeV) and during large solar flares, the energy of the particles released can be about $10^32$ erg (Foukal,
2008). More importantly, SPE doses inside astronauts’ spaceships can vary from 100 mGy/h to 500 mGy/h. On average, solar flares vary in duration from about 20 minutes to 3 hours (Grieder, 2010) (Table 1).

SPE radiation greatly affects the outermost tissues and especially the skin. *In vivo* studies have demonstrated that high doses of solar flare radiation can stimulate skin ulceration, keratinocyte necrosis, pigment incontinence, and damage in skin pigmentation (Wilson et al., 2011). Other studies indicated that skin lesions, hematological, and immunological dysfunctions were prevalent amongst groups exposed to comparable doses of SPE radiation as astronauts. Prolonged exposure to solar flare radiation can induce physiological damage and inhibit cancer and endogenous protective mechanisms (Hrushesky et al., 2011). Additionally, SPE radiation can cause disruptions in genes, increase immunologic stress, facilitate cervical epithelial cellular changes, induce tissue degeneration, inhibit DNA repair functions, and increase the incidence of infection and malignancy (Davis Jr & Lowell, 2006; Hrushesky et al., 2011).

Numerous studies have shown that SPE radiation can negatively affect pulmonary and cardiovascular function by increasing heart rate variability, inducing alterations in blood flow, and increasing systolic and diastolic blood pressure (Babayev & Allahverdiyeva, 2007; Dimitrova et al., 2004; Maghrabi & Maghrabi, 2020). During periods of high solar flares, an increased incidence of epileptic seizures, coronary disease, and myocardial infarctions were observed in exposed groups (Babayev & Allahverdiyeva, 2007; Dimitrova et al., 2004; Maghrabi & Maghrabi, 2020; Palmer et al., 2006; Vencloviene et al., 2013). *In vivo* studies showed that animals exposed to high doses of simulated solar flare radiation developed mycoplasmal pneumonia and radiation pneumonitis. Further studies suggested that delayed
effects of solar flare radiation exposure include immune dysfunction and dysregulation. Several studies have also found a positive correlation between solar flare radiation exposure and neurological system diseases (Mulligan & Persinger, 2012; Rapoport et al., 1998). Recent studies have even suggested that increased exposure to SPE may contribute to the development of mental and neurodegenerative diseases including schizophrenia, Alzheimer’s Disease, and multiple sclerosis (Lõhmus, 2018). Furthermore, SPE radiation can mediate chromosomal aberrations, affecting critical hormone production processes, causing alterations in melatonin levels, and affecting many other regulatory mechanisms (Halpern et al., 1995; Stoupel et al., 2006). Exposure to SPE radiation can also facilitate the accumulation of reactive oxygen species, damage DNA signalling pathways and stimulate inflammatory processes (Sridharan et al., 2015).

**Galactic Cosmic Rays**

Galactic Cosmic Rays (GCR) are made of alpha particles, protons, and ions with high atomic numbers that have been separated from their electrons (Onorato et al., 2020b). 85% of GCRs is composed of hydrogen nuclei or protons, while 13% of GCRs is composed of helium nuclei (Nelson, 2016). The nuclei in GCRs are accelerated to very high speeds and originate outside our solar system (Onorato et al., 2020b). GCRs pose a greater risk to biological functions than gamma rays and x-rays given that GCRs greater penetrative ability, being less significantly absorbed and attenuated by interaction with physical barriers (Table 1). Therefore, GCRs are capable of penetrating through very deep layers of organic and inorganic materials, including biological tissue (Onorato et al., 2020b). The high-energy particles radiated from GCR are thereby capable of inducing significant cellular DNA damage, since no shielding is effective in inhibiting their penetration into cells (Tucker et al., 2004; Wilson et al., 2000).
Secondary radiation presents an additional concern as radiation emitted following the absorption of primary radiation in exterior substances can still result in significant damage to more interior structures (Raman & Krishnan, 1928). Further, the forms of secondary radiation, triggered by GCRs, still possess significant penetrative potential and can accumulate more efficiently in biological tissues, as compared to primary GCRs (Slaba et al., 2017). GCR secondary radiation particles include beta particles, alpha particles, gamma rays, X-rays, neutrons, protons, and other particles with greater charge and mass (Mishra et al., 2018).

In vivo studies on low energy radiation from GCR observed stimulation of ovarian tumor formation in rat models (Mishra et al., 2018). Additionally, GCR’s high penetration capabilities make it possible for them to compromise central nervous system (CNS) structures and function. Neurons are extremely susceptible to the damaging effects of GCRs, often triggering apoptosis. This is because GCR particles can perturb synaptic structure and functions, stimulating neuroinflammation (Parihar et al., 2016). Thus, long-time exposure to GCR has the potential to compromise neural systems such as those related to hippocampus and prefrontal cortex functions. Furthermore, GCR can interfere with gene expression processes and protein phosphorylation mechanisms (Jeffery C Chancellor et al., 2014). When studying astronauts exposed to different levels of GCR, researchers found that the group exposed to higher doses of GCR had twice the amount of chromosome breaks (Maalouf et al., 2011). Another study found that astronauts habituated in the International Space Station (ISS) had intricate alterations in chromosomal positions (Cucinotta et al., 2011). Overall, this research indicates that galactic cosmic rays compromise genome stability and can induce chromosomal aberrations which could lead to chronic, detrimental biological processes and resulting conditions.
GCR penetrates through spacecraft metallic shielding which NASA employs for protection for its astronauts and is forecasted to be a severe biological problem in prolonged journey through space (Jeffery C Chancellor et al., 2014). This is in stark contrast to SPEs which can be blocked through effective aluminium shielding, already present in most modules sent to space (Parsons & Townsend, 2000). Solutions such as thicker shielding to counteract high doses of GCR’s don’t translate to space applications as the additional mass can prohibit successful space launch. Material science advancements such as metal hydrides and composite materials successfully reduce radiation penetration, though do not completely preclude penetration (Naito et al., 2020). Additionally, a major facet of material design towards mitigation of GCR’s, as with any form of heavy charged radiation particles, is the consequences of additional secondary radiation dosage originating from spacecraft shielding. This process, referred to as spallation, occurs when heavy charged particles fragment into numerous smaller radiation particles. The cascading particle shower can be even more biologically detrimental than the original GCR particles, as noted above.

The prospects of utilizing NPs to function as a radioprotectant against GCR has been closely evaluated in recent years. Extensive studies have found that nanomaterials synthesized with hydrogen show significantly enhanced radiation-shielding properties against GCRs and SPEs (Thibeault et al., 2015). Comprehensive studies conducted under simulated space-like conditions have also shown that single-walled carbon nanotubes are particularly effective in functioning as a radioprotectant under GCR ionizing space conditions.
Figure 2: The effects and extent of space radiation past LEOs and within spacecraft. The figure shows a) how both GCRs and SPEs have little impact on terrestrial and low earth orbit areas in space. This is due to Earth’s magnetic field that consists of both the Outer Belt and Inner Belt. Collectively, they are known as the Van Allen Belts. Current satellite structures including the ISS travel within the Van Allen Belts which mitigates the overall exposure to cosmic radiation. As space travel inevitably goes past the low earth orbit for long periods of time, the protection dissipates. Under these extreme conditions b) the spallation of GCRs on to spacecraft shielding is a point of concern. This illustration shows a very real possibility for astronauts traversing in this region where a GCR particle impacting the metal shielding of the spacecraft causes immediate spallation into smaller charged particles. The heavier radiation particles found in GCR can knock out smaller radiation particles creating an additional secondary radiation dosage. The resulting cascading particle shower occurring in the spacecraft is a major source of ionizing radiation and is more lethal than the original GCRs.

Skeletal System Overview

When discussing the effects of space on biological systems of human beings, the skeletal system is an important facet. In fact, the effects of microgravity alone cause hindered bone metabolism with a 1-2% decline in bone density loss each month in space (Genah et al., 2021). Space radiation can exacerbate these effects by activating bone resorption mechanisms which will increase with longer and more distant space travel (Willey et al., 2011). Furthermore, evidence of bone minerals absorbing radiation presents an important aspect to consider for human health in deep space travel. Effects from such absorption process can be explained by the greater radiation absorption cross-section of larger nuclei inorganic components and consequent hyperemia and activation of osteoclastic activity, detailed in later
discussion, (Yan et al., 2022). This effect will be a key component to understanding space radiation effects on biological systems, as well as an important point of consideration for radioprotectant design, and thus will be the focus of this review.

The skeletal system in the human body consists of cellular portion and non-cellular components. The cellular portion consists of three different cells that work in conjunction to regulate and maintain the skeletal system(Florencio-Silva et al., 2015). These three cell types are: osteoblasts, osteoclasts, and osteocytes(Gal et al., 2000). Additionally, the bone marrow produces a substantial amount of immune cells, especially CD4+ cells, help to defend the body from foreign substances through the release of cytokines(Luckheeram et al., 2012). These cells are essential to the adaptive immune response: coordinating recruitment and activation of additional immune cells such as macrophages, B cells, and CD8 lymphocytes(Lim et al., 2013). Osteoblasts function in the creation of hard bone tissue through the deposition of bone matrix as well as regulation of the bone resorption rate(Mohamed, 2008). In addition, osteoblasts cells can differentiate further leading to highly specialized osteoblasts specific to unique areas of the body(Ayukawa et al., 2009; Rutkovskiy et al., 2016). Osteoblasts produce bone by secreting collagen 1, a factor extremely important for the mineralization of several of the components for the bone creation process(Mizuno et al., 2000). The secretion of collagen 1 forms osteoids, a molecular composition used as the base binding in the creation of bones(Jaffe, 1935). The osteoblasts also cause calcium and other minerals to precipitate from the blood, which bond with the osteoids and creates the basis of bone tissue(Blair et al., 2011). Osteocytes can then induce the formation of bone tissue in necessary tissue sites through the process of bone remodelling. (Caetano-Lopes et al., 2007). Osteoclasts, on the other hand, perform antagonistic functions to the osteoblasts(Teitelbaum, 2007). They function to degrade bone tissue and reabsorb the minerals permitting bone re-modelling and/or dispersion into blood, re-
circulation (Blair, 1998). This process is accomplished through osteoclast localization to a particular bone site and the formation of an overlayer, with the interfacing bone region (Marino et al., 2014). They then absorb the calcified matrices within the covered region, allowing recycling of the minerals to be re-deposited into bone by the osteoblasts. In addition to resorption of bone, osteoclasts have newly discovered uses in the immunological aspects of the bone health and the monitoring of bone for harmful defects (BOYCE et al., 2007; McDonald et al., 2021). Osteocytes are mature bone cells that serve the purpose of recruiting osteoclasts to bone sites identified for de-mineralization, effecting the dynamic nature of the bone cycle (Del Fattore & Teti, 2012). Skeletal system in homeostasis are thereby characterized by equal rates of bone resorption and formation (Delgado-Calle & Bellido, 2015).

It is also imperative to investigate the composition of non-cellular parts of skeletal tissues to truly understand the impact of space radiation on bones. In fact, the bone matrix is composed of 60% mineral compared to 30% organic matrix, 8% water and 2% lipids. These values are averages over the collective bones of the skeletal system, with bone mineral content dependent on physical and structural factors; with the exception of cartilage-based tissues which contain higher fractions of organic matrix composition (Clarke, 2008). The inorganic component consists of mostly crystalline hydroxyapatite: \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \). However, impurities such as carbonate, potassium, magnesium, strontium, sodium, chloride and fluoride often substitute for pure phase components within the hydroxyapatite crystal. The organic component consists of nearly 90% of collagenous and only 10% of non-collagenous proteins (Feng, 2009). Although the specific composition of the various types of collagenous proteins depends on the bone type, type 1 collagen constitutes a dominant fraction. This collagen protein is structured in a triple-helix pattern with 3 different polypeptide chains (Tzaphlidou, 2008). This tertiary structure is characteristic for the different collagen...
types; with types I, II and III forming the fibril structures necessary for tissue rigidity. Minor fibrillar collagen includes types V and XI which play key roles in limiting the diameter of fibrils (Tzaphlidou, 2005). However, in terms of biological function, the 30 non-collagenous proteins found in the organic matrix play a significant role in the regulation between osteoclasts and osteoblasts (Landis, 1995). It is noteworthy that for bone tissues, lipid peroxidation and water hydrolysis will be quantifiably less significant in radiation effect compared to other processes. Therefore, radioprotective measures for bone will necessarily

While the dynamic nature of bone formation and coordinated action of component cells maintain healthy function in homeostatic and mild pathologic conditions, this system can become dysregulated under severe or chronic adverse stimulation, leading to negative impacts in the body. The most common dysfunction of skeletal cells involves osteoblast cells failing to maintain reabsorption and recycling rates of bone minerals (General, 2004). Osteopenia occurs when the reabsorption of bone is too fast for the reproduction of bones by the osteoblasts (Khosla & Melton III, 2007). Osteoporosis is similar to osteopenia, but more directly due to age and the slowing down of osteoblasts (Karaguzel & Holick, 2010). Both diseases cause bones to become brittle and susceptible to fracture under moderate loading; in severe cases, even from a sneeze or sudden movement. The onset of osteoporosis is very common in post-menopausal women due to a decrease in estrogen (Rachner et al., 2011). These diseases also occur commonly at increased rate among ISS astronauts due to their exposure to space environment including weightlessness and ionizing radiation (Ozdemir et al., 2009).
Reactive Oxygen Species Under Irradiation

Reactive oxygen species are naturally occurring species in the body, formed as a side product of oxygen metabolism in mitochondria (Figure 3). Further, these species contribute to regulatory process, with evolution of ROS species functioning as cell signalling molecules (Oest et al., 2015). However, abnormal generation of these species, due to internal (e.g., tumor site) or external (e.g., radiation absorption, radiolysis) factors, are damaging, producing an oxidative stress on cellular systems and structures (Cao et al., 2019). ROS evolution is especially dangerous to tissue function as these species can diffuse over characteristic distances and produce chemical modifications to biomolecules as indirect effects from a local radiation absorption event. Such effects include DNA damage (as chemical modifications including oxidation and base hydrolysis), cell neuropathy, and permanent nerve damage (Storz & Imlay, 1999). Ionizing radiation also has effects on endogenous antioxidant systems, such as expression of superoxide dismutase (SOD) (Leach et al., 2001). Down-regulation of antioxidant protein expression have been observed following radiation insult in vitro as well as concomitant increases in ROS production rate. Un-regulated or poorly regulated ROS generation then quickly imposes an oxidative stress causing cell death, DNA damage, and nervous tissue damage along with inhibition of osteoblast in the production of bone (Scott et al., 1989).

Additionally, oxidative stress has a very specific impact on the skeletal system. In a study done with 48 women and 53 men, increased oxidative stress was directly linked to a negative association between 8-iso-PGF2α, a common biomarker for oxidative stress, and bone mineral density. 8-Iso-PGF2α is produced when arachidonic acid is catalyzed by free radicals. The sensitivity and specificity of 8-Iso-PGF2α leads it to be one of the best ways to predict levels of oxidative stress “in-vivo” (Zheng et al., 2021). This leads to the conclusion that increased
oxidative stress is directly linked to a decrease in bone volume and bone density within the skeletal system. The increased oxidative stress resulting from exposure to ionizing radiation, leads to a similar result and therefore decreases bone density as well (Josson et al., 2006). In a separate study done on rats which had increased oxidized stress levels, the oxidative stress was even found to cause bone tumors as well as tumors around the body at a much more accelerated rate than compared with a control rat group. This effect has been seen in some retro studies done on humans, showing that radiation exposure may cause oxidative stress, leading to the more ready development of tumours on the affected areas (Kondo et al., 2010).

In addition, ionizing radiation triggers endogenous signals that facilitate oxidative damage to DNA, lipids, proteins, and other biomolecules. Ionizing radiation also reacts with water molecules in the body to form H₂O⁺ and free electrons. Ionizing radiation also results in the formation of secondary ROS including superoxide’s and hydrogen peroxides (Singh & Singh, 1982). Further reactions with these products yield more toxic ROS molecules such as the hydroxyl radical, peroxynitrite anion, nitrogen dioxide, and dinitrogen trioxide (Chiu et al., 1993; Darley-umsar et al., 1992). Furthermore, IR stimulates lipid peroxidation, which results in an increased membrane permeability, interferences with membrane proteins, and disturbances in ion gradients (Corre et al., 2010; Ebneth et al., 1998). Cumulative effects of IRn exposure include the slowing of mitosis, impairment to protein signalling networks as well as eventual membrane rupture (Singh & Singh, 1982).

Radiation-Induced Changes to cell and Skeletal System Tissue Function

Ionizing radiation can potentially induce hyperemia of skeletal tissues and eventual osteoradionecrosis through impaired bone circulation. While this phenomenon is rare in clinical settings, the high exposure to an ionizing radiation environment on a consistent basis
in space might be able to trigger this pathology course (Figure 2). As the ionizing radiation constricts vascularization around skeletal tissue to support other organs (hyperemia), osteonecrosis develops and creates interstitial edema in bone marrow. Within the bone marrow, there are extremely radiosensitive hematopoietic progenitor cells (HPCs) and self-renewing hematopoietic cells (HSCs). HSCs function to lay the foundation in hematopoiesis to generate lineages including erythrocytes, leukocytes, and platelets. HPCs are the more mature version of these cells. Both of these cells are used as a function for hematopoietic cell transplantation, which is used to treat hematologic disorders (Diegeler & Hellweg, 2017). These cells have a rapid turnover and therefore are extremely impacted by the exposure to ionizing radiation that comes into the cells. Exposure to ionizing radiation for these cells causes the stem cells to undergo cell death, establishing a cell block and producing a loss of clonogenic function, severely damaging the immune system and its capability to provide defence for the body ("Structure and Function of Bone Marrow Hemopoiesis: Mechanisms of Response to Ionizing Radiation Exposure," 2002).

Fat deposits replace hematopoietic cells in the marrow which further cause structural damage to bones. Additionally, the onset of bone marrow syndrome can begin to be noticed in the body. Progressive lymphopenia develops very early on and lymphocytes in the blood begin to depress. The lymphocyte impairment severely limits the body’s ability to resist infections. Other symptoms include granulocytosis and possible granulocytopenia which eventually culminates in death due to sepsis unless the apoptosis state of bone marrow cannot be reversed (Basu et al., 2001; "Structure and Function of Bone Marrow Hemopoiesis: Mechanisms of Response to Ionizing Radiation Exposure," 2002). Other damages to the bone can be seen through the damage of very fragile bone marrow cells, directly effecting its ability
to produce and maintain the immunological cells that it had been producing previously (Basu et al., 2001; Wauquier et al., 2009).

Figure 3. The schematic diagram shows how ionizing radiation can lead to osteoporosis in bones. The example displays the pelvic bone which undergoes osteoporosis within the tissue and cellular level. The cellular structure of skeletal cells undergoing radiation induced osteoporosis shows ROS affecting the osteoblast and osteoclast balance. The osteoblast cells will undergo apoptosis under ROS while a higher level of osteoclasts proliferate. This induces more destruction of the bones leading to osteoporosis from the cellular level. Ionizing radiation creates dysfunction within the skeletal system cells which ultimately affects the mineralization of the pelvic bone. Osteoporotic skeletal tissues start to degrade from their original matrix and ultimately lead to structural fractures. An astronaut exposed to the ionizing radiation originating from space will have to deal with this pathological issue unless a solution is developed.

**Ceria Nanoparticle as Radioprotectant Overview**

*Cerium oxide nanoparticles* (CNPs) have seen wide-spread application in research studies within the biomedical field due to their unique surface chemistry, the complex nature of the nanomaterial surface in aqueous environment, and the breadth of available synthesis
approaches. The unique properties of this rare earth metal oxide are due to the low energy difference between reduced Ce$^{3+}$ and oxidized Ce$^{4+}$ states which arises from the near-degeneracy of 5p and 4d electron states and shielding by the 4f orbitals (Bouzigues et al., 2011). However, unlike other elements within the lanthanide series, cerium exists in both the $^{3+}$ and $^{4+}$ state within the metal oxide crystal. The material maintains charge balance through the formation of oxygen vacancies, with reduced states localizing to vacancy sites. For nanoscale formulations, these defects are found at the particle surface and at greater densities as compared to those observed at bulk scale, due to the increased surface to volume ratio. Further, these sites are comparatively more active, as compared to bulk scale materials, due to the crystallite nanoscale dimensions and the greater specific surface area exhibiting surface oxygen vacancies (Tsunekawa et al., 1999).
Table 2 Various forms of ceria nanoparticles used as radioprotectants against different forms of radiation

<table>
<thead>
<tr>
<th>Nanoparticle and Functionalization</th>
<th>Objective</th>
<th>Rays Tested</th>
<th>Dose</th>
<th>Experimental System</th>
<th>Notable Results</th>
<th>Ref</th>
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<tbody>
<tr>
<td>Alendronate–polyethylene glycol (AL–PEG) was grafted onto the surfaces of ceria nanoparticles (CNPs)</td>
<td>Elevate superoxide dismutase 2 (SOD2) protein expression while maintaining cell viability under radiation environment using PEGylated CNP</td>
<td>$^{60}$Co gamma rays</td>
<td>0-20 Gy at a rate of 41.4 Gy/h.</td>
<td>Human normal liver cells (L-02)</td>
<td>PEGylated CNPs were compared to naked or weakly wrapped CNPs and were found to significantly decrease the toxicity that was found through naked CNPs as well as make a much more efficient radiation protectant for the body.</td>
<td>(Li et al., 2015)</td>
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<tr>
<td>Non-agglomerated CNPs in the range of 2-5 nm synthesized through a microemulsion method</td>
<td>Differentiate radioprotective properties of CNP between a tumor cell line and anormal cell line</td>
<td>X-Rays</td>
<td>0-10 Gy</td>
<td>Normal breast epithelial cells (CRL-8798) and breast carcinoma cells (MCF-7)</td>
<td>Radiation dosage on the naked cells killed almost 40-50 percent of them. However, with the usage of the CNPs in the well, the statistically significant protection was 100%. There was an almost 100% reduction and protection from radiation produced cell apoptosis.</td>
<td>(Roy W. Tarnuzzer et al., 2005)</td>
</tr>
<tr>
<td>CNPs synthesized through the microemulsion method</td>
<td>Determine whether the presence of CNPs can protect cells exposed to radiation and alter caspase 3/7 activity compared with control cells.</td>
<td>X-Rays</td>
<td>0, 5, 10, 15, 20, 25, 30 Gy</td>
<td>Lung fibroblast cells (L929)</td>
<td>CNPs were shown to significantly reduce radiation-induced cell death in the lung fibroblast cells.</td>
<td>(Ribeiro et al., 2020)</td>
</tr>
<tr>
<td>CNPs synthesized through the microemulsion method</td>
<td>Combat radiation-induced damage in murine models using CNP as a radioprotectant</td>
<td>X-rays</td>
<td>5 Gy per week for 30 Gy total</td>
<td>Non-tumor-bearing athymic nude mice</td>
<td>Lungs from the control group showed visible pneumonitis with macrophage invasion while the mice receiving CNPs showed no pneumonitis and appeared normal. This is due to the high concentration of oxygen vacancies and associated</td>
<td>(Colon et al., 2009)</td>
</tr>
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<td>3-5nm CNPs synthesized using the microemulsion process</td>
<td>Evaluate the functionality of CNPs to decrease radiation-induced dermatitis and xerostomia in head and neck region of mice.</td>
<td>IC160 X-Ray</td>
<td>0, 12.5, 15, 17.5, 20 Gy at a rate of 2.74 Gy/second</td>
<td>Female athymic nude mice</td>
<td>A statistically significant difference in radiation induced apoptotic death in acinar cells was discovered favoring the CNP group over the control group. An additional study showed no adverse effect of the CNP to the acinar cell.</td>
<td>(Madero-Visbal et al., 2012)</td>
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<tr>
<td>10-30 nm CNP</td>
<td>Deliver proper dosage of radiation to kill cancer cells while sparing healthy cells using CNP</td>
<td>X-Ray at 6MeV</td>
<td>18 Gy</td>
<td>Healthy adult male Sprague-Dawley rats</td>
<td>CNPs were found to be perfectly viable in the body of the rat. Additionally, CNPs were found to reduce some pathological damages of lung radiation.</td>
<td>(Kadivar et al., 2020)</td>
</tr>
<tr>
<td>Cerium Oxide nanopowder</td>
<td>Assess whether using CNPs will have selective radioprotective ability</td>
<td>6 MV photon beams</td>
<td>0, 10, 40, 100 cGy</td>
<td>Human fibroblast lung cells (MRC-5) and cancerous epithelial breast cells (MCF-7)</td>
<td>The mean cell viability for the ceria treated cells was found to be almost 15 percent higher than those left untreated. The use of CNPs as well as similar-function compounds can reduce the probability of deterministic and stochastic damages after exposure of ionization radiation.</td>
<td>(Nouraddin Abdi Goushbolagh et al., 2018)</td>
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<td>CNP with radius of 10nm was created using a precipitation-based synthesis</td>
<td>Determine whether testicular protection through the transfer of CNP in the body was found to help protect cells from direct ionizing radiation damage.</td>
<td>X-ray</td>
<td>0, 2.5, 5, 10 Gy</td>
<td>8-Weeks old C57BL/6J male mice</td>
<td>No cytotoxicity was observed due to CNPs in the body and being absorbed by the liver. The groups administered CNPs show more normal rates of spermatogenesis. CNPs were also measured to reduce direct cell damage including DNA damage.</td>
<td>(Das et al., 2018)</td>
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<td>50nm CNP dispersed in fetal bovine serum</td>
<td>Protect skeletal muscle cells against ROS originating from the space environment</td>
<td>Unspecified cosmic radiation</td>
<td>Unspecified dosage</td>
<td>Proliferating mouse skeletal muscle cells</td>
<td>It appeared that cosmic radiation had a reverse effect compared to microgravity effects at the transcriptional level. Due to insufficient cellular intake of CNP in space environment, the protective effects were not determined</td>
<td>(Genchi et al., 2021)</td>
</tr>
<tr>
<td>CNPs of 35nm size and 60% Ce³⁺ conformation using wet chemical synthesis</td>
<td>Determine effect of CNPs against ionizing radiation created damage in human bone marrow mesenchymal stromal cells</td>
<td>X-rays</td>
<td>7 Gy</td>
<td>Human bone marrow-derived mesenchymal stromal cells (hBMSCs)</td>
<td>CNP at a concentration of 1ug/mL increased cell autophagy, osteogenesis, and bone matrix deposition At concentrations 10ug/mL DNA damage further decreased 4-fold, bone matrix deposition increased 5-fold and cell autophagy by 3.5-fold.</td>
<td>(Wei et al., 2021)</td>
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<tr>
<td>3-5nm CNP with 60% Ce³⁺ conformation and 5-7nm CNP with 20% Ce³⁺ conformation</td>
<td>Determine whether CNP with higher trivalent surface conformation can mitigate ionizing radiation-induced loss in bone density and structure</td>
<td>X-Rays</td>
<td>7 Gy at 24 hours</td>
<td>Human bone marrow-derived mesenchymal stromal cells (hBMSCs)</td>
<td>Higher trivalent CNP confers higher ROS scavenging properties that enable protection of rats from radiation-induced DNA damage. Additionally, CNP worked to regulate osteoclast formation and improved osteogenesis by promoting proliferation of bone progenitor cells.</td>
<td>(F. Wei et al., 2023)</td>
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</table>
In fact, the vacancy oxygen vacancies (OV’s) allow for complex redox processes involving biomedically relevant chemical substrates. Specifically, these redox reactions occur upon substrate binding at vacancy sites, redox cycling of neighboring cerium sites, and the reformation of the vacancy (Künnewent et al., 2019). This ability to form/heal OV’s have been used most commonly in research literature to effect the scavenging of harmful ROS such as singlet oxygen, radical hydroxyl groups, and hydrogen peroxide. This ROS scavenging is catalytic and has been described in literature as *nanozyme* activity, due to the similarity between related surface reactions and those of native enzymes (Table 3).

One study found that CNPs could scavenge ROS, functioning as an exogenous antioxidant, and inhibit the effects of ischemic stroke, the second highest cause of death in the world(Kim et al., 2012). Broader implementation of CNPs in various formulations have highlighted the generalizability of this antioxidant property to the reduction of excess, pathological ROS levels across different cell types and disease state model systems. A separate study found that CNPs possess superoxide dismutase (SOD) mimetic activity, functioning as an analogue of the endogenous antioxidant enzyme(Hirst et al., 2009). The observed SOD activity was found to correlate with the surface Ce³⁺ state density at the CNP surface, with formulations possessing lower populations of reduced state (often described in literature as having lower Ce³⁺/Ce⁴⁺ ratio values) being less SOD active. Interestingly, it has similarly been found that formulations with lower reduced state density demonstrate higher catalase-mimetic (CAT) activity. Along with the inverse relationship demonstrated between reduced state densities and unique enzyme-mimetic character, these reactions have opposing bio-activities. In particular, SOD-active particles are often demonstrated to produce a more antioxidant nature, while CAT-active formulations are often noted
to produce more pro-oxidant character (Wang et al., 2017). While ROS scavenging reactions have been the most thoroughly studied and implemented, surface reactions between ceria and other biomolecules have been demonstrated such as oxidation of catechols, lipids, and organophosphates. This wider range of biochemical activities has allowed CNPs of varied formulations, to be used in wound healing, cancer and anti‐aging research applications (Thakur et al., 2019). In addition to these, a significant body of literature exists for the effects of various CNP formulations on components of the skeletal system in different conditions. These studies consider, in particular, the ROS scavenging character of specific CNP formulations as well as their interaction with hard bone.

Some of the earliest implementations of CNPs into bone therapeutic activities were as particle components in composite implant materials. CNP-modified Bioglass (particulate glass composition of SiO\(_2\), CaO, Na\(_2\)O, and P\(_2\)O\(_5\)) scaffolds were used to promote the growth and differentiation of human bone marrow‐derived mesenchymal stem cells (hBMSCs). The study demonstrates that the scaffold showed upregulation of OCN, ALP and COL-1 protein expression, which are associated with osteogenesis induction. Additionally, in vivo results on rat cranial structures indicated that the scaffolds increased collagen deposition and bone regeneration through osteoblast formation (Lu et al., 2019). In another study, cytocompatibility and cell proliferation of rat BMSCs correlated to the Ce\(^{3+}/\)Ce\(^{4+}\) ratio of CNPs embedded into titanium alloy structure for implants. Specifically, the lower Ce\(^{3+}/\)Ce\(^{4+}\) ratio of the studied formulations induced higher osteointegration of the implant, greater osteogenic differentiation, and increased regulation of the M2 phenotype of RAW264.7 murine macrophages (Li et al., 2018). Like the previous study, CNP was not directly embedded within the cells but was used to coat the titanium alloy, a common
element used in bone implants. The study suggested that lower Ce\textsuperscript{3+}/Ce\textsuperscript{4+} ratio containing particles counteract hydrogen peroxide created by leukocyte cells as this ratio is related to higher catalase-mimetic activity (Prestat & Thierry, 2021). A study conducted to determine CNPs role in precursor osteoblasts was accomplished without embedding with other materials. The naked NPs were incorporated into MC3T3-E1 osteogenic precursor cells where it was revealed that CeO\textsubscript{2} has a specific role in activating the Wnt pathway through nuclear translocation of β-catenin (Luo et al., 2022). While this study was accomplished in vitro, the conclusion that CNPs activate Wnt pathways is important as osteoblast differentiation can mitigate osteopenia (Zanotti et al., 2008).

In considering the use of CNPs for radioprotectant applications in space, background effects from the physical environment should ideally be understood first.

Nanomaterials that have experienced the space environment gives much more valuable information towards their efficacy in this harsh setting. CNPs can be considered within this rare group, having had a formulation sent on board the ISS to test effects on cell viability and proliferation in C2C12 mouse skeletal cells. Cell internalization results indicated limited cellular uptake, with poor interaction between CNPs and cell surface sites mediating internalization. This issue can be attributed to the active vs. passive internalization mechanism of cells which is known to be altered in microgravity conditions. Additionally, CNP-treated cells displayed fewer differentially expressed genes that did not correlate with corresponding gene ontology of enrichment (Genchi et al., 2021). While these initial results were inconclusive towards the beneficial effects of the nanoparticle in a space environment, it should be noted that the Ucp2 gene was deemed important in the cell response. Another study focused on the effects of microgravity on planarian worms. This experiment utilized a gravity simulator and discovered that CNPs were
able mitigate the growth delays for the worms that is usually associated under reduced gravity environment. This is most likely due to the oxidative stress that this environment results in and the nanoparticle’s role in mitigating this effect (Salvetti et al., 2021). While this experiment does not consider space radiation, the role of oxidative stress in the space environment should be considered in developing therapeutic solutions. It should be noted that while the application of CNP in space requires more in depth study to determine potential as a viable therapeutic, CNP synthesis has already been satisfactorily been performed on the ISS. Using a controlled-precipitation process, ceria was synthesized with high crystallinity and a nano-rod structure (Soykal et al., 2015). Biocompatibility studies were not performed, however, demonstration of a space-synthesized sample provides further support for the potential of CNPs in space-related applications.

Most studies on the radioprotectant ability of CNPs do not focus on space radiation but rather cell protection during chemotherapy applications. The primary focus is in regard to protecting skeletal system cells and tissues in both in vitro and in vivo experimental designs. This was the focus for a study that proposed CNPs to mitigate cellular damage in hBMSCs exposed to ionizing radiation therapy. The nanoparticle treatment reduced cellular aging associated with radiation and improved autophagy of cells through increased p53 expression. Skeletal system associated improvements included high bone matrix deposition, osteogenesis, and osteogenic differentiation that can be attributed to the high ROS scavenging properties of the CNPs. This material behaviour further supported cell health through reduction in DNA fragmentation (Wei et al., 2021). Another study from the same group tested a CNP formulation with a Ce$_{3+}$-dominant surface on rats exposed to sublethal doses of ionizing radiation. The same cellular senescence and autophagy factors were detected in vitro and in vivo due to the protective ability of CNP against
DNA fragmentation. Additionally, osteoclast activity levels that were previously associated with the effects of radiation were reduced, allowing for healthy osteogenesis under these conditions (Fei Wei et al., 2023). While further research can unveil specific mechanisms responsible for the observed bone differentiation properties, CNP formulations currently show significant promise as a radioprotective material for skeletal cells and tissues specific to the skeletal system.

Other research regarding CNPs radioprotective ability provides initial insight into specific mechanisms that are responsible for complete observed biological effects. A study using a mixed matrix membrane of CNPs and polysulfone exhibited resistance to radiation and overall longevity of the membrane component. The observed effect was determined to be due in part to the autocatalytic behaviour of CNPs which produced free radical scavenging (Bedar et al., 2019). Another study investigated the *in vivo* effects of CNPs in protecting germ cells of mice from x-ray irradiation. CNP treatment via tail vein injection showed a 13% decrease in tissue damage at 5 Gy radiation ascribed to the combined effects from radioprotection by direct and indirect effects from the ionizing radiation (Das et al., 2018). Additionally, CNPs have shown a concomitant increase in expression of superoxide dismutase 2 (SOD2) and related endogenous antioxidant species in a similar study. Interestingly, these studies highlight the value in pre-treating test animals with the CNPs, prior to irradiation. This consideration corroborates the potentiation of relevant gene product expressions which contribute to the total, conferred radioprotection. In this way, CNPs administered in these studies, and potentially for future application, provide a prophylactic and incidental exposure protection. In normal-type colon cells treated with CNPs, expression of SOD2 increased even under 2.74Gy/sec and shows potential for radiation protection of cells (Colon et al., 2010). It should also be noted that CNPs are well-tolerated by test animals within in vivo studies,
with no significant morbidity or mortality attributable to the nanoparticle treatment. Researchers used these nanoparticles to counteract radiation-induced pneumonitis during exposure of radiation (18Gy) (Colon et al., 2009). Another study investigated the efficacy of CNPs in eliciting a cytoprotective effect towards nanoparticle a pneumonitis model. While independent from the skeletal system, this pathology is associated with ionizing radiation exposure and may be of additional importance for space-related radiation health.

As a peripheral, though related consideration, several studies have investigated the radioprotective effects of CNP formulations towards cancer and healthy cell lines based on dosing parameters. Under 10 Gy irradiation cerium oxide nanoparticles displayed protection against normal breast line cells but not to MCF-7 human breast tumor line cells (R. W. Tarnuzzer et al., 2005). The authors noted a significant trend in cell type specific sensitivity to irradiation based on the dosing character. By increasing radiation from 100 to 500 cGy, a significant difference in radioprotective ability was determined against MRC-5. While the nanomaterial showed protective effects towards normal cell type at all doses, MCF-7 cancer cells saw a dose-dependent decrease in cell viability with increasing radiation doses (N. Abdi Goushbolagh et al., 2018). Popova et al. (Popova et al., 2020) released a report that took biomedical use of ceria nanoparticles a step further by encapsulating particles in multi-layered microcapsules: adding a time-release component to the therapeutic character.
CHAPTER THREE: DIRECT SYNTHESIS OF CERIUM OXIDE NANOPARTICLES IN REDUCING SUGARS

Introduction

Sustainable practices involve social, economic, and environmental applications working in harmony to create solutions that allow a better world for the future generation. Without incorporation of all three elements, a sustainable solution can’t be conceived 1. However, recent trends in research have enabled increasing the social and environmental applications of various topics within the science field. The field of nanotechnology has not been ignorant of this trend but rather allowed for full immersion of high-performance applications while retaining concern for the environment, health and economy 2. Green synthesis of nanoparticles is one way of utilizing sustainable practices in nanotechnology. This method relies on using nontoxic precursors and mild reaction conditions to create safe but also waste reduced nanoparticles. There are several approaches to this synthesis method with biological pathways gaining interest in recent years. This approach utilizes living organisms as a sort of nanoparticle factory by incorporating reduction enzymes. These enzymes can create specific nanoparticles through a bottom-up approach. However, pathogenicity issues as well as scale up obstacles such as large microorganism culture maintenance deem biological pathways as still rudimentary in its practicality 3.

Chemical pathways of generating nanoparticles often involve using harsh chemicals but this does not necessarily have to be the case. In fact, wet chemical synthesis of nanomaterials is an easy and highly manipulative route for nanoparticle creation. The principle behind this method involves using an elemental precursor and through a reducing agent, the ions generated from the precursor will come together to create nanostructures 4. The reducing agent in most wet-chemical
synthesis is often a harmful agent such as sodium borohydride. However, the need to develop less toxic reducing agents is an interesting scope to induce sustainable practices within nanotechnology. Recent research has investigated using light roasted coffee as a non-toxic solution for a reducing agent 5. This method provides a sustainable solution that invokes social, economic and environmental implications to create nanoparticles for the use of disinfectants. Nonetheless, an even more sustainable reducing agent can be developed with the utilization of a reducing sugar such as glucose.

Sugars are carbohydrates and are the most common biological macromolecule. They are used as energy storage and release, from building other macromolecules such as nucleotides to cell communication. In fact, many drugs are formulated from carbohydrates due to its high biocompatibility within cells 6. Sugars can be divided into reducing and non-reducing sugars which can help determine the chemical effect of the carbohydrate. Reducing sugars like glucose and fructose can be considered reducing agents which can convert metallic and non-metallic ions into structural compounds. In terms of structure, all monosaccharides and certain disaccharides are reducing sugars as they do not contain a glycosidic bond between anomeric carbons 7.

Glucose is not only the most common reducing sugar but also the most common carbohydrate. This allows for its application in nanotechnology to not only be economical but also accessible 8. Additionally, the low toxicity of glucose as a chemical reagent helps to address its environmental impact and provides a unique solution for creating nanoparticles that addresses the three pillars of sustainability. Other reducing sugars such as fructose and galactose share these same sustainable reagent characteristics and allow for this overarching category of reducing sugars to be useful 9.
As an element, cerium oxide (also known as ceria) is most often used in catalytic converters due to its amazing ability to either absorb or release oxygen based on the characteristic of the harmful emission. It can essentially turn environmentally detrimental carbon monoxide and nitrogen oxide into carbon dioxide and nitrogen gas respectively (23). However, ceria can be easily structured into a nanoparticle and this nanoparticle has wide applications for biomedical use. The increased surface to volume ratio can allow for high catalytic activity without interrupting cellular processes by having a smaller size (24). In fact, the vacancy oxygen (VO) defect of ceria nanoparticle allows for this particle to enhance redox processes. Th VO defect refers to the ability of a nanoparticle to accept oxygen to replace missing atoms in the crystal lattice (25). This ability to accept oxygen atoms can also be used to scavenge reactive oxidative species such as singlet oxygen, radical hydroxyl groups, and even hydrogen peroxide. This ability to be a very effective ROS scavenger has allowed ceria nanoparticles to be used in wound healing, cancer and anti-aging purposes (26). Popova et al. (16) released a report that took biomedical use of ceria nanoparticles a step further by encapsulating them in multilayered microcapsules. The microcapsule aids in releasing the ceria nanoparticle within the cell. Ultimately this nanoparticle containing structure has the purpose of acting as a radioprotectant by scavenging intracellular ROS. This nanoparticle layered capsule could potentially be used to protect humans against radiation in space.

The potential of utilizing reducing sugars for nanotechnology are endless for the creation of various nanoparticles. These structures have a multitude of applications from conductive ink to biomedical applications.
Materials and Methods Section

Materials

Materials included Dextran, Glucose, Sucrose, Galactose, Fructose, Cyclodextrin, Ammonia Hydroxide, Cerium Nitrate Hexahydrate,

Material Synthesis

Sustainable cerium oxide nanoparticles were created under room temperature conditions. Synthesis began with 3.387g of glucose added to 20mL of water under 1020 rpm stirring for 15 minutes. Additional 10mL of DI water was added and solution was kept stirring for 15 minutes. 9.77g of cerium precursor (cerium nitrate hexahydrate) dissolved in 20mL of DI water was added to the solution using a drip-by-drip process. The solution kept stirring for 2 hours. The pH of the solution was then recorded and 4mL of ammonia was added to reach physiological pH of 7.5. The solution was then stirred for 30 minutes to induce growth of nanoparticles. 20mL of the solution was then added to cellulose dialysis tubing (MW 3500) and put under dialysis process with DI water for 1 day. The DI water was exchanged at hours 1, 4 and 12. The collected solution was kept in a falcon tube under room temperature conditions.
**Structural Characterization of Sugar-CNP**

Synthesized solutions were characterized using UV-Visible and surface zeta potential characterization. FLUOSlab Omega (BMG Labtech) 96 well plate reader measured UV-Visible spectra between 250nm and 700nm. Peaks for Ce$^{3+}$ and Ce$^{4+}$ surface oxidation state were observed at TEM (Transmission Electron Microscopy) imaging was taken using TECNAI TEM. Images were processed and analyzed for nanoparticle size measurement and crystal diffraction pattern using ImageJ. XPS (X-ray photoelectron) measurements were taken using ESCALAB-250Xi spectrometer under ultra-high vacuum chamber (below 1.5 x 10^-9 mbar). An Al-Kα monochromatic radiation source was operated at 300 W (15kV, 20mA) power. C1s peak at 284.6 eV ± 0.2 eV was used to calibrate binding energies. Thermofisher Avantage software was used to identify and deconvolute chemical functional groups. Origin software was used to graph created plots to highlight various peaks.

**Reducing Sugar Potential Characterization**

Various sugars were tested for their reducing sugar capability using both a Benedict’s Reagent test and a 3-5 dinitrosalylic test (DNS) reagent test. The Benedict’s test was used to determine qualitative results to visibly show which sugars were reducing sugars. The protocol included using 25g of anhydrous sodium carbonate, 43.25g of sodium citrate and 4.325g of copper (II) sulfate pentahydrate mixed and filled to 250mL using DI water. 5mM concentrations of various sugars were created at 1mL volume and added to to each clean test tube. 2mL of Benedict’s reagent was also added to the test tube and the mixed solution was heated in 100C boiling water bath for
5 minutes. The test tubes were removed and the observed color change was compared to the control.

DNS reagent test was created using the same 5mM concentration of sugar in DI water at a range of 0mL to 3mL in different test tubes and all test tube volume was made to 3mL using DI water. The DNS solution was created by combing 1.5g of the precursor solution in 30mL of 2M sodium hydroxide. Additionally, 45g of sodium potassium tartrate was mixed in 75mL of water. Both the DNS solution and the diluted sodium potassium tartrate was mixed to 150mL using water. 1 mL of the created solution was added to each test tube and the mixed solution was heated in 100C boiling water bath for 5 minutes. The tubes were cooled to room temperature and the absorbance was measured at 540nm, using a microplate reader (FLUOstar Omega, BMG LABTECH).

**Antioxidative Functional Characterization of Sugar-CNP**

The antioxidative potential of Sugar-CNP samples were performed to measure SOD (superoxide dismutase) mimetic activity. The SOD mimetic activity was quantitatively determined using a SOD assay kit (Sigma Aldrich, Kit #19160-1KTF). Using a 96 well plate reader FloustarOmega (BMG Labtech), absorbance was recorded for 20 minutes duration to determine SOD inhibition rate using a kinetic method. Aq-CNP was used as a positive control in the SOD mimetic assay.

**Cell Culture**

Bone marrow-derived mesenchymal stem cells (MSCs, ATCC PCS-500-012™) were cultured in DMEM (ATCC) containing 10% of fetal bovine serum (FBS, ATCC) and 1%
penicillin/streptomycin (ATCC) at 37 °C using a humidified/5% CO2 incubator. After confluency the cells were collected and seeded in 96-well plates for MTT and Live/Dead assays and in 48-well plates for ALP and ARS experiments. The samples were diluted in cell culture medium and added to the wells containing cells. For ALP and ARS, the cell culture was additionally performed in osteogenic medium, prepared by incorporating 50 µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Merck), 20 mM β-glycerophosphate disodium salt hydrate (Merck) and 10 nM dexamethasone-water soluble (Merck) in the cell culture medium described above. The culture media containing samples were changed every 3-4 days. All cell studies were performed in triplicate.

**Cell Proliferation**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, brand) assay was performed after 1 and 3 days of MSCs culture. After each time point, a 5 mg mL⁻¹ MTT solution was poured on top of the wells and the plate was incubated for 4 h at 37 °C. After this time, the medium was removed and dimethyl sulfoxide (DMSO, Sigma Aldrich) was used to dissolve the purple formazan crystals formed after the reduction of MTT by metabolically active cells. The plate was incubated with DMSO for 20 min at 37 °C, and subsequently placed in a shaker (Stuart SSL4 see-saw rocker) for 10 min at 30 osc min⁻¹. Afterward, the absorbance was measured at 570 nm, using a microplate reader (FLUOstar® Omega, BMG LABTECH).

Live/Dead assay was also performed after 1 and 3 days of MSCs culture. The medium was removed, the cells were washed with PBS and the stock solution (from LIVE/DEADTM viability/cytotoxicity kit for mammalian cells, Thermo Fisher Scientific) diluted two times in PBS
was added. The plate was incubated at 37 °C for 30 min, and the cells were imaged in a fluorescent microscope (Nikon TS2-LS).

**Cell Differentiation**

Alkaline phosphatase (ALP) assay (ab83369, Abcam) was performed after 14 days of MSCs culture. After this time, the cells were washed with cold PBS and ALP assay buffer was added to each well. After incubation for 1 h on ice, the solutions on the wells were homogenized with pipetting and transferred to 2 mL flasks. The solutions were additionally homogenized for 30s using a motorized tissue grinder (Fisher Scientific). Afterward, the flasks were centrifugated at 4 oC at 4,400 rpm for 15 min and proceed to ALP activity quantification following the manufacturer’s instructions. The absorbance was measured at 405 nm on a microplate reader (FLUOstar® Omega, BMG LABTECH).

Alkaline phosphatase staining was also performed after 14 days of MSCs culture, using a commercial kit (ab284936, Abcam). The media was removed from the cultured cell wells, and the cells were washed with the buffer provided in the kit. The alkaline phosphatase staining reagent solution was added in the wells and the plate was incubate for 30 min at 37 °C. The samples were washed to times with buffer and imaged using a light microscope (Nikon TS2-LS).

Alizarin red S (ARS) staining and quantification was performed after 28 days of MSCs culture. After this time, the cells were fixed with 4% formaldehyde for 30 min at room temperature, and then washed two times with PBS. The cells were stained with 2% alizarin red S (Sigma Aldrich) for 60 min at room temperature, protected from light. After staining, the samples were washed two times with deionized water and imaged using a light microscope (Nikon TS2-LS). For
calcium deposition quantification, the dye was extracted from the stained monolayer of cells using a 0.5 N HCl containing 5% sodium dodecyl sulfate (SDS, Sigma Aldrich) solution for 30 min. After this time, the samples were placed in a 96-well plate, diluted to times with the HCl/SDS solution, and the absorbance was measured at 415 nm on a microplate reader (FLUOstar® Omega, BMG LABTECH)
Results

Physical Studies of Nanomaterials

TEM

Figure 3: TEM characterization of samples. The figure is involved in determining size and shape morphology of various sugar based CNP samples and comparing it with a control, (a) 7.5 pH CNP control; (b) Glucose-CNP; (c) Fructose-CNP; (d) Galactose-CNP; (e) Dextran-CNP; (f) Cyclodextrin-CNP; (g) Sucrose-CNP. The corresponding table shows the average size of each samples determined using ImageJ software. The various sugar based samples all show relatively small size in nanoparticles ranging from 3-5nm. Additionally, the standard deviation reveals the monodispersity of the samples. The control-CNP is quite different than the produced samples and imaging results show amorphous particles that are much larger than the 3-5nm range.

Various sugar reduced cerium oxide nanoparticles (Sugar-CNP) were synthesized using the facile wet chemical process detailed above. TEM was used to determine particle size of ranges as this information can determine successful production of nanomaterials. Figure 3 shows the resulting images and clear fringe patterns are visible in all cells. The control cell showcases amorphous structure and quite larger sized material compared to what is seen among the other sugar-based nanoparticles. The glucose-CNP and fructose-CNP samples are relatively smaller than
the other sugar-based samples as their average size is 3.83nm and 3.23nm, respectively. The other non-monosaccharide based CNPs show sizes larger than 4nm. More specifically, dextran-CNP exhibits average size of 4.48nm while cyclodextrin-CNP and sucrose-CNP showcase sizes of 4.54nm and 4.05nm respectively. The standard deviation percentage is less than 20% for all sugar based nanoparticles. The control CNP has a very high standard deviation at 55.
Figure 4: XPS characterization of Ce3d of various sugar samples. Deconvoluting peaks of Ce3d of various control and sugar CNP samples including (a) 7.5 pH CNP control; (b) 4.5 pH CNP control; (c) 6.5 pH CNP control; (d) 8.5 pH CNP control; (e) 9.5 pH CNP control; (f) Glucose-CNP; (g) Fructose-CNP; (h) Galactose-CNP; (i) Dextran-CNP; (j) Cyclodextrin-CNP; (k) Sucrose-CNP. The corresponding table shows the peak area ratio of Ce3+ higher oxidation state within various sugar-CNP formulations. The fitted Ce3d spectrum shows mixed oxidation state with both Ce3+ and Ce4+ indicated in the deconvoluted peaks. However, reducing sugar based CNPs show a more dominant Ce3+ peak compared to non-reducing sugar based CNP like cyclodextrin-CNP. The exception to this analysis is sucrose-CNP which shows a high 60.3% Ce3+ percentage. The highest Ce3+ percentage is found with fructose-CNP and the lowest is found within cyclodextrin-CNP. Results indicate that overall, the reducing sugar formulations have higher Ce3+ state than their non-reducing sugar-CNP counterpart.
XPS data on the Ce3d elemental peaks can verify the Ce\(^{3+}\) dominant conformation state of the sugar enhanced ceria nanoparticles. Cyclodextrin-CNP showed a Ce\(^{3+}\) state of 38%. This can be evidenced by the formation of the relatively high Ce\(^{4+}\) peak at 915 eV. Fructose enhanced CNP on the other hand showed a relatively high Ce\(^{3+}\) state of 70.9%. This can be evidenced by the nearly nonexistent Ce\(^{4+}\) peak at 914 eV. Galactose-CNP and Glucose-CNP show relatively high Ce\(^{3+}\) than the non-reducing sugar CNP at 68.8% and 65.1% of Ce\(^{3+}\) state (Table S1). Sucrose is a non-reducing sugar and shows lower Ce\(^{3+}\) percentage than the reducing sugar-CNP counterparts but still shows Ce\(^{3+}\) dominant conformation.

XPS data on the various pH formulations of CNP created without the addition of any kind of sugar was tested and analyzed to determine the effect of pH on the Ce\(^{3+}\) state conformation. More specifically, it was used to determine whether changing the CNP to a 7.5 pH would enhance Ce\(^{3+}\) conformation over the addition of the sugars. At relatively lower pHs of 4.5 and 6.5, there was relatively low Ce\(^{3+}\) state and was only measured to be about 28.4% and 33.1% respectively. At higher pHs of 8.5 and 9.5, there were low Ce\(^{3+}\) percentages at about 30.7% and 36.2% respectively. At the physiological pH of 7.5 which was also the pH calibrated for the sugar-enhanced CNP synthesis resulted in higher Ce\(^{3+}\) state of 43.7% compared to the other pH synthesized CNP controls. However, this formulation also was did not show dominance in the Ce\(^{3+}\) state as the percentage was lower than 50%.
UV/Vis

Figure 5: UV/Vis characterization of control-CNP compared to glucose-CNP. The Glucose-CNP sample was compared to various control CNP which did not include any sugar enhancement. It was also compared against the cerium nitrate which is a precursor to the Ce ions. Two dominant peaks were observed at 260nm and 310nm which indicates Ce\(^{3+}\) and Ce\(^{4+}\) oxidation states respectively. The glucose-CNP showed a more dominant Ce\(^{3+}\) formation as the peak is substantially higher at 260nm compared to 310nm. The cerium nitrate peak was shown to compare the lower pH samples to indicate that these samples contained high levels of the precursor and most likely did not translate to CNP.

UV/Vis data was used to confirm the Ce\(^{3+}\) dominant nature of reducing sugar enhanced CNP. It was also used to show how the sugar-CNP compares to the other CNP-controls at various pH. The two peaks that were displayed within the samples show Ce\(^{3+}\) state at 260nm and Ce\(^{4+}\) state at 310nm. Results showed that the lower pH synthesized CNP controls had a relatively similar peak formation to the cerium nitrate precursor formulation. Additionally, at pH of 7.5 and above, the CNP-control showed a dominant peak at 310nm indicating the formation of Ce\(^{4+}\). There is no indication of a Ce\(^{3+}\) state from this analysis for those control-CNP samples due to the low intensity peak at 260nm. The glucose-CNP formulation showed both a peak at both 260nm and a peak at 310nm. However, the intensity of the peak at 260nm was much higher than the peak found at 310nm which indicates the formation of a higher Ce\(^{3+}\) nature within the sample.
Reducing Sugar Potential Characterization

Benedict’s Reagent Assay

Figure 6: Benedict’s Reagent test assay. Benedict’s Reagent test was analyzed to determine qualitatively the presence of a reducing sugar or not. The indication of a color change was determined was determined with a (+) and no color change was indicated with a (−). This result showed that reducing sugars such as glucose and fructose showed intense color change in just water. This color change persisted with the formulated CNP that showed there was reducing sugar present in both glucose-CNP and fructose-CNP. Cyclodextrin on the other hand is not a reducing sugar and showed no color change when formulated in water and with CNP. Sucrose showed a different result with the sugar by itself showing no color change and indicating that sucrose is not a reducing sugar. However, with the formulation of the CNP, the results showed that there is presence of a reducing sugar through the qualitative result of the color change.

The Benedicts test is a qualitative test that can determine whether an unknown sugar is a reducing sugar or not. It uses the reaction of copper ions with free aldehyde or ketone groups to change color as the precipitate of cuprous oxide is brick-red in color. This test was used qualitatively where detection of a color change indicated presence of a reducing sugar. Sucrose and cyclodextrin in a water solution are considered non-reducing sugars and the results confirmed this theory as there was no change in the benedict’s solution. In contrast, both fructose, galactose and glucose (reducing sugars) showed substantial color change. In fructose only, the color change resulted in a deep red shift. In glucose only, the change in color was not as prevalent as it changed to a green color. However, due to the qualitative nature of the test, any color change determines the presence of reducing sugars. The presence of a green color change in the dextran sugar, also
indicates the reducing sugar properties of this sugar. However, the color change is not as prevalent as glucose, galactose or fructose sugars. These results were compared to the relevant sugar-based synthesis CNPs. There were marked reductions in the color change as the color change was more blue-green than a complete color change to red. As typical, the cyclodextrin-CNP did not result in any visible color change. Additionally, the reducing sugars resulted in color-shift to blue-green. Glucose-CNP turned into a lime-green color indicating higher reducing potential in comparison to fructose-CNP and galactose-CNP. Dextran-CNP continued the green color change in contrast to the other reducing sugars. Overall, the addition of the CNP did not change the outcome of the reducing sugar samples. However, sucrose with CNP showed changes in color under Benedict’s test. This was in stark contrast to what was determined from sucrose by itself. Sucrose-CNP turned into a blue-green color that is similar to what was seen with the fructose-CNP and galactose-CNP.
Functional Characterization

SOD Assay

Figure 7: SOD assay kit of various samples. SOD assay test was used to determine the superoxide scavenging ability of the various samples including a) 7.5 pH CNP control; (b) 4.5 pH CNP control; (c) 6.5 pH CNP control; (d) 8.5 pH CNP control; (e) 9.5 pH CNP control; (f) Glucose-CNP; (g) Fructose-CNP; (h) Galactose-CNP; (i) Dextran-CNP; (j) Cyclodextrin-CNP; (k) Sucrose-CNP. Each sample had 3 different concentrations tested of 1mM, 0.5mM and 0.25mM. All three SOD inhibition rates were analyzed with a higher inhibition percentage relating to higher SOD enzyme-mimetic properties. The table and graph (f) was provided to show the difference in SOD inhibition rate between the concentration and sample type of the various formulations.
Superoxide dismutase (SOD) catalytic activity assay was used to determine the superoxide scavenging ability of the various sugar-CNP formulations. This test relied on the quickly produced superoxide reaction getting scavenged by the samples at various concentrations. The test was analyzed through a 96 well-plate reader to detect the colorimetric that resulted in quantitative data. In the samples that did not contain sugars, the changes in pH were used to determine whether changes in pH were responsible for changes in SOD scavenging ability. It can be observed that 7.5pH-CNPs resulted in higher SOD scavenging ability. This was observed in all concentrations. It should be noted that in these control-CNPs, the higher concentration (1mM) samples were able to scavenge superoxide more than the lower concentrations (.5mM and .25mM). For the sugar-based samples, the reducing sugar-CNPs showed higher SOD inhibition percentages at 1mM than the non-reducing sugar-CNPs. Fructose-CNP shows the highest SOD inhibition rate at 98.3% at 1mM concentration. Additionally, higher concentration (1mM) of all samples resulted in higher SOD activity than the lower concentration (0.25mM) samples. Throughout all concentrations, the cyclodextrin-CNP formulation shows the least SOD inhibition rate which indicates its poor superoxide scavenging ability. Sucrose-CNP shows above 90% SOD inhibition rate even though sucrose is a non-reducing sugar.
Cell Studies

MTT Cell Viability Testing

![MTT cellular viability testing of sugar-CNPs on mesenchymal stem cells (MSCs).](image)

Figure 8: MTT cellular viability testing of sugar-CNPs on mesenchymal stem cells (MSCs). MTT cellular viability testing was conducted on mesenchymal stem cells (MSCs) using both a 10ug/µL sample and a 50ug/µL sample for each sugar-CNP solution. The images are taken after 3 day exposure to various nanoparticle solution. A bright field, a live fluorescence and dead fluorescence was taken of each sample and compared to the control cells.
Results from the MTT cell viability study is useful to determine the toxicity of the various samples. The results from both day 1 and day 2 were compared to a control cell sample that was not exposed to any solution except for the fresh media. Day 1 results indicate high cell viability in all sugar-CNP samples and concentration as they are above 80%. Likewise, day 2 results also show high cell viability above 80% in all samples and concentrations. Overall, the higher concentration (50ug/mL) samples showcase a higher cell viability than the lower concentration (10ug/mL). Additionally, the day 2 results indicate higher cell viability than what is observed in day 1 for both the control-CNPs as well as the sucrose-CNPs. This significant increase in cell viability is not indicated in the other sugar-CNPs samples. The control-CNP samples show significant cell viability compared to the control cells in day 1. This trend does not continue into day 2. The control CNPs at the 7.5pH within day 1 indicate the highest cell viability at 10ug/mL concentration (Figure S1).
Figure 9: ALP bone differentiation assay of sugar-CNPs. ALP assay was conducted using human mesenchymal stem cells in both 10ug/mL and 50ug/mL concentrations. Additionally, comparisons were made between osteogenic and normal media. The images were taken after 14 day exposure to various sugar-CNPs.
Alkaline phosphotase (ALP) activity was investigated to determine the bone stem cell differentiation properties of the created CNPs. ALP assays stain the phosphotase enzyme with a blue hue. ALP imaging and quantification show that the osteogenic medium resulted in higher enzymatic activity compared to the normal medium. This is clearly seen within the control cells as the ALP activity doubled with the introduction of osteogenic medium. Overall, there is substantial ALP activity with the formulations at 10μg/mL concentration compared to the higher 50μg/mL concentration. Within the normal media, a trend is observed where the highest ALP activity within control CNPs occurs with the 7.5pH synthesis method. Among the sugar-based samples, galactose-CNP has the highest ALP activity. When observing the results from the osteogenic medium, there is no significant statistical increase in ALP activity compared to control cells. Although the increase in ALP activity is not observed, the extent of decline found within sucrose-CNPs, cyclodextrin-CNPs and dextran-CNPs is observed. These complet-sugar based CNPs show lower ALP activity than more simple monosaccaride samples such as glucose-CNPs and fructose-CNPs.
Figure 10: ARS bone differentiation assay of sugar-CNPs. ARS assay was conducted using human mesenchymal stem cells in both 10ug/mL and 50ug/mL concentrations. Additionally, comparisons were made between osteogenic and normal media. The images were taken after 28 day exposure to various sugar-CNPs.
Alizarin red S (ARS) staining uses a red dye to determine calcium mineralization and is another study to determine bone differentiation. Between osteogenic medium and normal medium, there is no substantial difference in ARS activity within the control cells. Within the normal medium, all control-CNPs and sugar based CNPs resulted in higher ARS activity compared to control cells. Additionally, the control-CNPs showed increased ARS activity with higher concentration. Overall, the control-CNP synthesized in the 9.5pH environment resulted in the highest ARS activity among the control CNPs in the normal medium. Among the sugar-CNPs, sucrose-CNP at 10ug/mL is statistically significant than the other samples in the same concentration and medium. At higher concentration, sugar based CNPs show higher ARS activity. Within the osteogenic medium, the control-CNPs at pH 6.5, 7.5 and 8.5 indicate higher ARS activity than the more extreme pHs of 4.5 and 9.5. This trend was seen with both 10ug/mL and 50ug/mL samples without an increase in ARS activity associated with higher concentration. Overall, 10ug/mL concentration of sugar-based CNPs did not show significant increase compared to the control-CNPs. Galactose-CNPs showed the lowest ARS activity compared to other sugar-CNP samples. At 50ug/mL concentration, both dextran-CNPs and sucrose-CNPs indicated higher ARS activity associated with higher concentrations. Sucrose-CNPs showed substantial ARS activity at 50ug/mL in osteogenic medium. This was further evidenced through the imaging that indicates greater red staining. Overall, the sugar-CNP samples indicate greater red staining associated with higher 50ug/mL concentration in osteogenic medium.
Discussion

The structural characterization showed that the produced CNP were spherical in shape and confirmed the production of nanoparticles of range 3-5nm. Additionally, the particles showed high monodispersity due to the small change in size between each nanoparticle. The difference in size between the Cyclodextrin-CNP and the reducing sugar-CNP can be attributed to oxidation state of...
the CNP correlates with the major finding that reducing sugars enhance Ce$^{3+}$ formation. Smaller nanoparticles contain higher oxygen vacancies, surface defects and surface-to-volume ratio which correlate to a higher Ce$^{3+}$ conformation (Loschen et al., 2008). It is not yet known how the reducing sugars cause smaller sized nanoparticles but the correlation with size and those previously mentioned characteristics highlights potential properties to assess in the future.

As evidenced by the XPS and SOD catalytic assay data, using reducing sugars can enhance Ce$^{3+}$ conformation of CNPs. XPS results showed that the reducing sugars of glucose, fructose and galactose showed higher overall Ce$^{3+}$ state than the non-reducing sugar variations of sucrose-CNP and cyclodextrin-CNP (Figure 3). SOD data correlated with this finding as higher Ce$^{3+}$ conformations relate to higher superoxide dismutase activity (Heckert et al., 2008). This was especially true with the higher concentrations of the fructose, glucose, and galactose CNP formulations.

In these findings, fructose-CNP showed the highest Ce$^{3+}$ state and SOD activity while cyclodextrin-CNP showed the lowest ratio. Fructose has a greater ability to open its cyclic formation than glucose due to the instability of the 5 carbon pentagonal cyclic shape compared to the more stable 6 sugar hexagonal structure (Evans et al., 1928). This allows for stronger reducing properties that can enhance the Ce$^{3+}$ state and increase the SOD activity of the solution.

Benedict’s reagent (Figure 6) was used to determine whether a sugar was a reducing sugar or not. Each sugar was tested with just water and its formulation with CNP. The interesting results of sucrose-CNP showing reducing agent activity was in contrast with the sucrose solution in water and literature on the characteristics of this carbohydrate (Brouns, 2020). The sucrose solution in water showed no color change and determined the non-reducing characteristic of sucrose.
However, the sucrose-CNP showed a color change. This property can be explained by the synthesis of the CNP which involves cerium nitrate stabilizing at a low pH of 3.7 in aqueous solutions (Abellan et al., 2017). The addition of this cerium nitrate precursor is enough to hydrolyze the sucrose into its monosaccharide counterparts (fructose and glucose). Sucrose gets hydrolyzed under slight acidic environments (less than 6pH) which the addition of cerium nitrate precursor satisfies (Torres et al., 2007). This explanation also accounts for why sucrose-CNP shows relatively high Ce$^{3+}$ state in comparison to cyclodextrin-CNP. Both sugars are non-reducing sugars as evidenced by Benedict’s reagent but sucrose-CNP shows a much more dominant Ce$^{3+}$ state.

UV/Vis data was measured to show that reducing sugars can enhance Ce$^{3+}$ conformation. This was evidenced by the higher intensity of the 260nm peak that correlates to higher Ce$^{3+}$ state of the nanoparticles (Singh et al., 2021). While comparison was done with only glucose-CNPs and the control CNPs, a trend can be witnessed in where physiological pH (7.5) indicates higher Ce$^{3+}$ conformation. Synthesis of CNPs with glucose enhances this structural characteristic of the nanomaterial.

MTT cell viability testing is crucial in determining the biomedical applications as toxicity to the cell environment will mitigate any biological function of the nanomaterial. Overall, these results indicate high cell viability across the various formulations. Conducting bone differentiation studies on these CNPs helped uncover the potential skeletal system applications for the use of sugar-CNPs. ALP activity did not show any increase in activity with CNPs except in normal medium. However, due to nature of the bone microenvironment, it is crucial to emulate in vitro conditions to what is experienced in vivo{Shih, 2019 #198}. In the osteogenic media, no viable results were showing CNPs increased ALP activity.
CHAPTER FOUR: RADIOPROTECTANT CERIA NANOPARTICLES (CNPS) DRUG DELIVERY SYSTEM TO REDUCE ROS LEVELS AND MITIGATE SPACEFLIGHT OSTEOPENIA

Introduction

Space related bone loss (also known as spaceflight osteopenia) is a serious deterrent to long duration space flight missions. In fact, astronauts traversing through space experience bone mass loss close to ten times that of osteoporosis (Ohshima, 2015). The specific mechanism of this phenomena can be primarily attributed to the microgravity environment. The lack of load-bearing forces contributes to rapid bone resorption, muscle atrophy and cephalic fluid shifts which all lead to a dynamic loss in bone density (Amin, 2010). However, several factors contribute to this bone loss issue including spaceflight associated stress, changes in metabolism and continual gamma ray irradiation. These other factors enable the buildup of oxidative stress factors such ROS (Tian et al., 2017). Eliminating ROS is a vital aspect to consider when discussing space-related osteopenia.

Several solutions have been proposed to solve this dilemma from exercise to drug and hormone therapy. Bisphosphonate has emerged as a prominent solution with is history of treating osteoporosis within the general population. This molecule uses its structural moiety to bind and inhibit enzyme activity of farnesyl pyrophosphate synthase which is responsible for cellular apoptosis of osteoclasts. This effective drug has shown high efficacy in mitigating osteoporosis through either prophylactic or treatment applications (Lin, 1996). This led to NASA utilizing bisphosphonate combined with an exercise regimen to mitigate osteopenia among astronauts (Ohshima, 2015).

However, bisphosphonate as a viable solution in and of itself has some severe drawbacks. One prominent issue revolves around the inability of bisphosphonate to decrease high ROS levels
associated with bone cells reacting to microgravity environments. Additionally, bisphosphonate is taken orally with low absorption through the gastric epithelium (Fazil et al., 2015). These oral routes also prove to be a struggle as the specific biopolymers associated with these oral drugs can cause an increase in ROS under ionizing radiation environments found in space (Ashfaq et al., 2020). The high levels of ROS can reverse the effects of bisphosphonate by inhibiting osteoblast functions, compromising cellular signaling and eventual decline in bone strength.

In order to improve the drug delivery aspects of bisphosphonate while mitigating the high levels of ROS, ceria nanoparticles (CNP) shows evidence as a viable solution. CNPs work excellently as a drug delivery molecule as it has a high surface area to volume ratio which allows for greater conjugation with the drug while allowing dispersion through gastric epithelium (Deshpande et al., 2005). Additionally, nanoceria can function as an ROS-scavenging molecule that can prevent oxidative stress associated with radiation and/or microgravity.

This project will utilize CNPs as a bisphosphonate drug delivery system to prevent spaceflight osteopenia. By reducing ROS levels associated with ionizing radiation, CNPs will be a highly effective drug delivery molecule than biopolymers and the proposed project will investigate this hypothesis. Studies involving the synthesis of CNP nanoparticles, conjugation with bisphosphonate, ion beam radiation effects and in vitro bone cell response will be crucial to the discussion and will be conducted through experimentation.

The design and optimization of a CNP-bisphosphonate drug system could be beneficial in preventing spaceflight osteopenia. Bone issues are currently a substantial deterrent to long duration spaceflight and the prevention of this severe ailment for astronauts can alleviate this problem. Human space flight capabilities and space life research are topics that this project will address, and
which fall under The Human Exploration and Operations (HEO) Mission Directorate of NASA. Additionally, this project has relevant applications to address orthopaedical issues such as osteoporosis prevention and treatment for the general population. The earth surface does not experience the same extent of gamma radiation as in space, but reducing ROS from the myriad of day-to-day, oxidative stress inducing factors will be an effective therapeutic tool.

Materials and Methods Section

**Materials**

Cerium nitrate hexahydrate, hydrogen peroxide, 1,1’-Carbonyldiimidazole (CDI), Ethylenediamine (diamine), Polyethylenimine (PEI) and dimethyl sulfoxide (DMSO)

**Methods**

Material Synthesis

Cerium oxide nanoparticles were created using previously synthesized method (Wei, 2021 #116). Using a 5mM concentration of CNPs, 6 formulations were created which included CNP+CDI, CNP+CDI+drug, CNP+CDI+diamine, CNP+CDI+diamine+Drug, CNP+CDI+PEI and CNP+CDI+PEI+drug. In all 6 formulations, 250uL of CNPs was added to 1200uL of DMSO. 250uL of CDI (500mM/mL) was added to the solution and stirred. 50uL of PEI (10mg/mL) and 50uL of diamine concentrate was added to respective formulations. Finally, the drug solution (2mg/mL in DMSO) was added to the respective formulations. The solution was dialyzed against DMSO using 20K dialysis tube overnight. Solution was collected the next day.
Structural Characterization of CNP conjugated solutions

Synthesized solutions were characterized using UV-Visible and surface zeta potential characterization. FLUOStar Omega (BMG Labtech) 96 well plate reader measured UV-Visible spectra between 250nm and 700nm.

Antioxidative Functional Characterization of CNP conjugated solutions

The antioxidative potential of Sugar-CNP samples were performed to measure SOD (superoxide dismutase) mimetic activity. The SOD mimetic activity was quantitatively determined using a SOD assay kit (Sigma Aldrich, Kit #19160-1KTF). Using a 96 well plate reader FloustarOmega (BMG Labtech), absorbance was recorded for 20 minutes duration to determine SOD inhibition rate using a kinetic method. Aq-CNP was used as a positive control in the SOD mimetic assay.

Cell Culture

Bone marrow-derived mesenchymal stem cells (MSCs, ATCC PCS-500-012™) were cultured in DMEM (ATCC) containing 10% of fetal bovine serum (FBS, ATCC) and 1% penicillin/streptomycin (ATCC) at 37 °C using a humidified/5% CO2 incubator. After confluency the cells were collected and seeded in 96-well plates for MTT and Live/Dead assays and in 48-well plates for ALP and ARS experiments. The samples were diluted in cell culture medium and added to the wells containing cells. For ALP and ARS, the cell culture was additionally performed in osteogenic medium, prepared by incorporating 50 µM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Merck), 20 mM β-glycerophosphate disodium salt hydrate (Merck) and 10 nM dexamethasone-water soluble (Merck) in the cell culture medium described above. The
culture media containing samples were changed every 3-4 days. All cell studies were performed in triplicate.

Cell Proliferation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, brand) assay was performed after 1 and 3 days of MSCs culture. After each time point, a 5 mg mL-1 MTT solution was poured on top of the wells and the plate was incubated for 4 h at 37 °C. After this time, the medium was removed and dimethyl sulfoxide (DMSO, Sigma Aldrich) was used to dissolve the purple formazan crystals formed after the reduction of MTT by metabolically active cells. The plate was incubated with DMSO for 20 min at 37 °C, and subsequently placed in a shaker (Stuart SSL4 see-saw rocker) for 10 min at 30 osc min-1. Afterward, the absorbance was measured at 570 nm, using a microplate reader (FLUOstar® Omega, BMG LABTECH).

Live/Dead assay was also performed after 1 and 3 days of MSCs culture. The medium was removed, the cells were washed with PBS and the stock solution (from LIVE/DEADTM viability/cytotoxicity kit for mammalian cells, Thermo Fisher Scientific) diluted two times in PBS was added. The plate was incubated at 37 ºC for 30 min, and the cells were imaged in a fluorescent microscope (Nikon TS2-LS).

Cell Differentiation

Alkaline phosphatase (ALP) assay (ab83369, Abcam) was performed after 14 days of MSCs culture. After this time, the cells were washed with cold PBS and ALP assay buffer was added to each well. After incubation for 1 h on ice, the solutions on the wells were homogenized with pipetting and transferred to 2 mL flasks. The solutions were additionally homogenized for
30s using a motorized tissue grinder (Fisher Scientific). Afterward, the flasks were centrifuged at 4°C at 4,400 rpm for 15 min and proceed to ALP activity quantification following the manufacturer’s instructions. The absorbance was measured at 405 nm on a microplate reader (FLUOstar® Omega, BMG LABTECH).

Alkaline phosphatase staining was also performed after 14 days of MSCs culture, using a commercial kit (ab284936, Abcam). The media was removed from the cultured cell wells, and the cells were washed with the buffer provided in the kit. The alkaline phosphatase staining reagent solution was added in the wells and the plate was incubate for 30 min at 37°C. The samples were washed to times with buffer and imaged using a light microscope (Nikon TS2-LS).

Alizarin red S (ARS) staining and quantification was performed after 28 days of MSCs culture. After this time, the cells were fixed with 4% formaldehyde for 30 min at room temperature, and then washed two times with PBS. The cells were stained with 2% alizarin red S (Sigma Aldrich) for 60 min at room temperature, protected from light. After staining, the samples were washed two times with deionized water and imaged using a light microscope (Nikon TS2-LS). For calcium deposition quantification, the dye was extracted from the stained monolayer of cells using a 0.5 N HCl containing 5% sodium dodecyl sulfate (SDS, Sigma Aldrich) solution for 30 min. After this time, the samples were placed in a 96-well plate, diluted to times with the HCl/SDS
solution, and the absorbance was measured at 415 nm on a microplate reader (FLUOstar® Omega, BMG LABTECH).

![Diagram of synthesis method]

Figure 12: Summary of the synthesis method. Experimental process included synthesizing CNP using a hydrogen peroxide process and conjugating 1,1'-Carbonyldiimidazole (CDI). Some batches were further conjugated with Ethylenediamine (diamine) and Polyethylenimine (PEI). The drug was then attached to this functional CNP.
Results

Determination of Conjugation

Figure 13: Shows radiation effects of drug and 200ug of CNP, drug and 100ug of CNP, drug and 10ug of CNP and (drug by itself. These results indicate changes between products exposed to 60 seconds of microwave irradiation and no irradiation. With no CNP offering radioprotectant abilities, the drug control UV/Vis spectra indicates drastic changes in peak behavior. This is in stark comparison to the addition of 200ug of CNP that caused very little change in peak behavior when exposed to 60 second irradiation.

Successful determination of conjugation with CNP was determined using UV/Vis analysis. This process was used to highlight the changes in peak behaviour that is associated conjugation with the risedronic acid. The UV/Vis analysis indicates risedronate shows a prominent peak around 260nm with a secondary peak emerging near 300nm. When comparing the drug conjugated CNPs with the various linkers to formulations without the drug, the drug adds a change in the peak near the 260nm wavelength. Additionally, the UV/Vis analysis indicates a change in the 280nm peak of the CNP+linker formulations. The peak shifts towards the higher wavelength. This indicates a red shift in the peak.
Figure 14: Superoxide Dismutase (SOD) inhibition assay was used to determine ROS scavenging ability of the various conjugated materials.
Superoxide scavenging characterization was conducted using a SOD enzymatic assay. This test was done in order to determine the effect that different conjugations had on overall scavenging ability of the material. This is important as a successful radioprotectant drug delivery system would ideally be able to retain its ROS scavenging ability. Results from Figure 14 indicate that the scavenging properties of CNPs is increased with the drug formulations. Overall all drug-CNP formulations have an increase in superoxide scavenging ability compared to CNP by itself. Additionally, the CNP+diamine+Drug had the highest scavenging ability at nearly 99% SOD inhibition rate.
Irradiation Studies

Figure 15: Radiation studies were completed using microwave (MW) radiation for both 30 and 60 seconds of the drug with various concentrations of CNP.
The effects of radiation were conducted with intervals of 10s and 60s of microwave irradiation. UV/Vis analysis was conducted to determine what changes occur when the drug was exposed to microwave irradiation. Changes in the chemistry can easily be detected using UV/Vis analysis. These results were compared to various concentrations of CNP by itself. The purpose of this study was to determine the radioprotective ability of CNP against free drug molecules. The results indicate that the drug molecules exposed to microwave irradiation change peak behavior creating two additional peaks. However, the addition of 10ug/mL of CNPs reduced this effect substantially which indicates the radioprotective ability of the NPs. Higher concentration of CNPs resulted in identification of the ceria peaks rather than the drug molecules. However, under irradiation, the peak behavior of both 100ug/mL and 200ug/mL did not change. This indicates the stability of CNPs against microwave irradiation.
Cell Studies

Figure 16: Imaged cells were exposed to 50ug/uL concentration of drug, DMSO, CNP, CNP+PE+drug and CNP+Diamine+Drug under 60s irradiation.

Cell studies were used to determine cell viability as well as determine cytotoxicity of the NPs formulations. Human mesenchymal stem cells were utilized to determine whether the formulations can be used to increase bone mineral density. The results show that after day 1 of
exposure to the various formulations, cells maintained their cell viability and did not decrease below 80%. This result was indicated even under 60 seconds of irradiation of the CNPs. The CNPs with diamine drug formulation had increased cell viability even under irradiation. The highest cell survival was seen under 10 seconds of irradiation in this sample. A sample with just DMSO in the same concentration as the CNP formulations was used as comparison. This sample showed clear cytotoxicity associated with irradiation. As the irradiation time increased, so did the level of cytotoxicity. At 60 seconds, the irradiated DMSO caused the cell viability to decrease below 80%.

Day 2 results were also conducted and showed similar results to Day 1. The biggest change was the drastic change in cell viability of DMSO. The cell viability of this solution decreased to near 50%. CNP and the CNP with diamine drug formulation increased cell viability under no irradiation. However, 60 seconds of irradiation did decrease the cell viability below the non-irradiated samples. This result was still above 80% cell viability. The drug only samples increased cell viability with 60 seconds of irradiation.
Bone Differentiation Studies

Figure 17: Both Alzarin Red Stain (ARS) and Alkaline Phosphate (ALP) stains were used to determine bone stem cell proliferation. Cells were exposed to either irradiated or controlled drug conjugates to determine changes in cellular physiology.
Bone differentiation studies were used to determine bone growth which is important to assess bone mineral density. This study was used with both osteogenic and normal media. The ARS study showed an increase in calcium deposition under irradiation for CNPs only under osteogenic medium. Under normal media, this increase was not detected as prominently. When compared to the control cells, the drug only had marginal increase in ARS staining, and this was under 60 seconds of irradiation. The drug formulation of PEI showed an increase in ARS staining compared to the drug by itself. This was also marginally evaluated within the normal media. The ALP staining did not display the same results. Instead compared to the cell, the staining was reduced with all samples.
Discussion

Cerium oxide nanoparticles was created using cerium (III) nitrate hexahydrate through a water-based approach. Previous research from Seal’s research group shows the Ceria $^{3+}/^{4+}$ ratio of the synthesized nanoparticles to have a value of 1.68 or (62.6% Ce$^{3+}$)(Neal et al. 2021). This high ceria $^{3+}$ ratio allows for higher SOD enzyme-mimetic activity compared to catalase activity. Additionally, the enhanced SOD activity derived from the water-based CNP allows for enhanced radiation resistance as evidenced by previous studies on using amifostine (Grdina, Kataoka, and Murley 2000; Xie et al. 2018). However, the nano-scale and structure allows for greater surface area and thus higher oxygen vacancies that can prove to be a better radioprotectant than amifostine.

This aspect of the project required finding the optimal linker and process to allow for proper conjugation of the CNP with the bisphosphonate. Risedronate is a third-generation bisphosphonate that allows for excellent absorption through oral intake. However, as with any drug, the traditional biopolymer encapsulant degrades under radiation. Risedronate trisodium salt was selected for its high water and DMSO solubility which was necessary solvents to add the various combinations of linkers. The 3 methods include using a CDI linker only, CDI + PEI, and CDI + diamine. These 3 combinations of linkers were chosen to ensure that proper conjugation could occur in an aqueous environment. UV/Vis characterization shows the difference in elemental aspects between the water-based CNP, drug and the conjugated products. Adding the drug molecule to all three combinations results in variation in the peaks that determines properly conjugated products.

UV/Vis analysis showcased the conjugation of CNP with the various formulations. The change in peak behavior indicates successful conjugation of the drug with the CNPs. This is
indicated with the peak addition located around 260nm. Additionally, the peak shift in 300nm peak towards a higher wavelength indicates drug to nanoparticle conjugation. This is evidenced by previous research using alendronate (another kind of bisphosphonate) coated CNPs [Zhou, 2021 #191]. Additionally, SOD results show that conjugated products, especially with drug, result in higher SOD inhibition rate compared to water-based ceria. This matches previous results [Zhou, 2021 #191].

The conjugated product was subjected to microwave radiation to determine the changes in chemical structure and thus strength of the ceria nanoparticle encapsulant. Radiation studies were conducted against 30 seconds and 60 seconds of microwave irradiation with just various concentrations of the drug and CNP (10ug/mL, 100ug/mL and 200ug/mL). This study was conducted to determine whether CNP can prevent microwave degradation of the drug product. Results indicated that adding CNP not only changed the UV/Vis spectra but resulted in a higher $^{3+}$ ratio as indicated by the emergence of the peak at 250nm.
CHAPTER FIVE: CONCLUSION AND FUTURE OUTLOOK

In conclusion, ceria nanoparticle show promise as a radioprotectant material that can also work well as a drug encapsulant. In the synthesis work, using sugar based CNPs involved the creation of biocompatible Ceria Nanoparticles using sugar as reducing agent. The Reducing sugar enhancement of CNP allows for the higher Ce$^{3+}$ structural composition as evidenced by XPS results. This higher Ce$^{3+}$ composition translates to an enhanced SOD enzyme-mimetic activity as evidenced by SOD assay kit. Future biomedical applications can be achieved with an emphasis on sustainability.

By conjugating to various combinations of crosslinkers and drug, ceria nanoparticles proved to not only be able to create a stabilized product but also increase SOD inhibition. In fact, the conjugated products showed higher SOD inhibition rate than the CNP by itself. Radiation studies indicate that CNP is able to be used as a radioprotectant as it prevents changes in UV/Vis spectra after exposure to 60 seconds of microwave radiation. Further studies need to performed including XPS characterization, DSC and FTIR to show exactly how CNP can be used a radioprotectant material. Additionally, more extensive radiation studies will be conducted with X-ray and possible gamma ray irradiation.

While the technology and application for space radiation is still in the beginning, the challenges in furthering research should be addressed. One of the major challenges in future research involves stimulating the space environment. Heavy ion beams can be utilized in this regard to recreate typical radiations found in space such as GCRs and SPEs. The idea behind these
experiments involve exposure to high LET radiation exposure as evidenced by various conducted studies (Chew et al., 2019; Chou et al., 2022; Hidding et al., 2017). However, the linear aspect of exposure as well as the controlled nature of these experiments falls short to replicating GCRs in the space which involves random exposure and type of radiation (Schuy et al., 2020). Experiments conducted at the NASA Space Radiation Laboratory are attempting replicate GCRs by exposing cells to a range of ion species with different energies and exposure characteristics (Norbury et al., 2016). Additionally, research to stimulate effects of both radiation and microgravity are still in its infancy as well but could be a promising outlook for future studies (Takahashi et al., 2020). However, studies conducted in the space environment provide the best circumstances to study potential impact of NPs to relieve the hazardous factors in space. While studies are ongoing to determine impact of microgravity conditions, the low earth orbit of conducted astrobiology studies do not address radiation not blocked by the Van Allen belts (Yeung et al., 2020). Additionally, the complications associated with in vitro and eventual in vivo biological testing pose another problem as these experiments need to withstand the pressures associated with space launch (Bijlani et al., 2021). Addressing these challenges will inevitably increase the overall studies on using nanotechnology to protect against space radiation.

How nanotechnology advancements can be incorporated into clinical applications is an important factor to ensure future astronauts receive the proper healthcare that is needed. As mentioned previously, the only FDA regulated drug that is used for its radioprotective function is Amifostine. However, as a drug that is not dependent on dimension properties, Amifostine does not go through the same screening regulations as nanomedicine solutions. Essentially, the dimension characteristics that change as materials are created in the nano range scale require
additional regulation (Foulkes et al., 2020). This prevents the immediacy in translating nanotechnology into clinical applications. Additionally, information regarding the administration of these nanomedicines should also be addressed. The most common administration of drugs is through oral tablets which should be considered when translating nanomedicine solutions for space (Pavez Loriè et al., 2021). However, other drug administration routes should also be considered as possible solutions to effectively deliver the NPs. Addressing these factors can ensure that nanomedicine solutions are translated to the space environment as well as progress terrestrial clinical translation.

Nanotechnology provides the means to produce radioprotectants which can not only remain stable under ionizing radiation conditions, but also provide substantial ROS scavenging from radiolysis products. By incorporating these materials into space medicine and health to address the effects of radiation exposure may permit long-duration spaceflight by humans in the distant future.

Future outlook in this field should prioritize experiments done in the space environment; in particular, beyond low earth orbit. As of 2020, nearly 3000 experiments were conducted within the ISS and of that nearly half were related to biology and biotechnology (Witze, 2020). However, these results have only limited transferability towards our understanding and description of biological behaviour in the deep space environment where radiation is of greater intensity and energy. Experiments in a deeper space environment will provide vital understanding for the production of potential, high efficacy radioprotection. While these future plans are in development, current research towards the effects of ionizing radiation on the skeletal system remains an important focus, essential in current terrestrial and future deep space applications.
NPs formulations from a range of materials have shown efficacy, to varying extents, as radioprotectants against space radiation. Further, a subset of these formulations possessing radioprotective properties have further been shown to protect tissue components of the skeletal system, which is known to be especially sensitive to the effects of ionizing radiation. Results from such studies suggest radioprotectant mechanisms from these materials which rely on surface defects to confer reactive oxygen species (ROS)-scavenging properties, such as those observed for CNP and carbon-based nanomaterials, suggest greater potential for future use: outperforming other material compositions with respect to therapeutic efficacy. Gold, silver and silica NPs compositions are common within nanomedicine and well-studied in therapeutic applications. However, research into their use as radioprotective agents is limited and their performance within these studies suggests limited efficacy as radioprotectants. CNP and carbon nanomaterials have already been tested as radioprotectants for skeletal system bone loss in Earth gravity, however initial studies into ceria cellular uptake into bone cells showed limited activity in space environment. Further, the direct and indirect effects of microgravity on bone physiology will further complicate forecasting of such NPs formulations’ potential therapeutic efficacies.
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Thanks,

Balaashwin (Ashwin) Babu
UCF Nanoscience and Technology Center M.S
UCF AMPAC Graduate Research Assistant
NASA Florida Space Grant Consortium DTIF Recipient
Central Florida Brain Bee Coordinator
407-222-4009

From: Wiley Global Permissions <permissions@wiley.com>
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APPENDIX B: EVIDENCE OF REVIEW PAPER UNDER REVIEW BY PUBLISHER
NANOMED-883.R1 - “Nanotechnology Enabled Radioprotectants to Reduce Space-radiation Induced Reactive Oxidative Species”

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Tue 4/4/2023 10:46 PM
To: sudipta.seal@ucf.edu <sudipta.seal@ucf.edu>
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Dear Dr. Seal,

Your revised manuscript has been successfully submitted online and is presently being given full consideration for publication in WIREs Nanomedicine & Nanobiotechnology.

Manuscript ID: NANOMED-883.R1 (Please include the Revision number in all future correspondence regarding this submission)
Manuscript Title: “Nanotechnology Enabled Radioprotectants to Reduce Space-radiation Induced Reactive Oxidative Species”
CASRAI CRediT Taxonomy: Authors' contribution(s) to the manuscript are attributed as follows:
CRediT Taxonomy

Balaashwin Babu
Conceptualization-Lead, Data curation-Lead, Formal analysis-Lead, Writing – original draft-Lead, Writing – review & editing-Equal

Shreya Pawar
Conceptualization-Supporting, Data curation-Supporting, Formal analysis-Supporting, Writing – original draft-Supporting, Writing – review & editing-Supporting

Agastya Mittal
Conceptualization-Supporting, Data curation-Supporting, Formal analysis-Supporting, Writing – original draft-Supporting, Writing – review & editing-Supporting

Elayaraja Kolanthai
Conceptualization-Supporting, Data curation-Supporting, Formal analysis-Supporting, Writing – original draft-Supporting, Writing – review & editing-Equal

Craig Neal
Conceptualization-Supporting, Data curation-Supporting, Formal analysis-Supporting, Writing – original draft-Supporting, Writing – review & editing-Equal

Melanie Coathup
Conceptualization-Supporting, Data curation-Supporting, Formal analysis-Supporting, Writing – original draft-Supporting, Writing – review & editing-Equal
Sudipta Seal
Conceptualization-Supporting, Data curation-Supporting, Funding acquisition-Lead, Project administration-Lead, Supervision-Lead, Writing – original draft-Supporting, Writing – review & editing-Equal

I will first complete system and formatting checks and let you know if we need any additional information or files. Note that for revisions, we require a response to reviewers, figure permissions, and production-ready image files.

Once I verify that all the information we need is present, I will notify the editor to begin evaluation and, if needed, peer review. You can view the status of your manuscript at any time by checking your Author Center after logging into https://nam02.safelinks.protection.outlook.com/?url=https%3A%2F%2Fmc.manuscriptcentral.com%2Fnanomed%2Fdata=05%7C01%7Cashwin.babu%40knights.ucsf.edu%7Cf454c37d83d64ff5627008db35800227%7C5b16e18278b3412c919668342699eeb7%7C0%7Cf638162596166174634%7CUnknown%7CTWFpbGZsb3d8eyJwIzcjMC4wLiAwMDAiCiQjojV2liMzIuLJBTi6k1baWwJICJXCi6Mn0%3D%7C3000%7C7C%7C8sdata=ROAE9qGr%2Bd%2Bu5u%2Fc10GzCOxn35VD2o0t6SldG6HAO%3D&reserved=0.

Thank you for submitting your manuscript to WIREs Nanomedicine & Nanobiotechnology.

Regards,
Sumithra Elumalai
WIREs Nanomedicine & Nanobiotechnology Editorial Office
APPENDIX C: EVIDENCE OF ACCEPTANCE FOR REVIEW PAPER
Dear Dr. Seal,

It is a pleasure to accept your manuscript entitled “Nanotechnology Enabled Radioprotectants to Reduce Space-radiation Induced Reactive Oxidative Species” in its current form for publication in WIREs Nanomedicine & Nanobiotechnology. I am attaching the handling editor’s comment below for your perusal.

Please note that your manuscript will now undergo an integrity check for images (if applicable). Publication will only proceed on the condition that all final files comply with the journal integrity checks. In the event that any file does not comply with our integrity checks, the journal reserves the right to rescind this decision, or, alternatively, you may be contacted to resolve any concerns raised by these checks.

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Thank you for your contribution to the journal.

Sincerely,

Dr. Nils Walter
APPENDIX D: SUPPORTING INFORMATION FOR CHAPTER THREE
Table S1: The summary of the structural characteristics of sugar-CNPs compared to the 7.5pH CNP control

<table>
<thead>
<tr>
<th>Type of sugar-CNPs</th>
<th>Ce³⁺% (Ce⁵⁺ / (Ce³⁺ + Ce⁴⁺))</th>
<th>Size Information</th>
<th>SOD Inhibition (%)</th>
<th>Zeta potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Average size (nm)</td>
<td>% Deviation</td>
<td>1mM</td>
</tr>
<tr>
<td>7.5 pH Control-CNPs</td>
<td>43.7%</td>
<td>9.87</td>
<td>55%</td>
<td>57.18%</td>
</tr>
<tr>
<td>Glucose-CNPs</td>
<td>65.1%</td>
<td>3.83</td>
<td>13.1%</td>
<td>93.91%</td>
</tr>
<tr>
<td>Fructose-CNPs</td>
<td>70.9%</td>
<td>3.23</td>
<td>14.8%</td>
<td>98.30%</td>
</tr>
<tr>
<td>Galactose-CNPs</td>
<td>68.8%</td>
<td>3.54</td>
<td>12.9%</td>
<td>96.79%</td>
</tr>
<tr>
<td>Sucrose-CNPs</td>
<td>60.3%</td>
<td>4.05</td>
<td>11.2%</td>
<td>93.87%</td>
</tr>
<tr>
<td>Cyclodextrin-CNPs</td>
<td>38.0%</td>
<td>4.54</td>
<td>5.7%</td>
<td>13.03%</td>
</tr>
<tr>
<td>Dextran-CNPs</td>
<td>65.2%</td>
<td>4.48</td>
<td>7.4%</td>
<td>94.87%</td>
</tr>
</tbody>
</table>
Figure S1: MTT cellular viability testing of control-CNPs on mesenchymal stem cells (MSCs). MTT cellular viability testing was conducted on mesenchymal stem cells (MSCs) using both a 10ug/uL sample and a 50ug/uL sample for each sugar-CNP solution. The images are taken after 3 day exposure to various nanoparticle solutions. A bright field, a live fluorescence and dead fluorescence was taken of each sample and compared to the control cells.
LIST OF REFERENCES


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Witze, A. (2020). Astronauts have conducted nearly 3,000 science experiments aboard the ISS. *Nature*. https://doi.org/10.1038/d41586-020-03085-8